Molecular characterization of novel forms of β-1,3-glucanase gene and their promoter sequences with reference to abnormal leaf fall disease in *Hevea brasiliensis*

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By
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6th August, 2012

Certificate

This is to certify that the thesis entitled "**Molecular characterization of novel forms of β-1,3-glucanase gene and their promoter sequences with reference to abnormal leaf fall disease in *Hevea brasiliensis*"** is an authentic record of original research work carried out by Mrs. Supriya R, at The Rubber Research Institute of India, Kottayam - 686 009 under my supervision for the award of the degree of Doctor of Philosophy in Biotechnology under the Faculty of Science, University of Kerala, Thiruvananthapuram, Kerala. It is also certified that the work presented in this thesis has not been submitted earlier for any other degree or diploma elsewhere.

(Dr. A. Thulaseedharan)

DECLARATION

I hereby declare that this Ph.D thesis entitled "**Molecular characterization of novel forms of β-1,3-glucanase gene and their promoter sequences with reference to abnormal leaf fall disease in *Hevea brasiliensis*"** is an authentic and bonafide record of the research work carried out by me under the supervision of Dr. A. Thulaseedharan, Deputy Director (Biotechnology), at The Rubber Research Institute of India, Kottayam. The work presented in this thesis has not been submitted earlier for any other degree or diploma elsewhere.

Supriya. R

Kottayam
6th August, 2012

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Abbreviations

^{0}C	:	**d**egree **c**elsius
µg	:	**m**icrogram
µl	:	**m**icrolitre
µm	:	**m**icromole
ALF	:	**A**bnormal **L**eaf **F**all
AOX	:	**A**lcohol **O**xidase
***Avr* genes**	:	**A**virulence **G**enes
BA	:	6-**B**enzylaminopurine
β-glu	:	**β**-1,3-glucanase
bp	:	**B**ase **P**air(s)
BSA	:	**B**ovine **S**erum **A**lbumin
cDNA	:	**C**omplementary **D**NA
Ci	:	**C**urie
CTAB	:	**C**etyltrimethylammonium **B**romide
DEPC	:	**D**iethyl **P**yrocarbonate
dNTPs	:	**D**eoxynucleotide **T**riphosphates
DOPE	:	**D**iscrete **O**ptimized **P**rotein **E**nergy
EC	:	**E**nzyme **C**ommission
EDTA	:	**E**thylenediaminetetraacetic acid
ERE	:	**E**thylene **R**esponsive **E**lement
EST	:	**E**xpressed **S**equence **T**ags
g	:	**g**ram
gDNA	:	**G**enomic DNA
GEBP	:	**β**-glucan **E**licitor **B**inding **P**rotein
GIP	:	**G**lucanase **I**nhibitor **P**rotein
Gln	:	**G**lucanase
h	:	**h**our(s)
Hb	:	***Hevea brasiliensis***
IME	:	**I**ntron-**M**ediated **E**nhancement
IP	:	**I**nverse **P**CR
IPTG	:	**I**sopropylthio-β-D-galactoside

kb	:	**k**ilobase
kDa	:	**k**ilodalton
LB broth	:	**L**uria-**B**ertani **B**roth
M	:	**M**olar
MAS	:	**M**arker **A**ssisted **S**election
mg/l	:	**m**illigram per litre
mg/ml	:	**m**illigram per millilitre
min	:	**m**inute (s)
ml	:	**m**illilitre
mM	:	**m**illimolar
mRNA	:	**M**essenger **R**NA
MS	:	**M**urashige and **S**koog
NAA	:	**N**aphthalene **A**cetic **A**cid
NCBI	:	**N**ational **C**entre for **B**iotechnology **I**nformation
ng	:	**n**anogram
NR	:	**N**atural **R**ubber
OD	:	**O**ptical **D**ensity
ORF	:	**O**pen **R**eading **F**rame
PAGE	:	**P**oly **A**crylamide **G**el **E**lectrophoresis
PB	:	**P**rang **B**esar
PCR	:	**P**olymerase **C**hain **R**eaction
PEG	:	**P**olyethylene **G**lycol
PIP	:	**P**ost **I**nfection **P**eriod
PLF	:	*Phytophthora* **L**eaf **F**all
PR proteins	:	**P**athogenesis **R**elated **P**roteins
RAGE	:	**R**andom **A**mplification of **G**enomic **E**nds
RRII	:	**R**ubber **R**esearch **I**nstitute of **I**ndia
RRIM	:	**R**ubber **R**esearch **I**nstitute of **M**alaysia
RT	:	**R**everse **T**ranscriptase
SDS	:	**S**odium **D**odecyl **S**ulphate
SDS-PAGE	:	**S**odium **D**odecyl **S**ulfate **P**olyacrylamide **G**el **E**lectrophoresis
SOC	:	**S**uper **O**ptimal broth with Catabolite repression
SRPP	:	**S**mall **r**ubber **p**article **p**rotein

SSC	:	**S**odium chloride-**S**odium citrate buffer
TBE	:	**T**ris **b**orate **E**DTA buffer
TE	:	**T**ris: **E**DTA
Tris	:	**T**ris (hydroxymethyl) aminoethane
UTR	:	**U**ntranslated **R**egion
v/v	:	**v**olume per **v**olume
w/v	:	**w**eight per **v**olume
X-gal	:	5- bromo-4- chloro-3-indolyl-β-D-galactoside
X-gluc	:	5- bromo-4- chloro-3-indolyl-β-D-glucuronide

Chapter 1

Introduction

Natural rubber (Para rubber / Caoutchouc), is a high molecular weight elastic hydrocarbon polymer of isoprene (2-methyl-1,3-butadiene, $CH_2=C(CH_3)-CH=CH_2$) units derived from latex, a milky colloidal exudate produced in the specialized cells called laticifers or latex vessels of *Hevea brasiliensis*. The unique properties of the natural rubber (NR) make it used extensively for several applications and in many products. The flexibility of natural rubber to undergo vulcanization with sulphur under high temperatures is an important attribute, which provide NR the required strength and durability for wide industrial utilization. There is hardly any segment of life, which does not make use of natural rubber. Due to higher strength, elasticity, low heat build up and better resistance to wear and tear, about 80% of natural rubber is used in the automobile and electrical industry.

Natural rubber is found in the latex of as many as 895 species of plants belonging to 311 genera of 79 families such as *Euphorbiaceae, Apocyanaceae, Asclepiadaceae, Asteraceae, Monaceae, Papaveraceae, Sapotaceae* etc. (Archer and Audley, 1973; Backhaus 1985; Cornish *et al.,* 1993). Apart from this two major classes of fungi, Ascomycetes and Basidiomycetes, produce low-molecular weight rubber (Stewart *et al.*, 1955; Kurihara *et al.*,1962). However, the para rubber tree, *Hevea brasiliensis* (Wild. ex. Adr. de. Juss. Müell-Arg.), commonly known as rubber tree, a member belonging to the spurge family *Euphorbiaceae,* is the most exclusive contributor of NR produced world wide (99%) due to its good yield of rubber and excellent physical properties for the rubber products (Greek, 1991; Asawatreratanakul *et al.*, 2003). Other plants producing rubber include gutta-percha (*Palaquium gutta*), rubber fig (*Ficus elastica*), Panama rubber tree (*Castilla elastica*), spurges (*Euphorbia* spp.), lettuce, common dandelion (*Taraxacum officinale*),

Russian dandelion (*Taraxacum kok-saghyz*), *Scorzonera (tau-saghyz)*, and guayule (*Parthenium argentatum*).

Hevea brasiliensis is a native of Amazon River basin of South America. The tree is now grown in the tropical regions of Asia, Africa and South America. However, now a lion's share (92 %) of NR comes from tropical Asia. South America remained the main source of the limited amounts of latex rubber that were used during much of the 19th century. In 1876, Henry Wickham gathered thousands of para rubber tree seeds from Brazil, and these were germinated in Kew Gardens, England. The seedlings were then sent to Ceylon (Sri Lanka), Indonesia, Singapore and British Malaya. Malaya (now Malaysia) was later to become the biggest producer of rubber. About 100 years ago, the Congo Free State in Africa was also a significant source of natural rubber latex, mostly gathered by forced labour. *Hevea* trees now growing in South and South-East Asia are descended from the few seedlings transplanted from Brazil through Kew Gardens that have undergone several cycles of breeding.

Now the global rubber cultivation is in about 11.5 million hectars with an annual production of 11 million tonnes. The first commercial *Hevea* plantations in India were established at Thattekadu in Kerala by 1902. In India rubber is now cultivated in 0.71 million hectars with an annual production of about 0.86 million tonnes. India is one of the largest producers of NR in the world and is also considered to be one of the key players in global rubber business. Since 2006, India has been continuously holding the first position in terms productivity of NR. It is the fourth largest producer (first being Thailand) of NR during the year 2010 with a share of 8.2% of the global output. It is also the second largest consumer of NR in the world (first being China). Kerala alone accounts for 75% of the area occupied by the crop in the country. In the year 2010-11, India's NR consumption stood at 0.948 million tonnes, with a production at 0.862 million tonnes (Indian Rubber Statistics, 2011). This envisages the tremendous growth prospects of the rubber industry in India in the years to come.

The first use of rubber was by the Olmecs exclusively for many centuries, who then passed on the knowledge of natural latex from the *Hevea* tree in 1600 BC to the ancient Mayans. They boiled the harvested latex to make a ball for sports. 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 constructing roads. Other significant uses of rubber are door and window profiles, hoses, belts, and flooring. Gloves (medical, house hold and industrial) and toy balloons are also large consumers of NR. Significant tonnage of rubber is used as adhesives in many manufacturing industries and products, although the two most noticeable are the paper and carpet industries. Rubber is also commonly used to make rubber bands and pencil erasers. Additionally rubber produced as a fiber sometimes called elastic has significant use in the textile industry because of its excellent elongation and recovery properties. On account of the multifarious uses to which rubber can be put to, the consumption of rubber in India as well as in the world has been increasing steadily.

The wood from this tree is used in the manufacture of high-end furniture. It is valued for its dense grain, minimal shrinkage, attractive colour and acceptance of different finishes. It is also prized as an environment friendly wood as it makes use of trees that have been cut at the end of their latex producing cycle. Rubber seeds are also of economic importance. Seeds are the source of para rubber seed oil, recommended for manufacture of soap. They have potential use as a source of industrial oil as well as a protein supplement for use in livestock diets. Oil is also used in paints, varnishes and is effective against houseflies and lice. Natural rubber also contributes to the global environment preservation by reducing the amount of carbon-dioxide in the atmosphere. According to the International Rubber Research and Development Board, a hectare of rubber plantation with 300 mature trees in its 25 years of existence in the field could sequester about 138.9 MT of carbon-dioxide (Tilekerathne, 2007).

The role of the rubber tree in the sustenance of Indian economy is tremendous. However, loss due to several biotic and abiotic stresses usually occur in *Hevea* plantations are causing considerable loss of latex yield annually which ultimately affects the economic prospects of the country. Among the various stresses, crop loss resulting from diseases in rubber plantations are substantial. Timely plant protection operations ensure healthy growth and economic production. The major diseases affecting the rubber plantations include; abnormal leaf fall disease (*Phytophthora spp.*), secondary leaf fall (*Oidium hevea*), bird's eye spot (*Drechslera hevea*), leaf spot (*Periconia hevea*), pink disease (*Corticium salmonicolor*), red root disease (*Ganoderma philippi*), bark canker (*Phytophthora faberi*), shoot rot, powdery mildew, brown root disease (*Phellinus noxius*), black thread root disease (*Ustulina deusta*), etc.

In plants, leaves are the seat of photosynthesis which is highly essential for the growth and yield of the plant. Heavy infection of different pathogens upon leaf cause huge reduction of leaves and thereby affects the total photosynthetic area / rate available with the plant which ultimately 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 leads to quick defoliation. Hence control of the disease after its outbreak is difficult. So far the strategy adopted is prophylactic protection in which spraying of copper fungicides is done in anticipation of disease incidence.

The important leaf diseases of rubber are abnormal leaf fall, powdery mildew, Corynespora leaf fall, Colletotrichum leaf fall and bird's eye spot disease. Among them, the abnormal leaf fall disease (ALF) caused by the fungus *Phytophthora spp.* that occur annually during the monsoon period is the most damaging leaf disease of rubber in India. Rainfall is the most important pre-disposing weather factor. It is often noticed during June to August during which 70% of the annual rainfall is received. Disease incidence is less in Kanyakumari district of Tamil Nadu state but increasingly heavy northwards along the south-western coasts of Kerala. The abnormal leaf fall

(Sdoodee, 2004) and also causes low timber output (Viswanathan, 2005). In India, the attack of *Phytophthora* disease in rubber was first reported from the Palappilly estate in Thrissur in 1910. 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).

Though various crop husbandry techniques have been adopted to combat losses caused by this biotic stress, the most widely used management strategy is the application of agrochemicals. The intensive use of agrochemicals can lead to the evolution of pathogen populations with increased resistance and in some cases the chemicals are no longer used because of their high toxicity to non-target species. Nowadays, the focus is on strategies that allow crop protection with minimal use of agrochemicals and the most effective strategy involves breeding resistant crop cultivars. However, there are many pathogens for which no effective sources of disease resistance have been identified in many crops.

Genetic engineering or recombinant DNA technology, together with plant transformation and tissue culture techniques, has facilitated the development of transgenic plants with enhanced pest and disease resistance (Lurquin, 2002). Among the various approaches to make a plant disease resistant through genetic engineering, the most straight forward approach is to incorporate genes encoding antimicrobial proteins or peptides originating from plants or other organisms either alone or in combination. The use of pathogen-inducible promoters to regulate these antimicrobial factors will give an additive effect for this approach. The second way is to manipulate the plant's own defence mechanisms like induced resistance, basal resistance and race-specific resistance, which activate the plant's own antimicrobial defences. The third way is to manipulate the plant to prime defence responses *via* pathogen mimicry (genetic vaccination) (Collinge *et al.*, 2008).

Many reports (Makandar *et al.*, 2006; Yang *et al.*, 2008) show positive results relative to transgenic plants, expressing genes for fungal disease resistance. Here finds the exploitation of 'R genes' in conferring disease tolerance toward plant diseases. The so called plant disease resistance genes (R genes), a term coined by H.H. Flor (1956), encode proteins that detect pathogens. R genes have been widely employed for many years with great success through conventional programmes. These genes control many plant diseases caused by bio-trophic pathogens such as rusts and powdery mildew fungi. These genes have the advantage of conferring complete resistance against specific races of the pathogen. Definition of conserved structural motifs in R proteins has facilitated the cloning of useful R genes, including several that are functional in multiple crop species and/or provide resistance to a relatively wide range of pathogens. The enhanced understanding of R-gene structure and function make it possible to modify functional domains in future to tailor R-genes for use in providing broad-spectrum resistance to diseases in transgenic plants (Dempsey *et al.*, 1999).

Chitinases and β-1,3-glucanases are probably the best-studied antifungal proteins to date. They catalyse the hydrolysis of chitin and glucan, respectively, two major structural components in the cell wall of many fungi. They are coming under the pathogenesis related proteins (PR proteins). PR proteins are coded by PR genes, a group of R genes which are categorized under 18 families. PR proteins (initially named "b" proteins) were discovered in tobacco leaves hypersensitively reacting to TMV by two independently working groups (Van Loon and Van Kammen, 1970; Gianinazzi *et al.,* 1970). Subsequently PRs have been identified in numerous monocotyledonous and dicotyledonous plants across different genera, and hence it can be considered as ubiquitously distributed in plant kingdom.

β-1,3-glucanases (β-glu, (1,3-β-D-glucan 3-glucanohydrolase, (EC 3.2.1.39)) are PR-2 proteins that catalyse endotype hydrolytic cleavage of 1,3-β-D glycosidic linkages in β-1,3-glucans present in the cell wall of many pathogenic fungi especially the oomycete fungi whose cell wall is predominantly made up of β-1,3-glucan polymers and cellulose; unlike fungal

cell walls, they contain little chitin. *Phytophthora* cell walls do not contain a significant amount of chitin. Instead, β-glucans constitute 80 to 90% of the cell wall dry weight. The glucanases were also proved to be active on live *Phytophthora* mycelium, which they lysed in the absence of any antibiotic activity, indicating that they might play a significant role in plant disease suppression (Valois *et al.,* 1996). It has been proposed that it exhibits dual mode of action: directly, by degrading the cell walls of the pathogen, or indirectly by promoting the release of cell wall-derived materials that can elicit an active defense reaction (Boller, 1993). These abundant and highly regulated enzymes are widely distributed throughout the plant kingdom and have been intensively investigated at the physiological and molecular levels because of their well-proven role in plant defense response (Simmons, 1994; Leubner-Metzger and Meins, 1999).

The narrow genetic base along with a prolonged breeding cycle and juvenile period and a highly heterozygous nature have made conventional breeding of *Hevea* complex, labor intensive and time-consuming. Because classical molecular approaches to tag resistance genes, such as constructing segregating populations or near-isogenic lines are difficult to employ in rubber trees, there is a need to develop biotechnological approaches for improving rubber crops. Various investigations are going on to find a molecular solution to increase the tolerance or converting a susceptible clone tolerant towards the abnormal leaf fall disease.

In the earlier studies held at RRII, the expression patterns of the gene coding for β-1,3-glucanase enzyme in a tolerant (RRII 105) as well as a highly susceptible (RRIM 600) clone were examined following infection with ALF-causing *Phytophthora meadii*. The gene coding for β-1,3-glucanase was observed to be present in both tolerant and susceptible clones. The gene expression was found to get induced only when infected with the fungus. Twenty four hours after infection, the β-1,3-glucanase transcript level accumulation was almost equal in the tolerant and the susceptible clones. However a continuous accumulation of transcript level was observed for a longer period only in the tolerant clones. In the susceptible clones, 24 hours

after infection, the transcript level was drastically dropped. What controls this differential expression of β-1,3-glucanase gene in the tolerant and the susceptible clones of *Hevea* still remain unidentified. It was also observed that the purified β-1,3-glucanase could effectively inhibit the mycelial proliferation of *Phytophthora meadii* (Thanseem *et al.*, 2003; 2005). These observations raised the following assumptions about the mechanism of differential β-1,3-glucanase gene expression in the tolerant and susceptible clones of *Hevea*;

1) Is there any difference in the β-1,3-glucanase gene sequence and predicted amino acid sequences in different *Hevea* clones?

2) Whether any pathogen derived proteins are interacting directly with the β-1,3-glucanase gene promoter ?

3) Is there any difference in the promoter sequence of the β-1,3-glucanase gene between clones with different degrees of tolerance?

Finding the mechanism of differential expression of β-1,3-glucanase gene leading to tolerance against abnormal leaf fall disease will be helpful in the future attempts to develop tolerant clones of *Hevea* towards the ALF through transgenic and molecular marker assisted breeding approaches.

Objectives:

The present study was undertaken with the following perspectives:

1) To find out sequence variations, if any, in the β-1,3-glucanase gene in different *Hevea* clones which impart disease tolerance/ susceptibility to abnormal leaf fall disease.

2) To identify the sequence/ isoforms (if any) showing maximum antifungal activity.

3) To identify the cis-acting elements in the promoter region of β-1,3-glucanase gene in tolerant and susceptible clones.

Chapter II

Review of Literature

In a broad sense, the definition of plant disease includes anything that damages the plant health. The form of defence that provides resistance to a wide range of pathogens is called systemic immunity that occurs similarly in both plants and animals (Durrant & Dong, 2004; Vlot *et al.*, 2008; Iriti and Faoro., 2007). Unlike animals, plants lack mobile defender cells and a somatic adaptive immune system. They usually rely on the innate immunity of each cell and on systemic signals emanating from the infection sites (Jones and Dangl., 2006). Significant crop losses occur annually due to various plant diseases. An average of 12% of world crop production is lost per annum as a direct result of microbial infection. A detailed understanding of the plant's complex immune function will underpin crop improvement for food, fibre, latex and biofuel production. Among the various crop diseases caused by a diverse range of pathogens, fungal diseases are rated as the most prevalent biotic stress contributing to yield loss (Grover and Gauthaman, 2003).

In a survey held in 2003, contribution of fungal diseases towards total yield loss of important crops in India was 18–31% (Grover and Pental, 2003). Conventionally the incidence of plant diseases can either be controlled by the application of agro-chemicals which are expensive or by the breeding of new varieties that contain genes conferring biotic/abiotic resistance. Although, there has been success with conventional breeding and the production of disease resistant cultivars, the process is extremely time-consuming. The uncontrolled use of many agro-chemicals is environmentally hazardous and increases the input cost of farmers. The pathogen eventually becomes resistant to these chemicals also. This necessitates the development of crops which are resistant to disease, and minimum use of agrochemicals. The production of disease resistant transgenic plants of agricultural importance will thus play an increasingly important role towards the alleviation of this problem. An understanding of the molecular mechanism 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. An important part of the strategies designed to engineer increased resistance of plants to fungal diseases is the discovery and characterization of plant antifungal proteins and the isolation of their encoding genes (Yun *et al.*, 1997). Following the isolation of fungal resistance conferring genes they can be transferred to agriculturally important cultivars to provide resistance to specific pathogens (Cornelissen and Melchers, 1993). Thus, over-expression of antifungal proteins in transgenic plants is an alternative method to increase resistance towards fungal diseases.

Natural rubber is a strategic raw material highly essential in the manufacture of more than 40,000 products, like heavy-duty tyres and more than 400 medical devices such as tubing and latex products (Cornish, 2001; Hagel *et al.*, 2008). Natural rubber is an essential commodity for human life used in the manufacture of products ranging from simple rubber bands to spare parts of space shuttles. Besides, rubber is also useful in soil stabilization, vibration absorption, road surfacing, etc. Currently, more than half of the rubber we use is synthetic (manufactured from oil) but it is weaker, less elastic and conducts heat lesser than natural rubber. Increasing market demand and threat of global supply by plant disease makes natural rubber an important commodity for which there is no direct alternative. Among these, *Hevea brasiliensis* (*Euphorbiacaeae*), the para rubber tree is the most important source of rubber. Other species are *Glaziovii* (*Euphorbiacaeae*), *Ficus elastica* (*Moracaeae*), *Parthenium argentatum* and *Taraxacum kok-saghyz* (*Compositae*). Many other species such as *Euphorbia intisy* (*Euphorbiacaeae*), *Cryptostegia grandiflora*, and *Landolphia* sp. have been tried as possible minor sources of natural rubber. Though many varieties of plants produce polyisoprene in the form of latex, only the species of *Hevea*, the guayule plant and the *Taraxacum kok-saghyz* plant are capable of producing high molecular weight linear polymer with a 100 percent *cis* -structure typical of natural rubber.

2.1. *Hevea brasiliensis*

The Para rubber tree (*H. brasiliensis*), is highly preferred for natural rubber because it yields high quality latex over a sustainable period and is amenable to tapping. It was introduced to India by the British in the 19thcentury, and many plantations were successfully established by the early 20th century. The chief method for propagation of *Hevea* is bud grafting. Many clones with desirable traits have been developed through conventional breeding programs from the initial germplasm collections of the 19th century. However, many of the high-yielding clones are susceptible to a variety of diseases which cause serious economic loss. The narrow genetic base along with its long breeding cycle and high juvenility period, which extends up to seven years, makes the conventional crop improvement programmes rather difficult in rubber. The present goals of biotechnological research in *H. brasiliensis* include characterization of genes controlling important agronomic traits like tolerance to diseases and abiotic stresses, understanding the molecular mechanism of latex biosynthesis, characterization of lacticiferous specific promoters, removal of latex allergens and development of transgenic plants for better agronomic qualities and production of recombinant proteins (Thulaseedharan *et al.,* 2004). Among them the characterization of disease controlling genes is essential to understand the disease resistance mechanism so that a vital strategy can be followed to develop new disease resistant clones of *Hevea.*

The Climatic conditions necessary for the production of natural rubber includes an average temperature range of 25^0 to 30 ^{0}C and average annual rainfall of at least 2 meters evenly distributed throughout the year. Now, approximately 11.5 million hectares (28.4 million acres) of land were dedicated to the cultivation of natural rubber globally. In India, rubber cultivation started in 1902. In 2010-11, the production of natural rubber in India was 0.862 million tonnes. Presently, India occupies the first position in terms of productivity and fourth in the production of natural rubber (8.2 % of the global output). Thailand, Indonesia and Malaysia are the top three producers of rubber in the world.

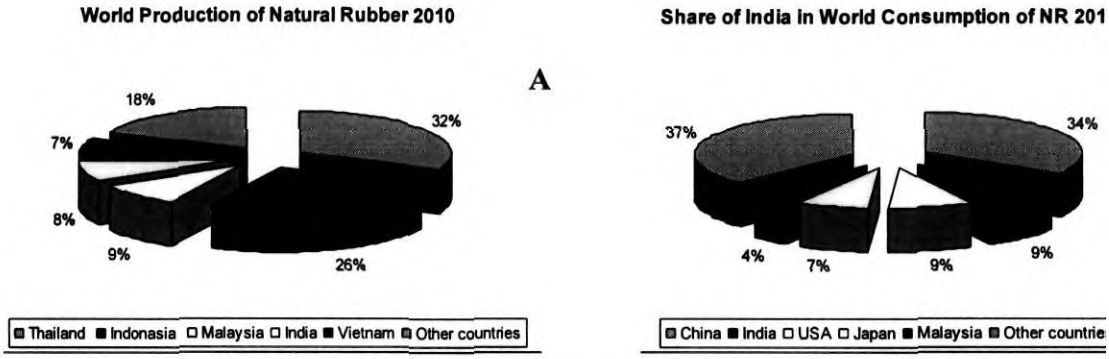


Fig.1. The percentage rubber production (A) and consumption (B) by different countries

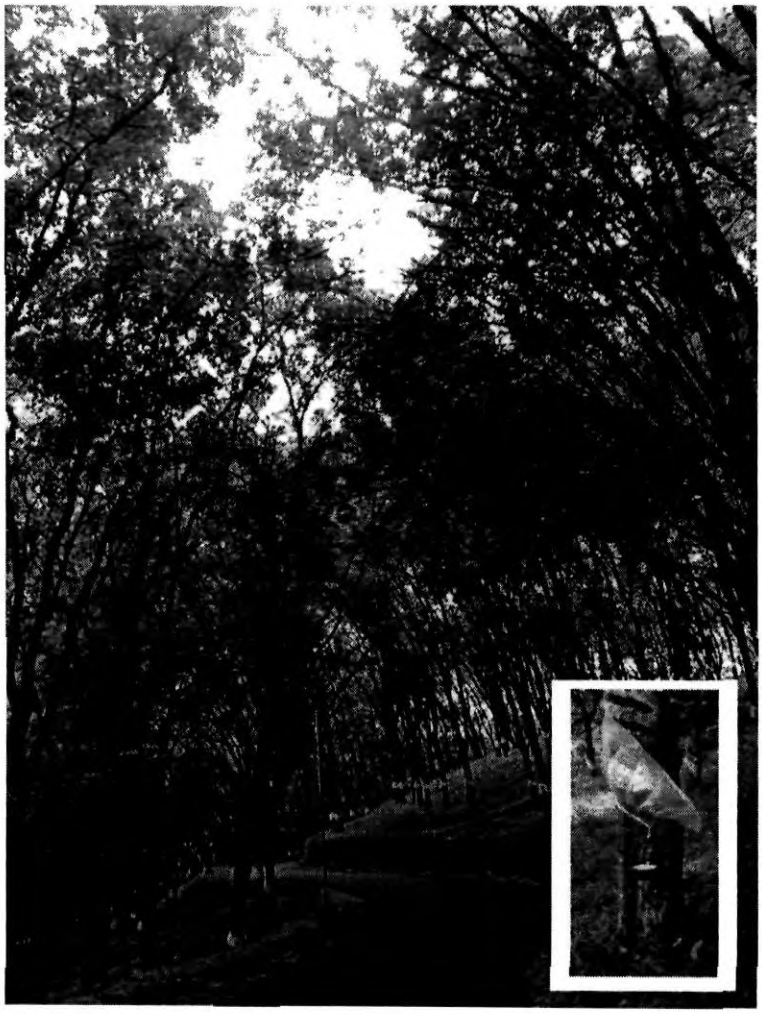


Fig.2. A rubber plantation with a clear view of tapping panel in the inset

India is also the second largest consumer of natural rubber in the world (8.8% of the global consumption (first being China – 33.8%). Kerala alone accounts for 75% of the area occupied by the crop in the country (Indian rubber statistics, 2011).

2.2. Important diseases affecting rubber plantations

Various drastic diseases, majority of them are fungal in origin, strike through the rubber plantations every year. These disease outbreaks are responsible for considerable loss in terms of latex yield every year. About 90 species of fungi are known to attack *Hevea* trees. The most prevalent ones being the following: *Botryodiplodia elactica* and *B. theobromae (die-back), Colletotrichum heveae* (leaf spot), *Fomes lamaensis* (brown root rot), *Gloeosporium heveae* (die-back), *Oidium heveae* (powdery mildew), *Pellicularis salmonicolor* (pink disease), *Phytophthora spp.* (causing fruit rot, leaf-fall, black thread, and die-back). The other diseases include *Polystichus occidentalis* and *P. personii* (white spongy rot), *Sphaerella heveae* (rim bright), *Sphaerostilbe repens* (red rot) and *Ustulina maxima* (charcoal rot). It is also attacked by *Bacterium albilineans* and parasitized by *Loranthus* spp. Nematodes isolated from *Hevea brasiliensis* include: *Helicotylenchus cavenessi, H. dihystera, H. erythrinae, Meloidogyne incognita acrita, M. javanica, Pratylenchus coffeae, P. brachyurus.* Insect pests include: Scale insects (*Aspidiotus cyanophylli* and *Parasaissetia nigra*). White ants cause serious damage to trees at all ages. Snails can be serious pests to young trees. Three types of root disease, classified as white, red and brown are controlled by cutting away diseased tissue and applying prophylactic coatings. Panel diseases classified as black stripe, moldy rot and panel necrosis are minimized by spraying or coating specific fungicides. Stem disease, consisting of pink disease, stem canker and dieback is reduced by brushing on specific fungicides. Leaf disease, consisting of abnormal leaf fall, *Gloeosporium* leaf disease, powdery mildew and bird's eye spot are controlled by a variety of sprays including copper oxychloride, sulfur dust and others applied by spray or dusting techniques (Rogers, 1981).

Though many fungal diseases are there, the abnormal leaf fall (ALF) caused by *Phytophthora,* powdery mildew by *Oidium hevea*, and leaf spot by *Corenyspora cassicola* are the major fungal diseases affecting rubber plantations. Abnormal leaf fall (ALF) or *Phytophthora* leaf fall (PLF) is the most economically significant fungal disease affecting the rubber plantations in India. Initial field trails indicated that this disease could cause yield loss of around up to 38 - 56%, when left unsprayed for one disease season (Ramakrishnan, 1960; Jacob, 2003).

Through the conventional breeding programmes, many promising clones with a fair degree of tolerance to ALF disease were evolved from the initial germplasm collections of the 19th century. But the emergence of a fully resistant clone to *Phytophthora* with high yield has so far not been developed. In the case of perennial tree crops like *Hevea*, such conventional breeding programmes are elaborate, labour intensive and time consuming. Millions of rubber plantations across Asia and Africa had actually originated from a minuscule of seeds collected from three specific localities of Amazon River basin in Brazil by Sir Henry Wickam in 1876 (Imle, 1978). Molecular studies revealed that none of the *R* genes isolated so far code for any toxic chemicals with direct fungicidal actions. This job is being done by another group of genes called defence genes. An understanding of the role of such an established defence-related gene like β-1,3-glucanase could be a viable alternative approach for the development of more sophisticated molecular tools to combat ALF disease in *Hevea*.

2.3. Plant immune system

Plants use diverse life strategies to ward off pathogens. Pathogenic bacteria proliferate in intercellular spaces (the apoplast) after entering through gas or water pores (stomata and hydathodes, respectively), or gain access *via* wounds. Fungi can directly enter plant epidermal cells, or extend hyphae on top of, between, or through plant cells. Pathogenic, symbiotic fungi and oomycetes can invaginate feeding structures (haustoria) and enter into the host cell plasma membrane. Haustorial plasma membranes, the extracellular

matrix, and host plasma membranes form an intimate interface at which the outcome of the interaction is determined. These diverse pathogen classes all deliver effector molecules (virulence factors) into the plant cell to enhance microbial fitness (Jones and Dangl, 2006). In these encounters, pathogens frequently reveal their presence to the plant's 'innate immune system' through microbe-associated molecular patterns (MAMPs) formerly termed pathogen-associated molecular patterns (PAMPS) (Hammond-Kosack and Kanyuka, 2007). Effectors from plant pathogens that are eukaryotic are poorly understood. Fungal and oomycete effectors can act either in the extracellular matrix or inside the host cell. For example, the tomato RLPs, Cf-2, Cf-4, Cf-5 and Cf-9 respond specifically to extracellular effectors produced by *Cladosporium fulvum* (Rivas and Thomas, 2005). The resistance shown by the plant can be pre-formed or induced. The plants can activate a very effective arsenal of inducible defence responses, comprised of genetically programmed suicide of infected cells (the hypersensitive response, HR), as well as tissue reinforcement and antibiotic production at the site of infection. These local responses in turn, trigger a long lasting systemic response (systemic acquired resistance, SAR) that primes the plant for resistance against a broad spectrum of pathogens (Dong, 2001; Metraux, 2001). The pre-formed resistance involves the factors, which are present in plants prior to the contact of pathogen. This includes surface features of plants such as thickness of the cuticle, configuration of cuticular wax, topography of leaf surface, degree of stomatal opening etc. Structural features like the presence of a thick cuticle, waxes on leaf and fruit surfaces forming a water repellent surface, and a thick , tough outer walls of epidermis cause delay in pathogen penetration, thereby contributing significantly to plant defence by allowing time for the induction of more sophisticated defence mechanisms.

In addition to these structural barriers, some plants exude a variety of chemical substances even before infection of a pathogen through the surface of their above ground parts as well as through the surface of their roots. Fungi-toxic exudates on the leaves of some plants like tomato and sugar beet has been found to inhibit the germination of spores of the fungi *Botrytis* and *Cercospora* respectively. Similarly, in the case of onion smudge caused by the

fungus *Colletotrichum circinans*, resistant varieties were generally found to have red scales containing phenolic compounds, protocatechuic acid and catechol, which could inhibit the growth of conidia (Nicholson and Hammerschmidt, 1992). The fungitoxic exudates were missing in white-scaled susceptible onion varieties. Several phenolic compounds, tannins and some fatty acid-like compounds such as dienes, present in high concentrations in the cells of young fruits, leaves or seeds are potent inhibitors of several hydrolytic enzymes, including proteolytic macerating enzymes of plant pathogens. Several other types of pre-formed compounds such as avenacin, a saponin (a group of phytoanticipins) from oats, exclude fungal pathogens from infecting host, which lack enzymes that breakdown saponin (Osbourn, 1996). Plants also display an innate pathogen specific resistance by producing responses like oxidative burst of the cell, change of cell wall composition that prevent the infection and *de-novo* synthesis of compounds like phytoalexins and pathogenesis related proteins.

There are two types of defence reactions, the hypersensitive reactions of the cell, directly affected by pathogen and other defence reaction that develops more slowly called the normosensitive reaction (Klement, 1982). Infection of resistant plants by a pathogen triggers a complex series of biochemical and cellular events at or near the site of infection, which is often referred to as hypersensitive reaction (HR) (Agrois, 1988). It is characterised by localized cell and tissue death at the site of infection (van Loon, 1988). The so called hypersensitive defence reaction of the cells more or less directly affected by the pathogen, occurs very rapidly with the plant cell's death. The second which is characterized by all other inducible defence reactions develops more slowly (Klement, 1982). The phenomenon of acquired resistance is paralleled with the appearance of a set of proteins collectively known as pathogenesis related proteins (van Loon, 1982). These proteins appear to be of acidic pH values, highly protease resistant and to be excreted to the intercellular fluid (van Loon, 1988). PR proteins p and q have chitinase activity where as PR-2, n and o are β-1,3-glucanases (Kauffmann *et al.*, 1987). These proteins are induced in several plant species upon infection with viruses, viroids, fungi etc. Since

these proteins are induced under specific pathological conditions, they are named as PR proteins. The pre mRNA's of the PR proteins is constitutively present, but not translated in non - stimulated leaves. Thus the expression of PR is controlled at the translational level.

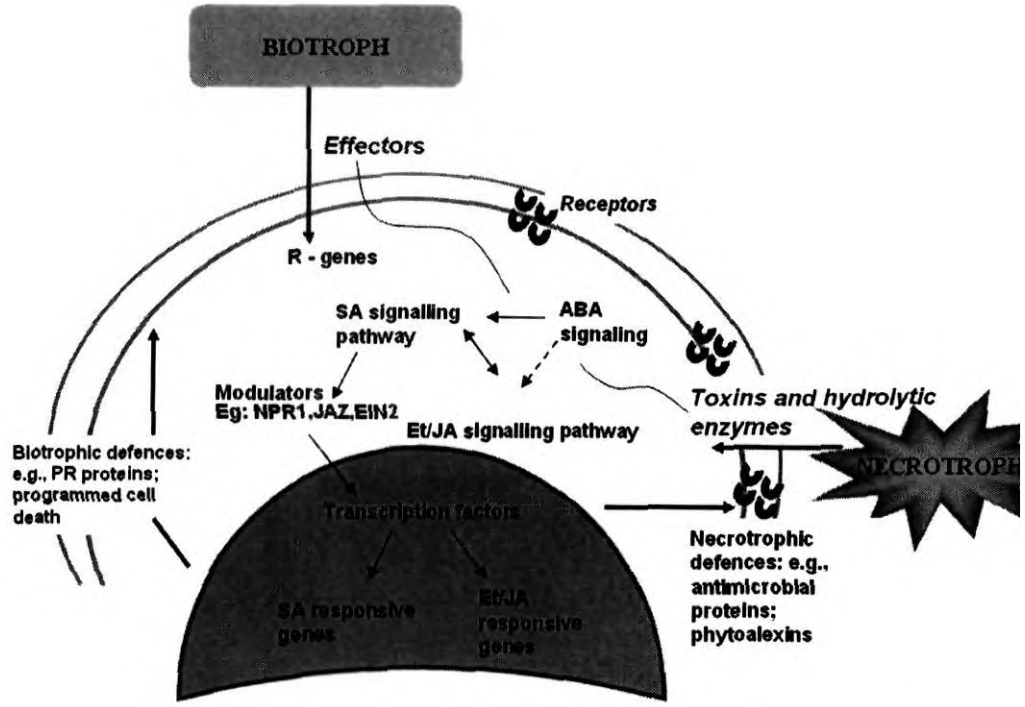


Fig.3. Schematic representation of the defence responses occurring in the host plant upon encountering biotic stress

Antifungal PR proteins are of great biotechnological interest because of their potential use as food and seed preservative agents and for engineering plants for resistance to phytopathogenic fungi (Dempsey *et al.*, 1998). Various studies have revealed that transgenic plants over expressing genes of the PR-1, PR-2, PR-3, and PR-5 families mediate host plant resistance to phytopathogenic fungi. Co-expression of multiple antifungal protein genes in transgenic plants seems to be more effective than expression of single genes (Bormann *et al.*, 1999).

2.4. Systemic acquired resistance

In plants most of the normosensitive reactions are based on the activation of genes coding for enzymes of phenyl propanoid metabolism, eg; phenyl alanine ammonialyase (PAL) and 4-coumarate-Co.A ligase and of

genes coding for enzymes synthesizing subsequent products such as phytoalexins (Mieth *et al.*, 1986; Fritzemeier *et al.*, 1987). This makes the pathogen to confine to the necrotic lesions near to the site of infection. A ring of cells surrounding the necrotic lesions become fully refractory to subsequent infection, referred to as localized acquired resistance (Baker *et al*, 1997; Fritig *et al.*, 1998). These local responses often trigger non-specific resistance throughout the plant. Thus, the whole plant becomes resistant to the challenge of infection by a broad range of pathogens after a previous and local interaction with a pathogen. This long lasting non-specific defence is known as systemic acquired resistance (SAR) (Ryals *et al.*, 1996). These responses can also be induced by exposing the plant to virulent, avirulent, and non-pathogenic microbes or artificially with low molecular weight and sometimes volatile molecules like salicylic acid, jasmonic acid (Delaney *et al.*, 1994; Xu *et al.*, 1994; Wu and Bradford, 2003) and 2,6-dichloro-isonicotinic acid or benzo (1,2,3) thiadiazole-7-carbothioic acid methyl ester (BTH) (Vallad and Goodman, 2004) causing the Systemic Acquired Resistance (SAR) or Induced Systemic Resistance (IAR).

The basic phenomenon in plants which prevent their colonization by pathogens can be assigned to two different defence levels and on each defence level different pathogen species or races may be affected. The first level of defence, the so called basic resistance results from the inability of pathogen to overcome the defence barriers of plants. The second level of defence that may be established in plants is the host resistance. This may be divided into vertical resistance or horizontal resistance (Prell and Day, 2001). Plants have induced cellular defences as well that prevent further colonization of the tissues, once the structural barriers of the host have been breached by the invading pathogen. Induced or active resistance involves substances either absent or present in very low levels before infection, and are produced, or activated during infection.

2.5. Genetics of host-pathogen interaction

There exists highly complex interactions between plants and phytopathogenic fungi. The defence mechanisms exhibited after recognition

of attacking pathogens are the result of a cascade of signal transduction events that affect activation of genes synthesizing proteins or enzymes which are engaged in active defence functions. The genetics of host-pathogen interactions was first studied in detail by Flor (1956) using flax (*Linum usifatissimum*) and flax rust (*Melampsora lini*) as a model system. The conclusion from this study was, "for each gene that confers virulence to the pathogen there is a corresponding gene in the host that confers resistance to the host and vice versa". This hypothesis is popularly known as 'gene-for-gene' hypothesis. Many plant/pathogen interactions are of incompatible interactions. The incompatible interactions (no disease) require a dominant or semi-dominant resistance (*R*) gene in the plant, and a corresponding avirulence (*Avr*) gene in the pathogen. *R* genes are presumed to (*a*) enable plants to detect *Avr*-gene-specified pathogen molecules, (*b*) initiate signal transduction to activate defences, and (*c*) have the capacity to evolve new *R* gene specificities rapidly (Hammond-Kosack and Jones., 1997). Numerous signal transduction components in the defence network have been defined, and several are being exploited as switches by which resistance can be activated against diverse pathogens. The introgression of R-genes into elite cultivars by conventional breeding is a lengthy process. However, recent molecular-level insights into the function of R proteins and downstream signal transduction pathways might provide strategies to remedy these deficiencies (McDowell and Woffenden, 2003). It is presumed that R genes enable plants to detect Avr-gene-specified pathogen molecules. Resistance genes involved in R- Avr interaction have been isolated from many crops and fungus-resistant transgenics are being produced by incorporating the R-genes in susceptible plants within a genus or a family, or even outside the family. Plant breeders apply the gene-for-gene concept every time they incorporate a new resistance gene into a desirable variety that is susceptible to a new strain of pathogen.

2.6. R- genes

The first *R* gene (Resistance gene) isolated was maize *Hm 1* gene (Johal and Briggs, 1992), that makes certain varieties of corn plants resistant to race 1 of *Cochliobolus carbonum* by inactivating the HC toxin produced by the fungus. During the last decade, more than 30 *R* genes, which confer

resistance against a wide range of pathogens including viruses, bacteria, fungi and nematodes have been isolated and cloned, from both monocots and dicots (Takken and Joosten, 2000; Hulbert *et al.*, 2001). Generally, in the host, the genes for resistance (*R* genes) are dominant over genes for susceptibility. In the pathogen, on the other hand, it is the genes for avirulence (*avr* genes) that are usually dominant, while the genes for virulence are recessive. Incompatible plant-pathogen interaction involves the recognition of the pathogen *avr* gene product (elicitor) by the host *R* gene product (receptor molecule). Most of the inducible defence-related genes are regulated by signal pathways involving one or more of the three regulators jasmonate, ethylene or salicylic acid (Delaney *et al.*, 1994; Reymond and Farmer, 1998). During SAR, salicylic acid levels rise throughout the plant. Defence genes such as pathogenesis related (PR) genes are expressed and plant becomes more resistant to pathogen attack. Plants that cannot accumulate salicylic acid due to the presence of a transgene that encodes salicylic acid degrading enzyme do not exhibit the systemic expression of defence genes (Glazebrook, 1999).

2.7. Pathogenesis- related Proteins

Among all induced responses, production of "Pathogenesis Related (PR) proteins" is the most important because they can lead to the increased resistance of the whole plant against a pathogen attack (Adrienne and Barbara, 2006). PR proteins were originally identified in tobacco plants infected by tobacco mosaic virus (van Loon and van Kammen, 1970). Later these proteins were subsequently detected in a wide range of plant species. Although, the term pathogenesis-related (PR) implies that the PR's are expressed in association with resistance responses it does not state that they have functional roles in defence (van Loon *et al.*, 2006). PRs can be induced systemically in non-infected distant leaves also as a result of a primary infection, which indicates a role in contributing to an enhanced level of protection (Ward *et al.*, 1991). The protective role of PRs can also be inferred from their accumulation in plant cell wall appositions formed against pathogen ingress, as well as from their release into fungal structures penetrating plant tissues (Benhamou *et al.*, 1991; Jeun, 2000; Jeun and Buchenauer, 2001).

Most of the identified PR proteins so far are acid-soluble, low molecular weight (6-43 kDa), extractable, stable at low pH (< 3) and protease-resistant proteins (Leubner-Metzger and Meins, 1999; Neuhaus, 1999). These proteins can be either acidic or basic depending on their isoelectric points (pI). The acidic PR proteins are predominantly located in the intercellular spaces. Eventhough the basic PR proteins have similar functions like acidic PR proteins, they are mainly located intracellularly in the vacuole (Legrand *et al.*, 1987; Niki *et al.*, 1998; Van Loon and Van Strien, 1999). PRs are distinguished by specific biochemical properties. The structure of a PR-1 family member (tomato PR1-b) was solved by nuclear magnetic resonance and found to represent a unique molecular architecture. The protein contains four α-helices and four β-strands arranged anti-parallel between helices. The tight packing of the α -helices on both sides of the central β-sheet (α -β-α sandwich structure) results in a compact, bipartite molecular core, which is stabilized by hydrophobic interactions and multiple hydrogen bonds (Fernández *et al.*, 1997). This compact structure probably determines the high stability of PRs and their insensitivity to proteases.

2.8. PR - gene family

Since the discovery of Pathogenesis-Related proteins (PRs) in 1970, 17 PR families have been identified (Table 1) based on amino acid sequences, serological relationship and/or enzymatic or biological activity (van Loon *et al.*, 1994; 2006) from different sources. Within each PR family, there are several classes comprising of different isoforms based on their high (basic) or low (acidic) pI values. The families of the PR proteins are numbered in the order in which they were discovered and new PRs were identified in different species are assigned to the existing recognized families and, if no similarity exists, a new family is created. While most of the families were originally identified from tobacco (dicot) some were identified from other plant species, including monocotyledons such as barley, wheat, rice and maize. More than 13 different pathogenesis related proteins are currently known (Borad and Sriram, 2008).

Table 1: Families of PR proteins

Families	Type member	Properties
PR-1	Tobacco PR-1a	Antifungal
PR-2	Tobacco PR-2	β-1, 3-glucanase
PR-3	Tobacco P, Q	Chitinase type I,II,IV,V,VI,VII
PR-4	Tobacco 'R'	Chitinase type I,II
PR-5	Tobacco S	Thaumatin-like
PR-6	Tomato Inhibitor I	Proteinase-inhibitor
PR-7	Tomato P69	Endoproteinase
PR-8	Cucumber chitinase	Chitinase type III
PR-9	Tobacco'lignin-forming peroxidase'	Peroxidase
PR-10	Parsley 'PR1'	'Ribonuclease-like'
PR-11	Tobacco 'class V'chitinase	Chitinase, type I
PR-12	Radish Rs-AFP3	Defensin
PR-13	Arabidopsis THI2.1	Thionin
PR-14	Barley LTP4	Lipid-transfer protein
PR-15	Barley OxOa (germin)	Oxalate oxidase
PR-16	Barley OxOLP	'Oxalate oxidase-like'
PR-17	Tobacco PRp27	Unknown

PRs from different plant species with the same family designation do not necessarily mean that they are identical proteins. A large number of small, basic and cysteine rich antimicrobial proteins are produced by many organisms throughout all the kingdoms. They display a great variety in their primary structure, species specificity and in their mechanism of action (Leiter *et al.*, 2005).

2.9. β-1,3-glucanases

Plant β-1,3-glucanases (β-1,3-Gs) (EC 3.2.1.39) comprises of large and highly complex gene families involved in pathogen defence as well as a wide range of normal developmental processes (Leubner-Metzger and Meins, 1999). It is also found in bacteria, fungi, algae, and in some invertebrates. They are included in the family of PR-2 proteins, which are abundant highly regulated enzymes widely distributed throughout the plant kingdom. β-1,3-Glucanase genes have been identified in a variety of plants, including tobacco (De Loose *et al.*, 1988), soybean (Takeuchi *et al.*, 1990), rubber tree (Chye *et al.*, 1995), banana (Peumans *et al.*, 2000), rice (Yamaguchi *et al.*, 2002), pea (Mauch *et al.*, 1988), tobacco (Kauffmann *et al.*, 1987), potato (Kombrink *et al.*, 1988), bean (Awade *et al.*, 1989), chickpea (Vogelsang and Barz, 1993), tomato (Joosten and De Wit, 1989), maize (Nasser *et al.*, 1990). *Arachis hypogaea* (peanut), *Cicer arietinum* (chickpea), etc. are having resistivity against various fungi like *Aspergillus parasiticus*, *A. flavs, Blumeria graminis, Colletotrichum lagenarium, Fusarium culmorum, Fusarium oxysporum, Fusarium udum, Macrophomina phaseolina* and *Treptomyces sioyaensis* (Rezzonico, 1998; Wu and Bradford, 2003; Hong and Meng, 2004; Wróbel-Kwiatkowska *et al.*, 2004, Liang *et al.*, 2005; Roy-Barman *et al.*, 2006). Not only different plant species have different β-1,3-glucanase genes, but also a single plant species may have several different copies of β-1,3-glucanase genes. For example, more than fourteen β-1,3-glucanase genes have been found in tobacco plants (Leubner-Metzger and Meins,1999). β-1,3-glucanases have a wide range of molecular mass range from 33 to 44 kDa (Hong and Meng, 2004; Saikia *et al.*, 2005) and isoelectric pH. Most of the basic β-1,3-glucanases are localized in vacuoles of the plant cells while the acidic β-1,3-

glucanases are secreted outside the plant cell. Wounding, hormonal signals like methyl jasmonate and ethylene (Wu and Bradford, 2003), pathogen attack like fungus *Colletotrichum lagenarium* (Ji and Ku, 2002) and some fungal elicitors released from pathogen cell wall (Boller, 1995) can also induce β-1,3-glucanases in the various parts of the plant (Wu and Bradford, 2003; Saikia *et al.*, 2005).

β-Glucanases are basically hydrolytic enzymes capable of causing lysis of cell walls composed of β-1,3-glucan. Glucan endo-1,3-β-D-glucosidase, commonly known as β-1,3-glucanases, are one of the major classes of *O*-glycosidic hydrolases. It also participates in the hydrolytic cleavage of the 1,3-β-D-glucosidic linkages of β-1,3-glucans, a major component of fungal cell wall (Simmons, 1994; Høj and Fincher, 1995), especially the oomycete fungi whose cell wall is typically made up of β-1,3-glucan followed by lysis and cell death. These enzymes are active on insoluble substrates, such as β-glucan components of the fungal cell wall, laminarin and pachyman. They can act both exogenously or endogenously. β-1,3-glucanases have also been reported as a part of plant's general or horizontal resistance, which is more durable, and hence commercially more attractive, compared to the race-specific resistance mediated by the *avr-R* gene interaction that will breakdown rapidly whenever a new race of virulent pathogen evolves.

On the basis of the type of glucosidic linkage cleaved by them, β-glucanases are further classified as β-1,3-glucanase, β-1,6-glucanase and β-1,4-glucanase. They play significant roles in the structural analysis of polysaccharides. There are reports of β-glucanases which have been found in higher plants fungi and bacteria. Six different β-1,3-glucanases have been purified from *Saccharomyces cerevisiae*. Strains of *Bacillus* and *Streptomyces* species are reported to be highly active in producing extracellular β-1,3-glucanases. Non-specific β-1,3-glucanases ie. β-glucosidase and β-1,3-glucanase enzymes have been purified from the fungus *Penicillium ochro-chloron* (Dake *et al.*, 2004). In sea urchins - *Strongylocentrotus purpuratus*, β-1,3-glucanases are reported as part of the embryo hatching enzyme (Bachman

and McClay 1996). Although, the major interest in β-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 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, and responses to wounding, cold, ozone and UV B.

The proteins encoded by different β-1,3-glucanase genes show differences in size, pI, primary structure, cellular localization, pattern of regulation and catalytic activity (Meins *et al.*, 1992). Based on these differences, especially primary amino acid sequence differences, β-1,3-glucanases in the genus *Nicotiana* were divided into four classes (Payne *et al.*, 1990; Ward *et al.*, 1991; Leubner-Metzger and Meins, 1999). These classifications have also been applied to β-1,3-glucanases of other plant species which have similar structural isoforms (Van Kan *et al.*, 1992; Beerhues and Kombrink, 1994; Domingo *et al.*, 1994; Oh and Yang, 1995). According to the classification proposed by Henrissat (1991), β-1,3-glucanases constitute the 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. Since β-1,3-glucanases are able to catalyse endo-type hydrolytic cleavage of the 1,3-β-D-glucosidic linkage in β-1,3-glucans they are also classified as PR-2 proteins under pathogenesis-related protein families.

In *Nicotiana,* the various isoforms have been classified into 4 distinct structural classes. In tobacco, the class I β- glu, are basic pathogen inducible forms. It is produced as a preproprotein 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 pre-protein is transported from the

endoplasmic reticulum *via* golgi compartment to the vacuole where the C-terminal extension is removed to give the mature, 33 kDa enzyme, which is not glycosylated. The C-terminal extension contains a signal for targeting to the vacuole. The class I glucanases are also involved in the normal development of healthy plants during seed germination. It is expressed in young flowers and in seed coat and is weekly expressed in the vegetative tissues during seedling development. The known mature β-1,3-glucanases (βGlu I) of tobacco and Gn2 of *Nicotiana plumbaginifolia* share 98 % amino acid identity. The 35-kDa tomato ßGLU I isoform GluB shares 90 % amino acid sequence identity with tobacco ßGLU I.

In contrast to class I, the class II isoforms are acidic and are secreted into the extracellular space. The tobacco class II isoforms PR-2a, PR-2b and PR-2c are pathogen- induced acidic proteins without C-terminal extension with an apparent size ranging from 34 – 36 kDa (Linthorst *et al.*, 1990). They do not appear to be pathogen-inducible, and hence are referred to as 'PR like' proteins. The pathogen-inducible acidic 35 kDa PR-2d (PR-Q') has been classified as class III β- glu (Payne *et al.*, 1990) in tobacco. They also form the secreted proteins localized in the extracellular spaces. The fourth class is an acidic secreted β-glu that is not pathogen-induced; but is expressed in the anther tapetum, for tetrad callose wall dissolution (Bucciaglia and Smith, 1994).

The acidic and basic β-1,3-glucanases are induced in the inoculated and virus-free leaves of the plant after infection with tobacco mosaic virus. The analysis of the cDNA clones showed that at least five genes for acidic β-1,3-glucanases are induced after tobacco mosaic virus infection. Later the southern blot analysis indicated that the tobacco genome contains approximately eight genes for acidic β-1,3-glucanases and a smaller number of genes encoding basic β-1,3-glucanases. These genes from both gene families were cloned and sequenced. Northern blot hybridization showed that genes encoding acidic and basic glucanases are strongly induced after tobacco mosaic virus infection or salicylate treatment of tobacco.

Table 2: Classes of β-1,3-glucanases identified from Tobacco

Class	Member name	Trivial name	Origin	MW (kDa)	pI	Localization
I	PR-2e	Glb	Nt (T)	33	Basic	Vacuole
I	PR-2e	Gla	Nt (S)	33	Basic	Vacuole
I	PR-2e	Gglb50	Nt (S)		Basic	Vacuole
I	PR-2e	Gln2	Nt (S)		Basic	Vacuole
I		Gn2 (d)	Np		Basic	Vacuole
I		Gn1 (d)	Np	34	Basic	Vacuole
II	PR-2a	PR-2 (I, b4)	Nt	35	Acidic	Secreted
II	PR-2b	PR-N (b5), G19	Nt	35	Acidic	Secreted
II	PR-2c	PR-O (b6(b), O')	Nt	35	Acidic	Secreted
II		PR-2d	Nt		Acidic	Secreted
II	Stylar ßGLU (e)	Sp41a	Nt	41	Acidic	Secreted
II	Stylar ßGLU (e)	Sp41b	Nt	41	Acidic	Secreted
III	PR-2d	PR-Q'	Nt	35	Acidic	Secreted
IV (f)	anther ßGLU (e)	Tag1	Nt	35	Acidic	Secreted

The cloning and analysis of these genes marked the first step towards the identification of regulatory elements involved in their coordinate induction. Though most basic glucanases are found to have a C-terminal extension which aids in the translocation of the protein, peach glucanases are

reported to have no C- terminal extension (Thimmapuram *et al.*, 2001). Table 2. shows the major classes of β-1,3-glucanases in tobacco.

2.9.1. β-1,3-glucanase isozymes

In plants, βGlu exists as multiple structural isoforms. Most, but not all of them were proved as effective anti-fungal agents both *in vitro* and when over-expressed in transgenic plants. The most detailed sequence information for these isoforms is available from cDNA and genomic clones of tobacco βGlu, since these were the first characterized ones from the tobacco plants. It forms a multigene family with considerable complexity in higher plants (Jin *et al.*, 1999). The structural isoforms have been reported from potato, tomato and many other plant species (Thimmapuram *et al.*, 2001).

Further, the β-1,3-glucanase isozyme patterns in infected plants are usually different from uninfected plants. Most studies showed that the quantity of isozymes (based on band intensity on native polyacrylamide gels) increased after infection with pathogens (Pan *et al.*, 1991; Churngchow *et al.*, 1995; Roulin *et al.*, 1997; Urquhart and Punja, 2002; Shinya *et al.*, 2004). In some cases, new isozymes appear in infected plants. For example, a study of tobacco plants showed that normal tobacco leaves contained one major β- 1, 3-glucanase isozyme, but an additional isozyme was detected in the tobacco mosaic virus-infected leaves. Further, the intensities of both isozyme bands were considerably greater than those of the uninfected tobacco leaves (Pan *et al.*, 1991). A study on ground nut showed that three isozymes of β-1,3-glucanase existed in the plant and that the activities of these isozymes increased after infection by *Cercospora arachidicola* (Roulin and Buchala, 1995). In the case of barley (*Hordeum vulgare* L.), the predominant isoforms in the normal leaves were the isozyme GI and to a lesser extent, the isozyme GIII. After infection with *Rhynchosporium secalis*, GI remained approximately constant, while the level of GIII increased. However, the most noticeable response to infection was the induction of a high level of the isozyme GII (Roulin *et al.*, 1997). Changes in isozyme patterns were also observed when plants were treated with other stimuli. For example, a study of

sugar beet plants showed that BTH induced synthesis of at least three acidic β-1,3-glucanase isozymes and a basic isozyme (Burketova *et al.*, 1999).

2.9.2. Gene structure of β-1,3-glucanases

The exon - intron architecture of the β-1,3-glucanse genes identified so far, perhaps with two exceptions, contain only a single intron. The intron shows a positional conservation in all these genes whether they encode an acidic or a basic enzyme (Shi, 2005). Usually the first exon of these genes encode major portion of the signal peptide that target these proteins to their corresponding locations (Von Heijne, 1983). The length of the intron in different β-1,3-glucanase genes can vary. The introns usually range in size from 400-600 base pairs, but some β-1,3-glucanase genes have shorter or longer introns. The curled-leaved tobacco β-1,3-glucanase gene has a 975-bp intron (Gheysen *et al.*, 1990). The introns in the three peach glucanases contained 160, 256 and 677 bp nucleotides each (Thimmapuram *et al.*, 2001; Ko *et al.*, 2003). The gDNA sequence of β-1,3-glucanases isolated earlier from *Hevea* clone RRII 105 also showed the presence of a single intron with 133 nucleotides. The introns contained 'CT' repeats also. The sequence also showed the presence of a signal peptide with a signal peptidase cleavage site between the 36^{th} and the 37^{th} nucleotide (Thanseem *et al.*, 2003). The β-1,3-glucanase gene (gn1) isolated from *Nicotiana plumbaginifolia* encoding 370 aminoacids contained two introns (Castresana *et al.*, 1990). Like the β-1,3-glucanase gene with one intron from the other reports, the first exon of gn1 encodes part (25 amino acid residues) of a signal peptide (30 amino acid residues). The first intron is 669 bp in length. The second intron, which is 199 bp in length, divides the rest of the gene into two more exons of 273 bp and 952 bp. Though the presence of intronless form of β-1,3-glucanases in *Hevea* has not yet been identified, the presence of glucanases without intron within the gene sequence has been identified in other crops like rice and barley. In barley, gene encoding the β-1,3-glucanase isozyme GI has no intron and this gene does not encode a signal peptide (Burton *et al.*, 1998). Since no vacuolar or endoplasmic reticulum targeting signals or any other obvious sequence motifs are needed for intracellular transport, GI has been shown to have

intracellular localization in barley leaves. In addition to GI, the barley β-1,3-glucanase isozyme GV (Xu and Fincher, 1992) and a rice β-1,3-glucanase (OsGLN1) (Akiyama and Pillai, 2001) are believed to have no signal peptide based on cDNA sequences of these two isozymes. It can be assumed that these two β-1,3-glucanase isozymes, like barley GI isozyme, might also be derived from an intronless gene ancestor. Two genomic clones, *FaBG2-1* and *FaBG2-2*, and a cDNA clone, *FaBG2-3*, encoding three different β-1,3-glucanases, were isolated from strawberry (Shi, 2005). *FaBG2-1* was comprised of two exons and one intron. The first exon of *FaBG2-1* encodes the major part of a signal peptide. *FaBG2-2* appears to be an intronless gene and does not encode a signal peptide. *FaBG2-3*, like *FaBG2-1*, also encodes a signal peptide, but is different from *FaBG2-1* in 3′ and 5′ non-coding regions.

2.9.3. Regulation of β-1,3-glucanase gene expression

β-1,3-glucanases show developmental regulation in response to treatment with hormones or infection with pathogens. In tobacco it has been identified that the pattern of β-1,3-glucanase (bGlu1) protein expression and the occurrence of steady state mRNA level in leaves and roots is very similar correlating it with the promoter activity of approximately 1.5-1.7 kb 5' flanking region (van de Rhee *et al.*, 1993; Vögeli- Lange *et al.*, 1994b). A number of various cis-acting regulatory elements are found to be involved in the expression of β-1,3-glucanase genes. For example, a TCA-like motif (TCATCTTCTT), known to bind a nuclear protein induced by SA, has been found in the upstream regions of tobacco (Goldsbrough *et al.*, 1993) and peach β-1,3-glucanse genes (Ko *et al.*, 2003). A highly conserved AGC element (AGCCGCC) has been found in the promoter regions of many β-1,3-glucanase and chitinase genes, and is known to be an ethylene-responsive element (Hart *et al.*, 1993; Ohme-Takagi and Shinshi, 1995, Ko *et al.*, 2003). Studies of a tobacco β-1, 3-glucanase gene showed that enhancer activity and ethylene-responsiveness also depend on the presence of a GCC box (GCCGCC) (Grimmig *et al.*, 2003; Brown *et al.*, 2003). The homologous 1.6 kb tobacco β-1,3-glucanase (Glb) promoter confers proper regulation to reporter gene expression in leaves, roots, seeds and cultured cells (Vögeli-Lange *et al.*, 1994b; Leubner-Metzger *et al.*, 1995; Rezzonico *et al.*, 1998). The detailed

analysis of the promoter also indicated that the distal -1193 to -1452 region is required for high levels of expression in leaves and also for responsiveness to ethylene and TMV infection (Vögeli-lange *et al.*, 1994b). A W-box sequence (TTGAC) also present in promoters of some β-1,3-glucanase genes, is recognized specifically by SA-induced WRKY DNA binding proteins (Yu *et al.*, 2001). Schematic representation of cis-elements present in the pathogen responsive glucanase gene in *Petroselinium crispum* and *N. tabacum* is shown in fig. 4.

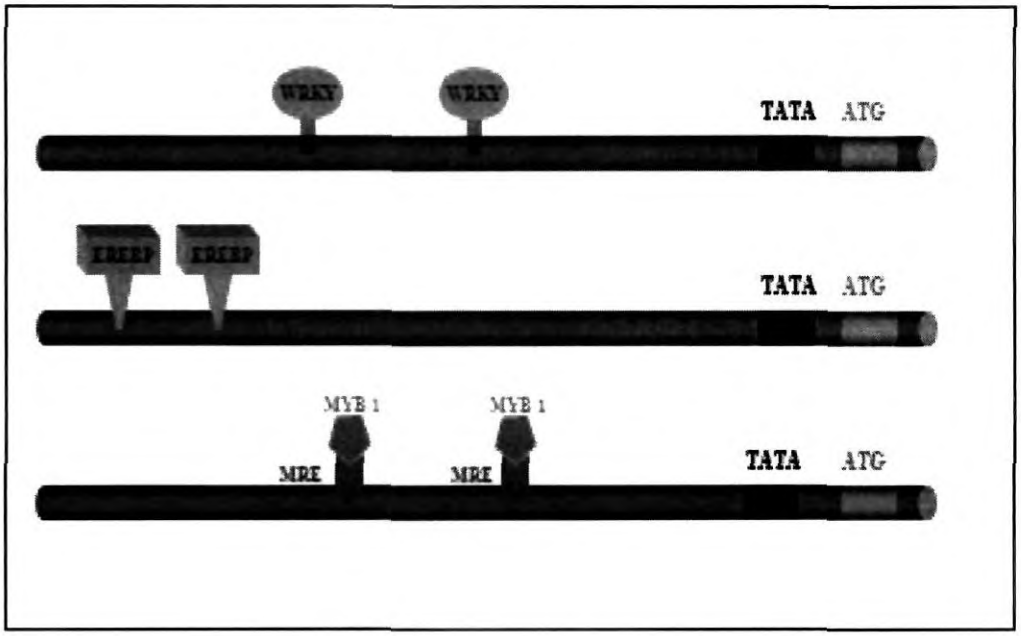


Fig. 4. Schematic representation of pathogen responsive *cis*-acting elements

(W-, P-, L-, G-, H-boxes, GCC-MRE-elements) and their cognate interacting factors (WRKY, , MYB,, EREBP). The promoter regions of the Petroselinum crispum PR1-1 (PcPR1-1) and the Nicotiana tabacum glucanase 2 (NtGln2), and PR1 (NtPR-1a) genes are shown. The relative positions of the interacting partners in relation to their respective TATA-boxes and the translation start sites (ATG) are given (ot drawn to scale).

Other cis-acting regulatory elements necessary for response to different stimuli have been found in the 5′ upstream region of some plant PR protein genes. For example, an ASF-1 binding site (TGACG) is present in promoters of some PR protein genes involved in transcriptional activation by SA (Despres *et al.*, 2003). In tobacco the hormones auxin and cytotkinin were found to have a negative regulation of the β-1,3-glucanase *(Glb)* gene

promoter while abscisic acid (ABA) at a concentration of 10 μM markedly inhibited the induction of β-1,3-glucanases but not of chitinases (Rezzonico *et al.*, 1998; Wu *et al.*, 2001).

Gus-reporter gene experiments with tobacco βGlu1, Gglb 50 and Glb promoters have shown that βGlu1 is transcriptionally induced upto about 10-fold in TMV infected leaves showing hypersensitive responses (Livne *et al.*, 1997). The promoters of the tobacco class II *PR-2b* and *PR-2d* are induced both locally around the necrotic lesions on TMV infected tobacco and systemically in non- infected leaves (Henning *et al.*, 1993). Since multiple regions present in the 1.7 kb tobacco of both promoters contained elements for the inducibility by SA and TMV. In the PR-2d gene, the portion includes a major cis-acting element in the region -364 to -288, conferring high- level expression to a core CaMV 35S promoter (Shah *et al.*, 1997). Linthorst *et al.*, (1990), found that healthy tobacco plants accumulate β-1,3- glucanases in their roots and in specific parts of the flowers showing the tissue specific regulation.

Multiple signal transduction pathways in tobacco and *Arabidopsis* and the cross-talk between these pathways seem to be utilized to signal the induction of different subsets of the PR genes in response to different pathogens. Studies on *Arabidopsis thaliana* suggested that the signaling events initiated by diverse pathogens are converged into a conserved mitogen 3 activated protein kinase (MAPK) cascade. The MAPK cascade in-turn leads to expression of WRKY proteins, transcription factors (Du and Chen, 2000) which bind to the W box elements (containing a TGAC core sequence) present in the promoters of many defence related genes, inducing their expression (Eulgem *et al.*, 2000; Du and Chen, 2000). Defence responses activated by the MAPK cascade are effective against both fungal and bacterial pathogens (Asai *et al.*, 2002). Most, although not all, plant promoters possess a TATA box and/or an initiator element either or both of which may be recognized by the TATA binding protein (TBP) in a complex with TBP associated factors (TAFs). Once the TBP/TAFs have bound, other components of the initiation complex assemble: TFIIA, TFIIB, RNApolymeraseII (polII) and TFIIF, TFIIE

and TFIIH. The spike on the RNA polymerase represents its C-terminal tail, which must be phosphorylated for transcription to commence. Diagrammatic representation of transcriptional initiation of a gene is shown in fig. 5.

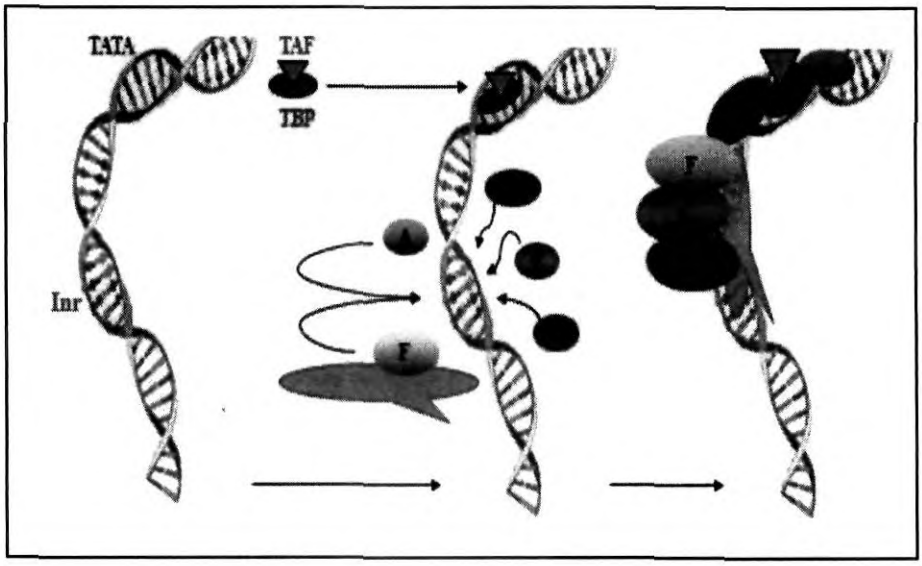


Fig. 5. An overview of transcriptional initiation

2.9.4. β-1,3-glucanases and their Importance in plants

There are considerable evidences to suggest that β-1,3-glucanase enzymes play an important part of both the constitutive and induced defence against many pathogenic fungi (Mauch *et al.*, 1988b; Mauch and Staehelin, 1989; Keefe *et al.*, 1990; Zhu *et al.*, 1994b; Jongedijk *et al.*, 1995). These enzymes are thought to act directly by digesting β-13-glucans in fungal cell walls especially in the oomycete fungi whose cell wall predominantly contains as well as indirectly by digesting fungal and host polysaccharides to produce elicitors capable of evoking the hyper sensitive (HR) responses (Ebel and Scheel, 1992). Mutants deficient in these enzymes generated by antisense transformation showed markedly reduced lesion size, lesion number, and virus yield in the local-lesion response of Havana 425 tobacco to tobacco mosaic virus (TMV) and of *Nicotiana sylvestris* to tobacco necrosis virus indicating decreased susceptibility (Beffa *et al.*, 1996). These mutants also showed decreased severity of mosaic disease symptoms, delayed spread of symptoms, and reduced yield of virus in the susceptible response of *N. sylvestris* to TMV. The isolation of a potato (*Solanum tuberosum L. cv Huinkul*) acidic 39 kDa β-

1,3-glucanase (GLU-39) with antifungal activity against *Phytophthora infestans* and analysis of its expression in potato cultivars showed a very high level of field resistance after 72 h of infection with *Phytophthora infestans* when β -1,3-glucanase activity was markedly increased (Tonon *et al.*, 2002). Nazeem *et al.*, (2008) reported that the resistant genotype *Piper colubrinum*, possessed higher enzyme activities than with the *P. nigrum* varieties towards the foot rot disease caused by *P. capsici*. The study also confirmed the role of β-1,3-glucanase and related enzymes in the defence mechanism of black pepper against foot rot disease.

2.9.4.1. Role in developmental processes

Though β-1,3- glucanase plays a significant role in the response of plants to microbial pathogens, there is compelling evidence that these enzymes are also implicated in diverse physiological and developmental processes in the uninfected plants including cell division (Waterkeyn *et al.*, 1967; Fulcher *et al.*, 1976), microsporogenesis (Worral *et al.*, 1992; Bucciaglia and Smith., 1994), pollen germination and tube growth (Roggen and Stanley, 1969; Meikle *et al.*, 1991), fertilization (Lotan *et al.*, 1989; Ori *et al.*, 1990), embryogenesis (Dong and Dunstan., 1997; Helleboid *et al.*, 1998), fruit ripening (Hinton and Pressey., 1980), seed germination (Vögeli- Lange *et al.*, 1994a; Leubner-Metzger *et al.*, 1995), mobilization of storage reserves in the endosperm of cereal grains (Fincher and Stone., 1993), bud dormancy (Krabel *et al.*, 1993), and responses to wounding, cold, ozone and UV-B (Thalmair *et al.*, 1996; Linthorst, 1991). These diverse physiological functions might force these enzymes to occur as multiple structural isoforms that differ in their size, iso-electric point, primary structure, cellular localization and pattern of regulation (Meins *et al.*, 1992).

In higher plants, at a critical stage of microspore development, there is a large increase in the β-glu activity that results in the degradation of callose wall composed of β-1,3 glucan surrounding the tetrads of haploid microspores, The anther specific β-1,3-glucanases cause the release of microspore into the anther locule, where they develop into mature pollen grains (Steiglitz, 1977).

Further evidence for the role of β-glu in callose wall dissolution and microsporogenesis has come from the sense transformation experiments. Transgenic tobacco plants over-expressing a vacuolar β-glu under the control of tapetum- specific promoters in the tapetum tissues exhibited premature degradation of callose, production of abnormal microspores and partial to complete male sterility (Worrall *et al.*, 1992).

In 1990, Ori *et al.* identified a 41 kDa tobacco β-glucanase (acidic glyco protein) called *Sp41* which was found principally in the stylar transmitting tissue of the tobacco flower, important for guiding pollen tube towards the ovules for successful fertilization. The same protein was neither found to be expressed in leaf, fruit, sepal, petal, anther, pollen sac, and ovary nor induced by the biotic/ abiotic elicitors in the leaves and styles. The reporter- gene experiments showed that 2.5 Kb 5'- non-coding region of *Sp41* gene is needed to confer the stylar transmitting- tissue specific expression.

β-Glu weakens the endosperm by digestion of cell-wall material and this promotes the radical protrusion to facilitate the seed germination (Datta and Muthukrishnan, 1999) The first hint that β-glu might play a role in regulation of germination came from the observation by Leubner-Metzger *et al.*, in 1995. These endosperm glucanases were found to get induced exclusively in the micropylar region of the endosperm where the radicle will penetrate. Class I β-glu mRNA and enzyme activity were also observed in the micropylar endosperm of tomato seeds prior to radicle protrusion (Wu *et al.*, 2000). Levy *et al.*, (2007) identified the first β-1,3-glucanase *Arabidopsis* enzyme that is associated to the macromolecular Pd complex, termed AtBG_pap which form a plasmodesmatal gate keeper for the intercellular communication.

2.9.4.2. Defence-related functions of β-1,3-glucanases

β-glucanases were proposed to be involved in plant defence as early as 1971 (Abeles *et al.*, 1971). Synchronously, the PR proteins were first

described as a novel set of abundant proteins accumulated in leaves of resistant tobacco cultivars reacting with HR to TMV infection (Van Loon and Van Kammen, 1970). β-glucanases were later included as PR-2 family of proteins. There is abundant evidence that it is involved in the defence mechanism of plants against the pathogen infection (Simmons, 1994). 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 release of cell wall derived materials that can act as elicitors of defence reaction (Boller, 1993). 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. It was observed that, β-glu expression is induced upon pathogen attack, 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. The location of β-1,3-glucans in fungi is shown in fig.6.

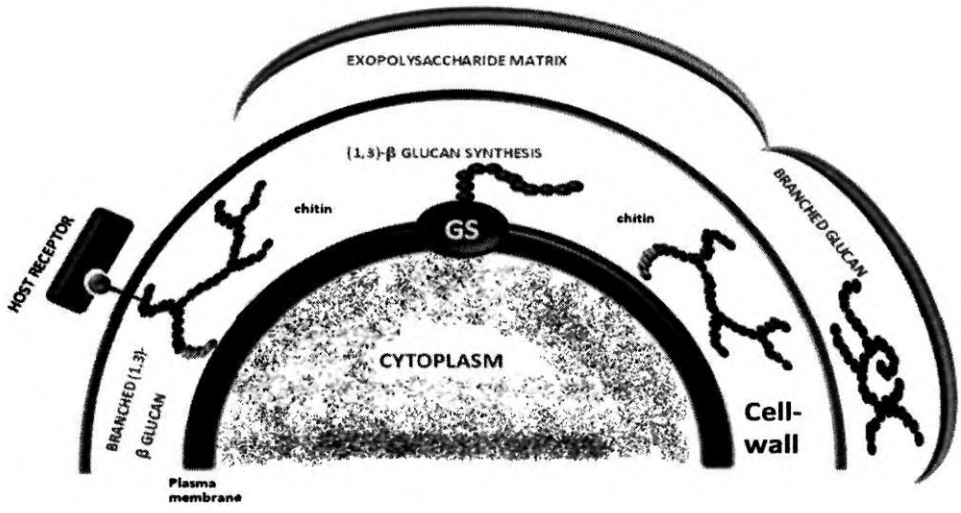


Fig. 6. Location of (1,3)-β-glucans in fungi

Linear (1,3)-β-glucans are synthesized at the apex and are re-modelled and cross- linked to other polysaccharides in the sub-apical regions.

Induction of β-1,3-glucanases by pathogens also depends on the host plants. The different clones of *Hevea brasiliensis* showed considerable variability during pathogenesis when the production of a β-1,3-glucanase upon infection with *Corynespora cassiicola* was compared. Increased enzyme activity was found in the tolerant clone, while a decrease was observed in the susceptible clone (Philip *et al.*, 2001). There are many other examples which show that the expression levels of these enzymes increased after infection with pathogens, such as barley infected by powdery mildew (Ignatius and Chopra, 1994), maize infected with *Aspergillus flavus* (Lozovaya *et al.*, 1998), soybean infected with *Pseudomonas syringae* (Cheong *et al.*, 2000), pepper infected with *Xanthomonas campestris* pv. *vesicatoria* and *Phytophthora capsici* (Jung and Hwang, 2000), wheat infected with *Fusarium graminearum* (Li *et al.*, 2001), chickpea infected with *Ascochyta rabiei* (Pass.) *Labr.* (Hanselle and Barz, 2001), and peach infected with *Monilinia fructicola* (Zemanek *et al.*, 2002).

2.9.5. Over-expression of β-1, 3-glucanase in response to pathogen attack in transgenic plants

Since many years, the over-expression of PR proteins in transgenic plants has been practiced as a defence strategy against pathogens. The most desirable and effective strategy for disease resistance in plants is to incorporate the disease resistant genes into the commercially acceptable cultivars. Agronomically-viable levels of durable resistance in crop plants against a relatively broad range of pathogens could be achieved by biotechnological and molecular approaches. This includes the expression of fungal avirulence genes to provoke non-specific, hypersensitive resistance when combined with the corresponding plant resistance genes, expression of genes involved in the synthesis of phytoalexins toxic to fungi, and expression of genes encoding inhibitors of fungal enzymes or known antifungal proteins. These transgenic approaches have been tried in several advanced laboratories all over the world as alternative approach to conventional crop improvement programmes. Evidences suggest that expression of anti-fungal β-glucanase transgenes alone or in combination with chitinase transgenes could reduce the

susceptibility of plants to infection by certain fungi. Broglie *et al.* (1991) constitutively expressed bean chitinase gene in tobacco and *Brassica napus*; and the plants showed enhanced resistance to *Rhizoctonia solani.* This was the first report on the success of such an approach in developing fungus resistant transgenic plants. In recent years, a resistance gene effective against all known strains of blight has been identified and successfully copied from a wild relative of the potato, *Solanum bulbocastanum*, and introduced into the genome of cultivated varieties of the potato. This is also an example of cis-genic genetic engineering (Song *et al.*, 2003). Earlier studies shows that the genes *Pto* and *Xa21* conferred resistance to the fungal and bacterial pathogens in tomato and rice (Martin *et al.*, 1993; Song *et al.*, 1995). But for most times the resistance conferred by these genes is specific to particular host and in some cases to a limited assortment of pathovars. The potential initial candidates for single gene defence mechanism approach are the ones coding chitinases and β-1,3- glucanases because of its ability to hydrolyse the β-1,3-glucan and chitin in the fungal cell walls respectively. In response to infection by tobacco mosaic virus was examined in tobacco messages for the acidic glucanases were induced similarly to the mRNAs for other PR proteins, while basic glucanase showed a different response suggesting differential regulation of the various isoforms in tobacco at the mRNA level (Ward *et al.*, 1991).

β-1,3-glucanase genes, alone or together with chitinase genes or other defence related genes, have been transferred to a number of plant species and expressed constitutively in these plants. The defence involving these genes are particularly interesting because unlike many other secondary metabolites, which are complex in type and expression, and often toxic to pathogens and humans alike, enzymes such as β-glu are thought to be non- toxic and are produced by relatively direct gene-to-enzyme pathways. Since these active anti-microbial agents are individually encoded by single genes, it is easier to manipulate these enzymes to bio-engineering plants towards improved resistance through manipulation of these enzyme expression systems. The *Gns I* gene of rice, which encodes 1,3;1,4-β-glucanase was over-expressed in transgenic rice under the control of CaMV 35S promoter (Nishizawa *et al.*,

2003). When these transgenic plants were inoculated with virulent blast fungus (*Magnaporthe grisea*), they developed many resistant type lesions on the inoculated leaf accompanied by an earlier activation of defence-related genes. Chen *et al.*, (2006) identified that the over- expression of a tobacco β-1,3-glucanase gene along with an alfalfa defensin gene resulted in enhanced resistance in tomato against *Ralstonia solanacearum*. Jach *et al.* (1995) observed that transgenic tobacco plants individually over-expressing barley chitin, class II β-glu and type I ribosome inactivating protein resulted in increased protection against *R. solani*. The co-expression of all the transgenes together significantly enhanced protection against fungal attack. Sela-Buurlarge *et al.* (1993) transformed tobacco plants with transgenes encoding modified class I tobacco β-glucanase and chitinase. They found that the extracellular wash fluids from the leaves of plants expressing β-glucanase and chitinase showed strong anti-fungal activity against *Fusarium solani*, whereas this effect was less for plants expressing either transgene alone. The constitutive expression of an inducible β-1,3-glucanase reduces the disease severity caused by the oomycete pathogen *P. megasperma f.sp medicaginis*, but does not reduced the disease severity of the chitin containing fungi (Masoud *et al.*, 1996). In most cases, the resulting transgenic plants exhibit enhanced levels of fungal disease resistance or delayed symptom development as compared to the control plants (Zhu *et al.*, 1994b; Jach *et al.*, 1995; Jongedijk *et al.*, 1995). In some cases, the expression of β-1,3-glucanase transgenes alone can reduce the susceptibility of the plants to infection by certain fungi. The transgenic tobacco plants that expressed a soybean β-1,3-glucanase showed reduced symptoms when infected with *Alternaria alternata* (Yoshikawa *et al.*, 1993). Soybean β-glucanase was also used to transform kiwifruit, and some of the transformants showed increased resistance towards *B. cinerea* which causes the grey mold disease (Nakamura *et al.*, 1999).

Transgenic tobacco plants that expressed a tobacco class II β-1, 3-glucanase showed reduced symptoms when infected with *Phytophthora parasitica* var *nicotinae* or with *Peronospora tabacina* (Lusso and Kuc, 1996). Potato plants transformed with soybean β-glu were found to have increased

resistance against *P. infestans* (Borkowska *et al.*, 1998). Ito *et al.* (1995) transformed egg plants with soybean β-glu and found that the transgenic plants showed a statistically significant degree of disease resistance to *P. capcici*. Transgenic rice plants that expressed a β-1,3; 1,4-glucanase gene *Gns1* showed less severe symptoms comparing to the control plants when infected with virulent blast fungus *Magnaporthe grise*a (Nishizawa *et al.*, 2003). *Brassica napus* plants transformed with β-glu and chn genes showed increased resistance to *Sclerotinia sclerotiorium* (Lan *et al.*, 2000). Anand *et al.*, (2003) developed a transgenic wheat line, which showed stable inheritance and co-expression of β-glucanase and chitinase isolated from the cDNA library of *F. gramineorum*-infected wheat spikes of scab resistant cultivar 'Sumai 3', showed a delay in the spread of the infection under green house conditions against *F. gramineorum.* Other studies showed that plants transformed with β-1,3-glucanase alone did not exhibit resistance to certain pathogens or showed less resistance compared to plants which were transformed with both the β-1,3-glucanase and chitinase genes. Similarly, plants transformed with chitinase gene alone also did not show an adequate level of resistance, because plant chitinases alone usually affect only the hyphal tip and are unable to effectively degrade harder chitin structures of fungi. In addition, like β-1, 3-glucanase, chitinases inhibit only a limited number of fungal species. However, when the two enzymes are combined, a synergic effect can usually be observed. For example, tobacco plants transformed with a barley class II basic chitinase along with a barley class II basic β-1, 3-glucanase gene showed enhanced levels of protection against *Rhizoctonia solani* as compared to plants transformed with a single gene (Jach *et al.*, 1995). Potato plants transformed with soybean β-glu were found to have increased resistance against *P. infestans* (Borkowska *et al.*, 1998). Tomato plants expressing tobacco class I β-1, 3-glucanase and chitinase transgenes showed reduced susceptibility to infection by *Fusarium oxysporum* f.sp. *lycopersici* (Jongedijk *et al.*, 1995). Other examples of transgenic plants which expressed both types of genes and exhibited strong defence effects are tobacco plants against *Rhizoctonia solani* (Broglie *et al.*, 1991), *Fusarium solani* (Sela-Buurlage *et al.*, 1993), and *Cercospora nicotianae* (Zhu *et al.*, 1994b), and Alfalfa plants against *Phytophthora megasperma* f. sp

medicaginis (Masoud *et al.*, 1996). Over-expression of an elicitor releasing β-1,3-glucanase from soybean in transgenic tobacco plants was shown to result in enhanced resistance to *Alternaria alternate* and the oomycete, *P. parasitica var nicotianae* (Yoshikawa *et al.*, 1993).

2.10. Glucanase inhibitor proteins (GIPs)

During invasion of their plant hosts, species of the oomycete genus *Phytophthora* secrete glucanase inhibitor proteins (GIPs) into the plant apoplast, which bind and inhibit the activity of plant extracellular endo-β-1,3-glucanases (EGases) (Damasceno *et al.*, 2008). The first cloning and sequencing of GIPs (Rose *et al.*, 2002) from the soybean pathogen *P. sojae* revealed homology to serine proteases (SP) and, specifically, to the chymotrypsin (S1) subclass.

2.11. Pathogen- inducible elicitors

Many studies show that plant β-1,3-glucanases can be induced by pathogen elicitors alone without the presence of a pathogen. For example, when cultured parsley cells (*Petroselinum crispum*) were treated with heat-released soluble cell-wall fragments (elicitors) from fungi, β-1,3-glucanase activity increased strongly in elicitor-treated cells (Kombrink and Hahlbrock, 1986). Another study showed that a *Phytophthora infestans* derived elicitor can induce β-1,3-glucanase activities in potato leaves (Kombrink *et al.*, 1988). Similar results were also found with pea β-1,3-glucanases which can be induced by the fungal pathogen, *Fusarium solani f. sp. phaseoli* and by autoclaved spores of the pathogen (Mauch *et al.*, 1984).

Pathogen elicitors usually have receptor molecules at the host cell surface. When a particular receptor molecule in plant binds with the pathogen elicitor, it is assumed that the plant recognizes the pathogen. The newly emerged tools of molecular biology refines this model and leads to the hypothesis that a ligand produced by the pathogen interacts, presumably directly, with a corresponding plant receptor, which triggers the activation of defence responses. The β-1,3- glucanases digest cell walls of penetrating

pathogens thereby liberating glucan fragments which then act as exogenous elicitors for triggering plant defence reactions (Prell and Day, 2001). All these evidences suggest an active role for β-1,3-glucanases as an antifungal agent that confer the plant several lines of defence against the invading microorganisms. One of the examples of defence response induced by an elicitor is the generation of the oligoglucoside, β-1,3-β-1,6-heptaglucoside, from the pathogenic oomycete *Phytophthora megasperma* f. sp. *Glycinea* by soybean class III β-1, 3-glucanases. This elicitor then induces accumulation of the phytoalexin glyceollin (Sharp *et al.*, 1984). The protein that binds this elicitor is called β-glucan elicitor binding protein (GEBP) and is located in the plasma membranes of soybean root cells. This protein may be a receptor of the elicitor, because the binding affinities of the oligoglucoside for the binding protein correlate well with its abilities to induce phytoalexin accumulation in the soybean cotyledon tissue (Cheong *et al.*, 1993).

2.11.1. Elicitor induced expression of β-1,3-glucanase gene

In addition to induction by pathogens, β-glu and other PR or stress related proteins are also expressed in response to numerous varied agents called elicitors, which may be either biotic or abiotic in origin. Molecules derived from pathogens that can induce stress related responses in plants are called biotic elicitors. They often are simply crude extracts from pathogens. For example, pea pod β-glu expression was induced by the autoclaved spore extracts of the pathogenic fungus *F. solani* (Mauch *et al.*, 1989). Parsley β-glu is expressed in response to elicitors from phytopathogenic fungi (Kombrink and Hahlbrock, 1986). Two β-glu were induced in potato upon treatment with a fungal elicitor. In barley leaves, there was an enhanced expression of β-glu in response to elicitor derived from *R. secalis*, the pathogen responsible for barley leaf scald (Mascerenhas *et al.*, 1990). A specific factor in the crude elicitor preparation, such as proteins, lipids or carbohydrates is actually responsible for inducing the PR gene expression. An indirect defensive role for β-glu was suggested by the observation that β-1,3/ 1,6–glucan oligosaccharides termed oligosaccharide elicitors or oligosaccharins, which are released from pathogen walls by the action of host glucanases can induce

wide range of plant defence responses (Ebel and Cosio, 1994). This was well documented for interactions between soybean and the β-glucan elicitor from the pathogenic oomycete, *P. megasperma*. Yoshikawa *et al.* (1981) found that soybean tissues contain a factor capable of releasing soluble and highly active carbohydrate elicitors from mycelial walls of *P. megasperma* .sp.*glycinea*. This factor was shown to be β-1,3-endoglucanase, which is constitutively present at low levels in soybean tissues (Takeuchi *et al.*, 1990). β-1,6 linked glucans of various chain lengths with frequent β-1,3 linked glucan side chains are constituents of the fungal cell wall. Upon contact of the fungal cells with host tissues during the infection process, the β-1,3-linked glucan side chains are presumed to be attacked by the host β-glu resulting in the release of elicitors. The smallest β-glucan released with elicitor activity was a β-1,3-β-1,6-heptaglucoside and structural requirements for the elicitor activity of these oligosccharides have been investigated (Okinaka *et al.*, 1995).

2.12. *In vitro* Studies

The ability of β-1,3-glucanases and chitinases alone or in combination to co-operatively degrade and kill fungi *in vitro*, indicates their role in destroying pathogenic fungi, whose cell wall is often made up of chitin and β-glucan. These enzymes destroy the fungi by degrading chitin and β-glucan in the cell wall present at the hyphal tip which ultimately leads to its thinning and bursting (Arlorio *et al.*, 1992). Several class I β-1, 3-glucanases from tobacco have been shown to inhibit the growth of *Fusarium solani in vitro* (Sela-Buurlage *et al.*, 1993). In *Hevea* it was shown that the basic glucanase Gln1 inhibited the mycelial proliferation of *Phytophthora meadii* causing ALF *in vitro* (Thanseem *et al.*, 2005b). Though β-glu and chitinase (chn) are induced in pathogen-infected tissues, they are usually highly and constitutively expressed in roots and lower stems as these plant parts are continuously exposed to the soil microflora including the plant pathogens. The β-1,3-glucanases are coordinately expressed along with chitinases after fungal infection. This co-induction of the two hydrolytic enzymes has been described in many plant species, including pea, bean, tomato, tobacco, maize, soybean,

potato, and wheat (Mauch *et al.*, 1988a, b; Vogelsang and Barz 1993; Jach *et al.*, 1995; Bettini *et al.*, 1998; Lambais and Mehdy, 1998; Petruzzelli *et al.*, 1999, Cheong *et al.*, 2000, Li *et al.*, 2001). However, in the case of oomycetes like *Phytophthora*, β-glu alone may cause hyphal lysis as their cell wall is principally made of β-glucan and devoid of chitin. A 34 kDa basic β-glu purified from soybean hypocotyls infected by an incompatible race of *P. sojae* inhibited spore germination and hyphal growth of the chitin negative fungus *P. sojae*, but did not show any antifungal activity against the chitin-containing fungi *Alternaria mali, Colletotrichum gloeosporioides, and Magnaporthe grisea* (Yi and Hwang, 1997). The purified β-1,3-glucanase from cucumber alone significantly inhibited spore germination and inhibited the growth of *C. lagenarium in vitro* (Ji and Kuc, 1996). The isolated and purified β-1,3-glucanase from wheat leaves showed antifungal activity against both *Alternaria longipes* and *Rhizoctonia cerealis* on tested plates, and inhibited the germ tube elongation and spore germination of *Verticillium dahliae* and *Fusarium oxysporum f.sp cucumerinum* (Sun *et al.*, 2004). A 39 kDa β-glu purified from resistant potato cultivar produced a direct inhibitory effect on the germination of sporangia of *P. infestans* (Tonon *et al.*, 2002). However, when purified basic β-glu was applied to the hyphae of the arbuscular mycorrhizal fungus *Glomus mosseae* grown *in vitro*, no effect was observed (Vierheilig *et al.*, 2001). It has been demonstrated that some phytopathogenic fungi, whose growth is inhibited by chn and β-glu *in vitro* are able to overcome the effects of these enzymes over a period of several hours, indicating the ability of those fungi to adapt to these enzymes (Ham *et al.*, 1997). They isolated a glucanase inhibitor protein (GIP) from the culture fluid of fungal pathogen *P. sojae* that can inhibit only specific isoforms of β-glu, but not all. Some reports regarding the inconsistency of endoglucanases as inhibitors of fungal growth *in vitro* and in transgenic plants might be attributed to this ability of certain phytopathogens to secrete inhibitors of plant endoglucanases (Rose *et al.*, 2002).

An exo-α-1,3-glucanase isolated from *T. harzianum* could effectively inhibit spore germination as well as hyphal growth of phytopathogens like *A.*

niger, Botrytys cinerea and *Penicillium aurantiogriseum* (Ait-Lahsen *et al.*, 2001). However, the enzyme showed no activity against fungi like *Rhizoctonia solani* and *P. syringae*. The difference in sensitivity of the various fungi to the enzyme might be due to the different molecular architecture of spore and hyphal cell walls and/ or the presence of specific inhibitors of enzymes. *Clonostachys rosea* produces β-1,3-glucanase *in situ*, which can degrade hyphae of *Fusarium* and *Pythium* and contribute to biological control efficacy against the *Fusarium* root and stem rot and *Pythium* damping-off diseases on cucumber plants (Chatterton and Punja, 2009). The dual culture experiments of the aflatoxin forming fungus *Aspergillus flavus* along with the maize callus on the same plate resulted in the inhibition of the fungal growth. The inhibition of fungal growth was correlated with the activity levels of β-glu in the callus and in the culture medium. *In vitro*, class II β-1, 3-glucanases from tobacco and tomato have no inhibitory activity on fungal growth (Joosten *et al.*, 1995; Anfoka and Buchenauer, 1997).

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

experiments by destroying the pathogen faster, and thereby giving better protection for grapes against *B. cinerea* (Rey *et al.*, 2001).

2.13. *Phytophthora* and Abnormal leaf fall disease

Phytophthora species are a group of oomycete fungi which are currently classsifed as the stramenophile (Chromistan) eukaryotes (Tylor *et al.*, 2006). 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. *Phytophthora* species resemble fungi but have number of biological characteristics that are relatively uncommon in other fungi (Griffith *et al.*, 1992). They differ from fungi in having the cellulose instead of chitin which is the common cell wall component of fungi outside oomycetes (Bartnicki-Garcia and Wang, 1983) and also posess aseptate mycelia. They also have diploid nuclei in the vegetative phase unlike the haploid nuclei in most fungi. Early studies have shown that major components of the oomycete cell wall are β-1,3-glucan, β-1,6- glucan and cellulose, the β-1,3-linked glucans, which constitutes 80 – 90 % of the cell wall dry weight. Chitin has been reported from one of the oomycete, *Apodachlya* (*Leptomitaceae*) (Lin and Aronson, 1970). While they are most closely related to aquatic organisms, such as brown algae and diatoms, they can exist on land also. However, *Phytophthora* organisms are often referred to as water molds because they do need water to complete their life cycle.

There are 107 recognized species of *Phytophthora* as mentioned in the *Phytophthora* database (http://www.phytophthoradb.org), 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 multi billion dollar losses in world cash crops. Thus they are having high economic importance. 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 – 49) caused by *P. infestans* (Erwin and Ribeiro, 1996).

Phytophthora infestans was the infective agent of the potato blight that caused the famine, and still remains the most destructive pathogen of potato crops. The other example is the soya bean root and stem rot agent *Phytophthora sojae*, which has also caused longstanding problems for the agricultural industry. *Phytophthora spp.* are mostly pathogens of dicotyledons, and are relatively host-specific parasites.

As mentioned before, the diseases caused by *Phytophthora* have a large economic impact on the nursery industry. *Phytophthora syringae* results in more than $1 million in annual losses to shade tree production in Oregon alone. *Phytophthora* diseases on *Rhododendron* may affect up to 10% of plants; if a nursery becomes infected with *Phytophthora ramorum*, it could lose $90,000 to $1.3 million in costs for crop destruction and cleanup along with the loss of sales.

The cytoplasm of *Phytophthora* spp. contains two reserve carbohydrates, mycolaminarin, a soluble β-1,3 linked glucan, and cellulin an insoluble granular inclusion 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* causing diseases of fruit, foliage, crowns and roots of plants are generally considered to be multicyclic that results in severe epidemics. In general, plant diseases caused by this genus are difficult to control chemically; 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. Thus the growth of resistant cultivars is the main management strategy to control the disease occurrences in plants.

In *Hevea brasiliensis, Phytophthora* causes the severe abnormal leaf fall disease (ALF). The first report of ALF in India was in 1910 from the estates near Palippilly in the Thrissur district of Kerala state (McRae, 1918). Meanwhile the disease spread to all the other traditional rubber growing

districts. Later the disease had spread to other rubber growing areas of the country and subsequently to other rubber growing countries like Sri Lanka, Myanmar, Cambodia, Vietnam, Nigeria, Cameroon, Brazil, Costa Rica and Venezuela. Outbreak of the disease was also noticed in major natural rubber producing countries like Malaysia and Thailand. The causative agents for the ALF is mainly the *Phytophthora* oomycetes- *Phytophthora palmivora* Butl., *P. meadii* McRae. & *P. nicotianae var. parasitica* and *P. botryosa* ((Chee, 1969). However the species most common in the traditional areas is *Phytophthora meadii*. Rainfall is the most important pre- disposing factor for the appearance and spread of the disease. A continuous spell of 250- 350 mm rain for 7-10 days without intermittent hot sinshine favours the occurrence of the disease. The temperature range should be 22- 25 ^{0}C and 26^0- 30 ^{0}C respectively. The relative humidity of above 90 % is most suitable for the outbreak of the disease. The disease spreads rapidly and assumes epidemic proportions under these favourable conditions. Under the normal monsoon conditions, the disease starts by the middle of june and reaches the peak by the middle of July. When the monsoon is late, very heavy incidence is noticed from the middle of July to the middle of august (Priyadarshan, 2011). During favourable conditions, *Phytophthora meadii* produces microscopic (42-55 X 25-32 µm) oval, papillate to semipapillate, hyaline asexual spores or sporangia. Sporangia are deciduous and fall from the colony with a short stalk attached to the base of the sporangia. In water, sporangia release numerous swimming zoospores. Without sufficient water, sporangia germinate by producing germ tubes that penetrate the host. Zoospores are able to swim for many hours and are attracted to organic matter or host tissue. These zoospores encyst, germinate by forming a small germ tube, and penetrate the host. The infection can also be caused by the directly germinated sporangia which produce the germ tubes. The pathogen gains entry into the host tissue through the stomata (Thankamma *et al.*, 1975). This pathogen is heterothallic and produces sexual spores, called oospores, when two mating types (A1 and A2) are grown together. Occurrence of two mating groups in *Phytophthora meadii* causing abnormal leaf fall disease of rubber in South India (Rajalakshmy *et al*, 1985).

During the late 1990's, 33 *Phytophthora meadii* isolates were reported from different *Hevea brasiliensis* clones grown in different climatic regions in Sri Lanka. Growth, morphology and pathogenicity of the isolates were compared. Growth and pathogenicity levels varied among the isolates. Some isolates obtained from moderately susceptible rubber clones were highly pathogenic, compared to isolates obtained from resistant clones. Highly pathogenic isolates produced a higher number of sporangia on agar at 27 ± 2°C. It was impossible to group isolates according to the clone or the region from where they were obtained. (Jayasuriya *et al.*, 1999). Heterothallism has been demonstrated in Indian isolates of *Phytophthora meadii* from rubber.

In rubber clones, the initial symptoms are seen on the immature green pods which are most susceptible to the disease. Usually the inoculum development starts with the germination of previous season's oospores, which serve as resting spores, present in infected dry pods, leaves and twigs deposited in the soil. Small lesions appears initially by the 'pin head' black globules of latex, usually at the basal end of the pod, which later gets enlarged with continuous wet weather into brown water-soaked areas. Correspondingly the globules of latex become bigger and more apparent. The pod surface soon gets covered with a white mass of sporangia which are washed by rain down onto the leaves, petioles, young shoots and stems where they cause further infections. On petioles also the infections are usually indicated by a globule of latex at the point of fungal entry from which a grey-to-black lesion develops. At this stage the leaves get abscised with the petiole attached to it, though leaflets often become reddish-brown before this. On the leaves, circular, brownish water-soaked lesions appear on the lamina with fine droplets of coagulated latex in concentric rings. Lesions eventually coalesce to form large, irregular necrotic areas. Leaflets are easily shed on vigorous shaking. Infection of shoot apices leads to extensive shoot dieback. In mature plantations, extensive defoliation results in considerable crop loss. Infections elsewhere on green shoots result in depressed dark brown areas often with a white mat of sporangia. Tapping wounds become infected during the rains when lesions on pods are actively sporulating. Sunken, vertically parallel depressions develop just above the tapping cut and sometimes also below it. When infected bark is

removed, characteristic, distinct vertical black lines are seen on the wood corresponding to the external depressions (black stripe or black thread). Increase in plugging index, reduction in dry rubber content of latex, and more weed growth are other secondary effects of the disease (Jacob *et al.*, 1989). Unless treated, infections result in uneven regeneration of the bark giving panel burrs which make subsequent tapping difficult or impossible. In advanced stages of infection the bark splits open, causing gaping wounds (canker) with foul-smelling pads of coagulated latex between the bark and wood.

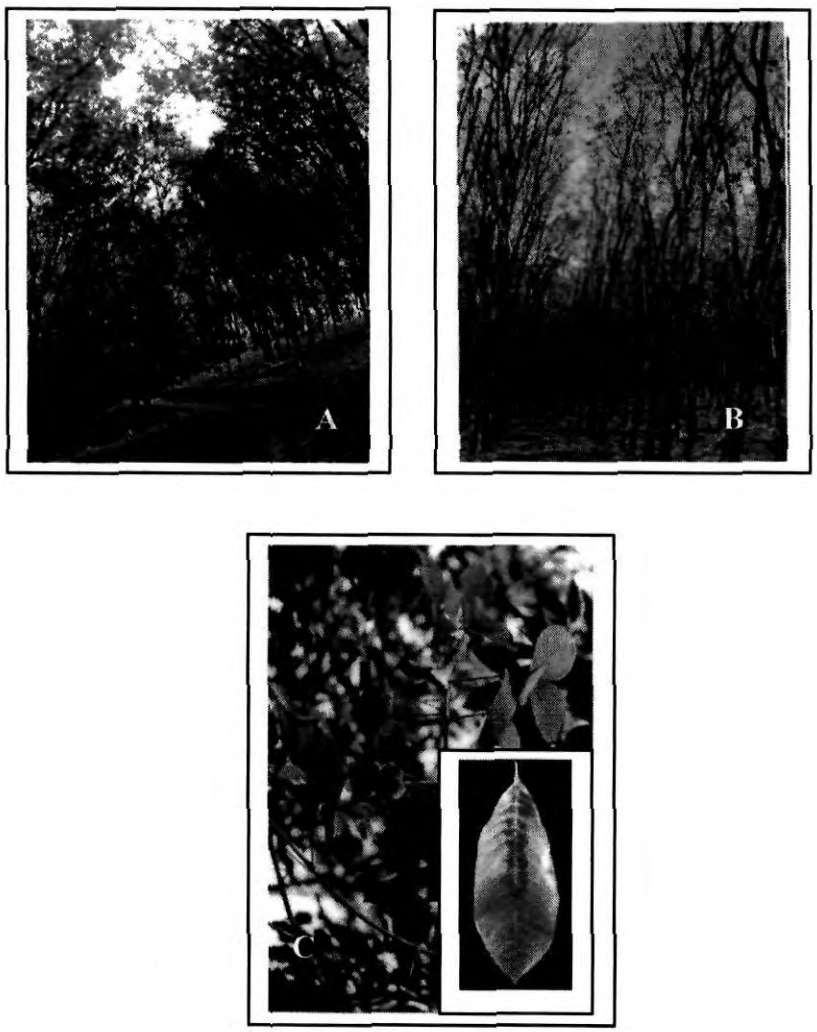


Fig: 7. Incidence of ALF disease in *Hevea*. *A*-Healthy plantation, B-ALF disease affected plantation, C-Infected pod. (Inset– enlarged image of an infected leaf of *Hevea*). (B & C ref: Thanseem, 2004)

The management of the disease includes the prophylactic spraying of the foliage prior to the onset of south west monsoon with (1) Bordeaux mixture using high volume sprayers or (2) Oil-based copper oxychloride dispersed in diluent spray oil employing either low volume airblast sprayers from the ground or through aerial application. For aerial spraying, 6.2 litres of 40% oil based copper oxychloride paste in 37 litres of diluent oil or 8 kg oil dispersible copper oxychloride powder 56% in 40 litres of oil is used per hectare. Spraying should be done as close to the monsoon as possible. *Trichoderma* spp. was tried as a biocontrol agent and was shown to inhibit the growth of *P. meadii* (Vanitha *et al.*, 1994). This offers yet another opportunity to manage *Phytophthora* incidence in rubber plantations using the bio-control agents. The other control measure is the replacement of the crown of a susceptible high yielding clone with that of a resistant variety like FX 516, but with limited success (Pillay *et al.*, 1986).

2.14. Molecular studies on ALF disease in *Hevea brasiliensis*

The past three decades witnessed tremendous advancements towards understanding of the molecular mechanisms of plant growth and development in different crops. The recent developments in recombinant- DNA and *in vitro* plant regeneration techniques could provide a direct route for the introduction of specific genes controlling agronomic traits into the crop plants. But most of these studies were carried out in annuals and only a very few in the tree species. As a perennial tree species, genetic improvement through conventional breeding in rubber tree is rather a slow and difficult process. The major constraints in rubber tree breeding are long juvenile period, seasonal flowering, lack of any early selection parameters especially for estimating traits such as latex yield, susceptibility to TPD and wind damage, low fruit set, very long period of field experimentation, and pronounced interaction of genotype and environment and presence of high amount of phenolics, terpenoids, tannin *etc.*, which can react with proteins and nucleic acids have made molecular studies in *Hevea* rather difficult (Thulaseedharan *et al.*, 2000, 2008). Chye and Cheung (1995) described the isolation and characterization of β-1,3-glucanase gene from *Hevea* cDNA library prepared from the latex of

Hevea brasiliensis. Nucleotide sequence analysis revealed that the 1.2 kb cDNA encoding a basic β-1,3-glucanase showed 68% nucleotide homology with other glucanase genes in the database and was expressed at higher levels in latex than in leaf. Te-Chato and Suranilpong (1995) identified resistant callus lines to *Phytophthora spp.* culture filtrate in rubber. Subsequently, Thanseem *et al.,* (2003) cloned and characterized β-1,3-glucanase gene from an Indian *Hevea* clone RRII 105. Further, they also demonstrated that a prolonged accumulation of β-1, 3-glucanase mRNA transcripts with abnormal leaf fall (ALF) disease tolerant RRII 105 clone compared with susceptible one. Another defence related protein is *Hevein* (a chitin-binding protein) that play a crucial role in the protection of wound sites from fungal attack and is also involved in the coagulation process. It belongs to a multigene family and the specificity of its expression in the latex is under investigation (Broekaert *et al.* 1990; Pujade-Renaud *et al.* 2005). Chrestin *et al.* (1997) described that over-expression of chitinase (hevein) gene was due to the partial de-glycosylation of the hevein receptor and the resulting delay in coagulation.

β-1,3-Glucanase activity was observed in the lutoid extracts of *Hevea* latex. Churngchow *et al.*, (1995) isolated two β-1,3-glucanase isozymes GI and GII from fresh *Hevea* latex using ion exchange and affinity chromatography Five isoforms consisting of three basic and two acidic isozymes were detected in native IEF gels after activity staining (Breton *et al.*, 1995). A cDNA clone encoding class I β-glu was isolated by screening a *Hevea* latex cDNA library with a heterologous probe from *N. plubaginifolia* (Chye and Cheung, 1995). Expression of *Hevea* glucanase in different tissues was examined through northern hybridization, and it was observed that the gene is constitutively expressed in the laticifers. *In situ* hybridization studies were carried out to examine the distribution of β-glu mRNAs in the petiole sections of *Hevea,* and it was found that anti-fungal β-glu is expressed exclusively in the laticifer cells of the petiole. If current progress in the *in vitro* culture and genetic transformation combined with molecular biology applications continue, the future may witness superior *Hevea* tree clones tailored for special agronomic and economic characteristics especially with increased disease tolerance.

Chapter III

Materials and Methods

3.1. Isolation, cloning and characterization of genomic DNA sequences coding for β-1,3-glucanase from different *Hevea* clones

3.1.1. Genomic DNA isolation from 10 *Hevea* clones

For many downstream applications, high quality genomic DNA without extraneous/ intraneous contaminants is essential. Young, uninfected and fully expanded leaves are ideal for a good quality genomic DNA isolation. Leaves of the ideal stage were selected from the *Hevea* clones maintained in the germplasm nursery of Rubber Research Institute of India. The clones selected for the present study were FX 516, RRII 105, GT1, RRII 33, GL 1, PR 107, PB 86, RRIM 600, RRII 414 and RRIM 701. The leaves were collected in polythene bags, stored in ice buckets and immediately brought to the laboratory for DNA isolation. All laboratory reagents used were of molecular biology or analytical grade. Stock solutions and media were sterilized by autoclaving or by filter sterilization using Millipore disposable filters (0.2-0.4 μm). The leaf samples were washed thoroughly in tap water and rinsed with distilled water. For DNA isolation, modified hexadecyltrimethyl ammonium bromide (CTAB) method, an efficient DNA isolation method described by Doyle and Doyle (1990) was followed. The cationic detergent CTAB solubilises membranes and forms a complex with DNA (Sghaier and Mohammed, 2005). This modified CTAB procedure consists of the following steps:

i. About two gram leaf tissue was pulverized in liquid nitrogen using a mortar and pestle.

ii. This freshly ground tissue was homogenized with 20 ml, 2X CTAB extraction buffer.

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

iii. The sample was then kept at 55 ^{0}C for 20 min in a 50 ml centrifuge tube with frequent mixing.

iv. The homogenate was centrifuged at 10,000 rpm for 10 min, pellet was discarded and the supernatant was transferred to a new tube.

v. Equal volume of Tris-saturated phenol-chloroform-isoamyl alcohol (25:24:1) was added to the supernatant and mixed gently by inversion of the tube.

vi. The sample was spun at 10,000 rpm for 10 minutes and the aqueous phase was transferred to a new tube.

vii. RNA in the sample was degraded by incubating at 37 ^{0}C for 1 h after the addition of 5 µl of *DNase* free *RNase* (10 mg/ml, M/S Sigma Aldrich).

viii. To inactivate the *RNase* and other residual proteins *Proteinase K* (20 µl from the stock of 20 mg/ml- (M/S Bangalore Genei)) was added. The incubation was continued for another 1 h at 37 ^{0}C.

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

x. Aqueous phase was transferred to a fresh tube.

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

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

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

xiv. The tube was kept in ice for 20 min and the precipitated DNA was pelleted by centrifuging at 5000 rpm for 10 min at 4 ^{0}C.

xv. The DNA was washed in 70 % ethanol, air-dried the pellet and suspended in TE (10:1) buffer.

TE Buffer:
1mM Tris- HCl and 0.1mM EDTA

xvi. Finally the DNA samples were stored at –20 ^{0}C.

3.1.2. DNA quantification

The quality and quantity of the isolated genomic DNA was checked in a UV spectrophotometer (M/S Beckman, USA) by measuring amount of UV

irradiation absorbed by the bases in the DNA. For quantitating DNA, the ratio of absorbance at the wavelengths 260 nm and 280 nm (260/280) is taken. Pure preparations of DNA have OD_{260}/OD_{280} values of 1.7 to 1.8 which indicates good quality DNA without protein contamination. The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. DNA quantification was done according to the formula:

1.0 OD at 260 nm for double-stranded DNA = 50 ng/µl of dsDNA.

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

3.1.3. Agarose gel electrophoresis

For qualitative analysis of the isolated DNA, 0.8% agarose was prepared in 0.5X TBE (pH-8.0; prepared from 5X TBE (0.45 M Tris Borate, 0.1 M EDTA)). Heated the solution in a microwave until all the small translucent agarose particles were dissolved. The melted agarose was cooled to approximately 60 ^{0}C and ethidium bromide solution (1 mg/ml- stock) was added to give a final concentration of 0.5 µg/ml. The gel mixture was poured into a clean gel casting tray, a suitable comb was inserted and left to solidify. When the gel was solidified, the comb was removed carefully. The gel was placed into electrophoresis tank filled with running buffer just to cover the wells. DNA sample mixed with 5X gel loading buffer (Bromophenol Blue - 0.25%, Xylene Cyanol - 0.2%, Glycerol - 30% in distilled water) was loaded into the wells of the gel and electrophoresis was carried out. After electrophoresis, the gel was removed from the apparatus and separated DNA was viewed under a UV trans-illuminator and documented using 'EDAS 290' (Electrophoresis Documentation and Analysis System- Kodak, USA).

3.1.4. PCR Amplification

Primers: The primer sequences were designed based on the reported genomic DNA sequence of β-1,3-glucanase from *Hevea brasiliensis* clone RRII 105 by Thanseem *et al.*, 2003 (NCBI Acc. No. AY325498).

1F - 5' GGC AAA GAG GGA AGA TTT CG 3' (Forward primer)

1R -5' CCA TAA GCA TAT CTT GAG TTG G 3' (Reverse primer)

The primers were synthesized at M/S Sigma Aldrich, Bangalore, India in a 3OD scale.

PCR mix (20 µl reaction volume):

Buffer	-	2 µl of 10X buffer
MgCl2	-	1.5 mM (pH 8.3)
dNTPs	-	100 µM
Forward and reverse primers	-	250 nM each
Taq polymerase (M/S Bangalore Genei, Bangalore)	-	0.5 units
Template DNA	-	20 ng

Thermal cycling profile:

94 ^{0}C -	1min	-	1 cycle
94 ^{0}C -	2 mins		
55 ^{0}C -	1 min	-	36 cycles
72 ^{0}C -	1min		
72 ^{0}C -	10 mins	-	1 cycle

3.1.5. Elution of amplified products from agarose gels after electrophoretic separation for cloning

Electrophoresis of PCR amplified products were carried out in 1.0 % low melting point agarose gels and the DNA bands were excised from the gel after quickly viewing over UV light so as to avoid nicks. Elution of DNA bands from agarose gels had been performed using the GFX^{TM} PCR, DNA and Gel band purification Kit (M/S GE Health Care, USA) according to the manufacturer's instructions.

3.1.6. Cloning of amplified genomic DNA

The universal TA cloning method has been adopted for cloning of the amplified β-1,3-glucanase gene from the different *Hevea* clones. The procedure exploits the terminal transferase activity of *Thermus aquaticus* (*Taq*) DNA polymerase. The non-template dependent activity of *Taq* polymerase preferentially adds a single adenosine to the 3'-ends of a double stranded DNA molecule, and thus majority of the molecules PCR amplified by *Taq* DNA polymerase possess single 3'-A overhang. The use of a linearized

"T-vector" which has single 3'-T overhang on both ends allow direct ligation thereby providing high-efficiency cloning of PCR products, facilitated by complementarity between the PCR product containing 3'-A overhang and vector 3'-T overhang. Here the cloning was done using TOPO TA® Cloning Kit for sequencing, provided by M/S Invitrogen Life Technologies, USA as per the manufacturer's instructions.

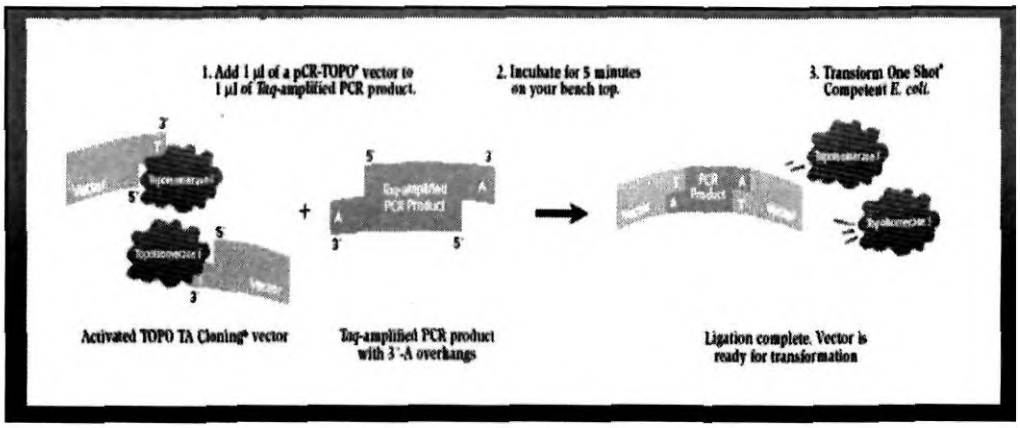


Fig. 8. Illustration showing Topo TA cloning procedure

3.1.7. Transformation of *E. coli*

Transformation was carried out with the high efficiency competent DH5α cells supplied with the TOPO TA® cloning Kit. Following the manufacture's instruction, 2.0 µl cloning reaction was added into a vial containing 50 µl of one shot chemically competent *E. coli* cells and mixed gently and incubated in ice for 10-15 min. The cells were subjected to a heat shock for 30s at 42 ^{0}C and was transferred immediately to ice and further incubated for a brief time. 250 µl SOC medium was added and incubated at 37 ^{0}C for 1h to allow the bacteria to get express the β-lactamase gene contained in the plasmid to confer ampicillin resistance. The transformed mixture was plated on LB ampicillin (50 µg/ml) medium and kept for 16 hours incubation at 37 ^{0}C.

3.1.8. Confirmation of cloning (colony PCR and plasmid PCR)

For screening putatively transformed white colonies based on PCR amplification of insert few colonies were picked randomly and suspended

individually in 20 µl PCR cocktail. The patches of colonies were preserved on original plates for further applications. An initial denaturation was done at 96 ^{0}C for 10 min to lyse the cells and inactivate nucleases. 30 cycles of amplification was done following the PCR conditions standardized for the amplification of the insert. Same insert specific forward and reverse primers were used. PCR products were electrophoresed in 1.0% agarose gel and observed under UV trans - illuminator. Colonies giving right amplification of insert DNA were located from the original plate and inoculated in LB medium and incubated overnight at 37 ^{0}C for plasmid isolation.

3.1.9. Recombinant plasmid isolation and PCR

Plasmids were isolated from positive colonies using Perfect prep Plasmid mini Kit (M/S Eppendorf, USA). The presence of the insert in the plasmids were confirmed by PCR amplification (as described in section 3.1.4) of recombinant plasmids with the insert specific forward and reverse primers. About 2.0 ng of the plasmid DNA was used as a template in the PCR reaction. Amplification was later checked in 1.5 % agarose gel.

3.1.10. Sequencing of the insert in recombinant plasmids and analysis

The recombinant plasmid DNA obtained were sequenced at the DNA sequencing facility at M/S Macrogen (Korea) with M13 forward and reverse primers. Sequences obtained were analysed and compared with published genomic DNA sequence of the clone RRII 105 through BLASTn analysis and ClustalW.

Blastn - http://blast.ncbi.nlm.nih.gov/Blast

ClustalW - http://www.ebi.ac.uk/Tools/msa/clustalw2/

3.2. Amplification of promoter regions of β-1,3-glucanase gene through inverse PCR

In 1988, Ochman *et al.*, first described the Inverse PCR (IPCR technology). This technique is very useful for identifying unknown flanking

DNA sequences of known genomic sequences. The inverse PCR method involves a series of restriction digestion and ligation, resulting in a looped fragment that can be primed for PCR from a single section of known sequence. IPCR works on the principle that digested DNA at very low molecular concentrations are able to self ligate to give a circular DNA product. The procedure consists of four steps, restriction digestion of DNA, self-ligation of the digested DNA, linearization of the self-ligated product with a second restriction enzyme and finally the PCR. IPCR comprises the standard PCR. However it has the primers oriented in the reverse direction rather than the usual orientation. The template for the PCR is the restriction fragment that has been self ligated and linearized. Fig.9 illustrates the principle behind the working of inverse PCR

Here inverse PCR has been performed to obtain the upstream promoter fragment of the already reported sequence of β-1,3-glucanase (NCBI Acc. No. AY325491) by Thanseem *et al.*, (2003). The full length coding DNA sequence had been chosen for restriction enzyme analysis and primer designing specifically for inverse PCR.

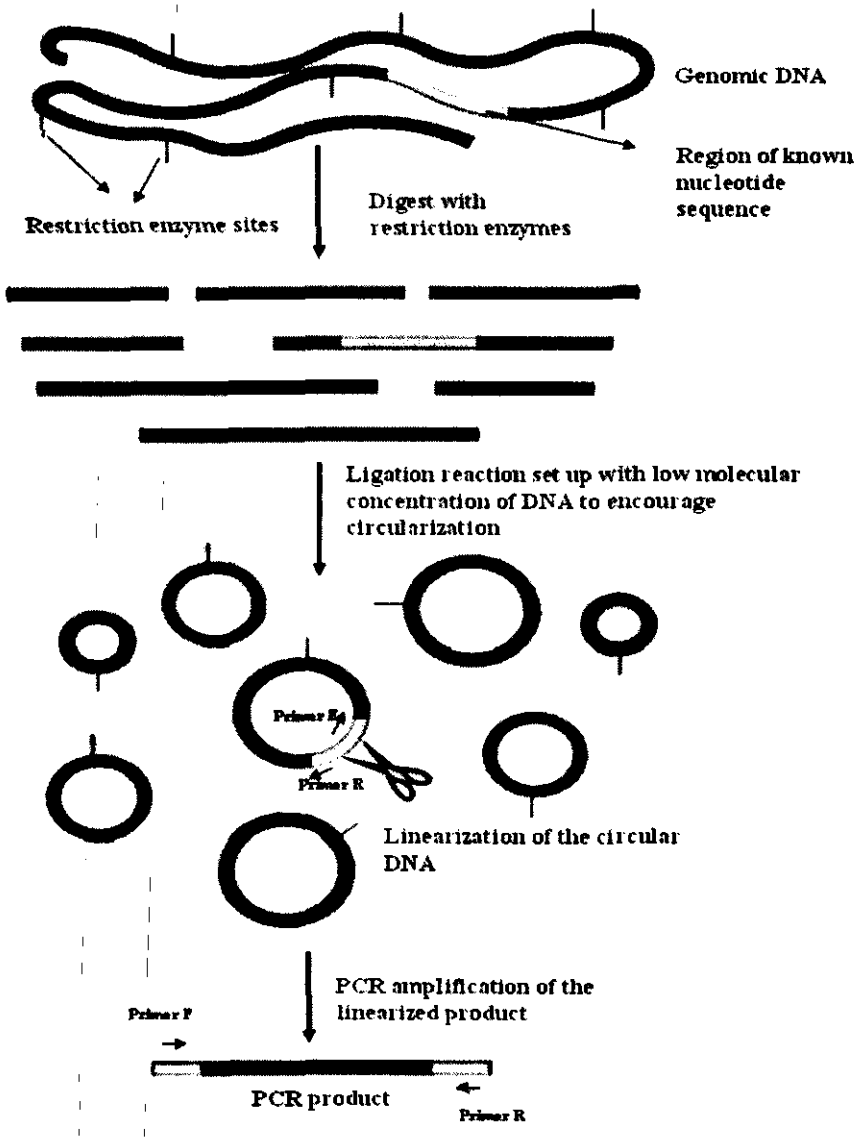
3.2.1. Genomic DNA isolation

DNA extraction was done following the modified CTAB procedure of Doyle *et al.*, (1990) (section. 3.1.1)

3.2.2. Restriction enzyme digestion, circularization and linearization of DNA

For identifying the restriction enzyme sites and thereby to design the primers for inverse PCR, the restriction map of the reported β-1,3-glucanase gene (Acc.No.AY325498) was made through the Web cutter software (http://bio.lundberg.gu.se/cutter2/). The restriction enzyme which is having only one site in the genomic sequence of the reported β-1,3-glucanase gene had been selected for the restriction digestion and linearization of the genomic DNA.

Figure. 9. Schematic representation of the working of inverse PCR



i) Restriction enzyme (RE) digestion

The enzymes *Ssp* 1 and *Bgl* II were used for the digestion and linearization of genomic DNA respectively. The RE site of the *Ssp* 1 enzyme starts at the 768th nucleotide and that of the *Bgl* II enzyme is at the 414th nucleotide within the reference β-1,3-glucanase gene sequence (AY 325498).

a) *Ssp I (*for digestion of the genomic DNA)

Recognition Site: **5'....AAT⇩ATT....3'**
3'....TTA⇧TAA ...5'

The enzyme *Ssp* I (M/S Bangalore Genei, Bangalore, India) was used to digest 5 µg of DNA in a 100 µl reaction at 37 ^{0}C overnight. Fractionated on a 1% agarose gel to check whether the DNA has been digested. The enzyme was heat inactivated by incubating at 65 ^{0}C for 20 minutes.

ii) Circularization *via* ligation

The circularization of the digested products was carried out using 2U of T_4 DNA ligase enzyme (M/S New England Biolabs, England). 0.5 µg of the digested DNA was used in a 500 µl reaction. The sample was incubated at 16 ^{0}C overnight. Circularized DNA was ethanol (2.5 vol) precipitated, washed with 70 % alcohol and air dried.

iii) Linearization of the circular DNA

The circularized DNA was linearized using the enzyme *Bgl II* (M/S New England Biolabs, England) in a 10 µl reaction volume containing 0.5 µg DNA, 1 U of enzyme and 1 µl of 10 X buffer.

Recognition Site: 5'....AGA⇩TCT....3'
3'....TCT⇧AGA....5'

One microlitre of the linearized product was used for a 20 µl PCR reaction.

3.2.3. Inverse PCR

The following primers were designed from the genomic sequence of β-1,3- glucanase gene (Acc. No. AY325498) for performing inverse PCR; Forward: **5' TAG GAA ATT CCT ACC CTC CTT CTG 3'**, Reverse : **5' CCT GTT ATA CCA AGG CTT GCT G 3'**. Fig. 10 shows the sequence details, restriction enzyme sites and the orientation of the forward and reverse primers chosen for inverse PCR.

Figure: 10. Details of genomic DNA sequence, restriction enzyme sites and designed primer locations in the β-1,3-glucanase gene (NCBI Acc. No. AY325491)

β-1,3-glucanase (NCBI Acc. No. AY325491)

1257 base pairs

Forward primer

Reverse primer

***Ssp*1 site**

***Bgl* II site**

```
Ksp632I    SpeI
             BseRI      Eco57I
atggctatctcctcttcaacttcaggaactagtagttccctgccctcaagaactactgtcatgcttcttctgatt base pairs
taccgatagaggagaagttgaagtccttgatcatcaagggacgggagttcttgatgacagtacgaagaagactaa 1 to 75
                Eam1104I   AclNI
                EarI



              EcoT14I
              BssT1I
              Eco130I          BsaI
ttctttacagcaagccttggtataacaggtctctctctctctctctctctctctctctctctccatctatc base pairs
aagaaatgtcgttcggaaccatattgtccagagagagagagagagagagagagagagagagaggtagatag 76 to 150
              StyI             Eco31I
              ErhI

                    HindII
tactctcatgttaaagttgacgatgctctttttttctctccttgttctaaaaggtttcaactaatacctgtattt base pairs
atgagagtacaatttcaactgctacgagaaaaaaagagaggaacaagattttccaaagttgattatggacataaa 151 to 225
                  HincII

   ApoI                          Mva1269I
   EcoRI                         BsaMI
aggaattcagatgcccaggtaggtgtttgctatggaatgcaaggcaacaaccttccacctgtttcagaggtcata base pairs
tccttaagtctacgggtccatccacaaacgataccttacgttccgttgttggaaggtggacaaagtctccagtat 226 to 300
   AcsI                          BsmI

                                      BsaBI
                                      MamI
                                AcsI  BsrBRI
gctctctataaacaatctaacatcaagagaatgagaatttatgatccaaatcgagcagtattggaagcccttaga base pairs
cgagagatatttgttagattgtagttctcttactcttaaatactaggtttagctcgtcataaccttcgggaatct 301 to 375
                                ApoI  Bsh1365I
                                      Bse8I

                                   BstYI
                                   XhoII
                                   BstX2I
ggctcaaacattgaactcatactaggtgttccaaactcagatctccaaagccttaccaatccttccaatgcaaac base pairs
ccgagtttgtaacttgagtatgatccacaaggtttgagtctagaggtttcggaatggttaggaaggttacgtttg 376 to 450
                                   BglII
                                   MflI


tcatgggtacaaaaaaatgttcgtggcttctggtcaagtgtcctgttcagatatatagcagttggcaacgaaatt base pairs
agtacccatgtttttttacaagcaccgaagaccagttcacaggacaagtctatatatcgtcaaccgttgctttaa 451 to 525
```



PCR has been carried out at an annealing temperature of 52 ^{0}C with 34 thermal cycles. PCR components and the rest of the PCR profiles were as shown in section 3. 1. 4. The amplified products of the PCR had been fractionated by electrophoresis in a 1.2% agarose gel at 50 V. The elution of DNA bands from agarose gels had also been performed using the GFX^{TM} PCR, DNA and Gel band purification Kit (M/S Amersham Pharmacia, USA) according to the manufacturer`s instructions.

3.2.4. Cloning of the PCR product and sequence characterization

The PCR amplified products after purification were used directly for cloning. Cloning of the amplified genomic DNA of the selected *Hevea* clones was carried out using TOPO TA® cloning kit for sequencing (M/S Invitrogen Life Technologies, USA) following the manufacturer's protocol (section.3.1.6). The cloned upstream gene sequences were later transformed into the chemically competent *E. coli* (DH5α) cells supplied along with the kit. The transformed cells were plated on the LB agar plates containing 50 µg/ ml ampicillin. A few colonies were selected after 16 hours of incubation and colony PCR was done to identify positive bacterial colonies as described in section.3.1.8. Plasmids were isolated from positive colonies using Perfect prep Plasmid mini Kit (M/S Eppendorf, USA). The confirmation of transformation was done by PCR amplification of the plasmids using β-1,3-glucanase specific primers. Amplification was checked through 1.5 % agarose gel electrophoresis. The recombinant plasmids obtained were sequenced at M/S Macrogen, Korea. Sequences of β-1,3-glucanase from the clone RRII 105 were aligned using the ClustalW software at the EMBL-EBI site. Homology of the sequence with other sequences in the public database (NCBI) was done to identify similar sequences.

3.2.5. *In silico* identification of promoter cis-elements

In order to identify putative transcription factor binding sites and conserved plant cis-acting regulatory elements in the promoter sequences of β-1,3-glucanase, a data base analysis with PLACE software has been done. PLACE (Plant Cis- acting Regulatory DNA Elements) is a database of motifs found in plant cis-acting regulatory DNA elements mainly among the vascular plants. (http: www.dna.affrc.go.jp/htdocs/PLACE/). It is a database of 319 cis-acting regulatory DNA elements collected from previously published reports (Higo *et al.*, 1999).

3.2.6. Screening for nucleosome forming potential

The isolated promoters of β-1,3-glucanase were screened for nucleosome forming potential since the interaction of TF with DNA is a dynamic process. The distributed nucleosome positioning signals may be crucial in determining chromatin architecture *in vivo* in the regulatory regions. The nucleosome positioning (Kiyama and Trifonov, 2002) controls both the nucleosome packaging in discrete chromatin regions and the accessibility of transcription factor binding sites (TFBS). This was carried out using RECON program, which was accessed through the internet (http://www.mgs.bionet.nsc.ru/mgs/programs/recon/).With this programme, it is able to construct profiles of nucleosome potential and to characterize the probability of nucleosome formation along DNA sequences.

3.2.7. Identification of CpG islands

Detection of regions of genomic sequences that are rich in the CpG (p= phosphodiester bonds) pattern is important because such regions are resistant to methylation and tend to be associated with genes which are frequently switched on. Regions rich in the CpG pattern are known as CpG islands. Unlike CpG sites in the coding region of a gene, in most instances, the CpG sites in the CpG islands of promoters are unmethylated if genes are expressed. This observation led to the speculation that methylation of CpG sites in the promoter of a gene may inhibit the expression of a gene. Methylation is central to imprinting alongside histone modifications (Feil and Berger, 2007).The presence of CpG islands in the promoter region had been analysed through EMBOSS CpG at the EMBL-EBI site. (www.ebi.ac.uk/Tools/emboss/cpgplot/).

3.3. Characterization of the promoter-gene combinations of β-1,3-glucanase obtained through inverse PCR

3.3.1. PCR amplification of promoter- gene combinations of β-1,3- glucanase gene

In the previous attempts to isolate the promoter sequence of β-1,3-glucanase gene, different forms of promoter sequences were obtained from a single *Hevea* clone, RRII 105 through inverse PCR. Here a polymerase chain reaction has been performed to confirm that the different promoter sequences obtained earlier belong to different genomic forms of β-1,3-glucanase gene and also to obtain the full length gene sequences upto the stop codon.

Genomic DNA isolated from *Hevea* clone RRII 105 was used as the template for the PCR. The forward primer was designed from the 5' region of specific promoter sequences isolated and a reverse primer specific to the earlier reported genomic sequence of β-1,3-glucanase gene (Acc. No. AY325498). The reverse primer (1R) contained the stop codon also. Another reverse primer (2R) had also been designed from the inner regions of the gene which does not contain the stop codon (table.3).

PCR mix and the thermal cycling profile

The PCR components were taken as mentioned earlier for a 20 µl reaction (section.3.1.4). The thermal cycling profile composed of an annealing temperature of 50 ^{0}C for 36 cycles. The rest of the cycling profile were similar as mentioned earlier in section 3.1.4. Along with the forward primers (IPF1, IPF2, IPF4 and IPF5; IR reverse primer was used for PCR. With IPF3 forward primer, 2R was used to get the product amplified. The PCR amplification of the primer combination IPF5 (forward) and 1R (reverse) was done with the proof reading enzyme - 0.5U XT-5 Polymerase (M/S Bangalore Genei, Bangalore).

Table: 3 List of primers used for PCR

Forward primer:
5' AGT TTT AAT TTG GCG ATA G 3' - IPF1 (designed from 198 bp promoter)
5' GGA CAT TCA TCT GTC CAA CG 3' - IPF2 (from 553 bp promoter)
5' CTC AGT GTA TAT TGC TGT GC 3' - IPF3 (from 582 bp promoter)
5' CAT AAC TGT TTA ATA G 3' - IPF4 (from 913 bp promoter)
5' GCA TTC TTT TAC GTA TTT A 3' - IPF5 (from 913 bp promoter)
Reverse primer:
5' CAC ACA TAT CAC TCT TAA GG 3' - 1R
5' CCT GTT ATA CCA AGG CTT GCT G 3' - 2R

3.3.2. Cloning and transformation of the amplified products

The PCR amplified products after purification using GFX Illustra DNA Gel Band Purification Kit (M/S Amersham Pharmacia, USA) was used directly for cloning. Cloning of the amplified genomic DNA of the selected *Hevea* clones was carried out using the pGEM®-T Easy cloning vector for sequencing (M/S Promega, USA).

3.3.2.1. Ligation of the vector and the insert

The pGEM®-T Easy Vectors are approximately 3kb and are supplied at 50 ng/µl. The calculation of the appropriate amount of PCR product (insert) to be included in the ligation reaction was done using the following equation.

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

The ligation reactions were set up as shown shown below:

T4DNA Ligase 10 X buffer	-	1 µl
pGEM® -T Easy Vector (50 ng)	-	1 µl
PCR product	-	1 µl
T4 DNA ligase (3 weiss units/ul)	-	1 µl
Deionized water to a final volume of	-	10 µl

Mixed the reactions by pipetting and inubated the reactions overnight at 4 ^{0}C. Centrifuged the tubes containing the ligation reactions to collect the contents at the bottom.

The amplified product obtained from the primer combination IPF5 (forward) and reverse primer (1R) has been cloned in Zero Blunt® Topo® PCR cloning kit (M/S Invitrogen, USA) for the cloning of the blunt ended fragments according to the manufacturer's instructions. The ligation mix was transformed to the competent DH5α cells prepared and blue/white screening of the colonies was used to identify positive transformants.

3.3.2.2. Competent cell preparation

E.coli DH5α cells were used for cloning purpose. Competent cell preparation and transformation were done as follows (Ausubel *et al.* 1999).

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

ii. Single colonies from the plate was picked and cultured in 3 ml LB overnight at 37 ^{0}C in an environmental shaker (New Brunswick Scientific, USA).

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

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

v. The cells were harvested by spinning at 5000 rpm for 3 min at 4 ^{0}C.

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

vii. The tubes were then spun at 5000 rpm for 3 min at 4 ^{0}C and the supernatant was discarded.

viii. The cells were re-suspended in 200 µl 0.1 M ice-cold $CaCl_2$ and either quickly frozen to –70 ^{0}C for storage or kept in ice for immediate use.

3.3.2.3. Transformation of the competent cells

i. For transformation, 2-4 µl of the ligated mix was added to the competent cells and incubated in ice for 10 min.

ii. Heat shocked the cells at 42 ^{0}C for 2 min.

iii. After the heat shock, the tube was immediately transferred to ice and chilled for 5-10 min.

iv. 900 µl room-temperature SOC medium was added and the culture was incubated at 37 ^{0}C for 90 min with shaking at 150 rpm.

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

vi. The plates were incubated for 12 – 16 h at 37 ^{0}C.

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

SOC medium (100 ml)

2.0 g Bacto®-tryptone, 0.5 g Bacto®-yeast extract, 1 ml 1M NaCl, 0.25 ml 1M KCl, 1 ml 2M Mg^{2+} stock (filter sterilized), 1 ml 2M 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 20 mM. Brought to 100 ml with sterile, distilled water. The final pH was7.0.

2M Mg^{2+} stock

20.33 g $MgCl_2 \cdot 6H_2O$

LB medium (per liter) with ampicillin

10 g Bacto®-tryptone, 5 g Bacto®-yeast extract and 5 g NaCl
Adjusted pH to 7.0 with NaOH.

Added 15 g agar to 1 liter of LB medium and autoclaved. Allowed the medium to cool to 50°C before adding ampicillin to a final concentration of 100 µg/ml.

Poured 30–35 ml of medium into 90 mm petri dishes. Allowed the agar to harden.

IPTG stock solution (0.1M)

1.2 g IPTG

Added water to 50 ml final volume. Filter sterilized and stored at 4^0C.

X-Gal (2ml)

Took 100 mg 5-bromo-4-chloro-3-indolyl-β-D-galactoside. Dissolved in 2 ml N,N′-dimethylformamide. Covered with aluminum foil and stored at -20^0C.

100 µl of 100 mM IPTG and 20µl of 50 mg/ml X-Gal was spread over the surface of an LB ampicillin plate and allowed to absorb for 30 minutes at 37^0C prior to use.

3.3.2.4. Colony PCR, plasmid isolation and sequencing

The colony PCR of the transformed colonies were performed using insert specific primers as described in section. 3.1.8. Apart from the initial denaturation of 94 ^{0}C for 10 minutes, the remaining PCR profile was the same as described in section 3.1.4. The recombinant plasmids were isolated from the positive colonies and sequenced later at M/S Macrogen, Korea using the M13 forward and reverse primers.

3.4. PCR amplification of 3'UTR regions of β-1,3-glucanase isoforms

An attempt had been made to find out the variability if any with the 3' UTR regions of the obtained sequences *via* PCR. Forward primers specific to the promoter sequences and reverse primers complementary to the 3'UTR region based on the reported mRNA sequence of β-1,3-glucanase by Chye and Cheung (1996) (NCBI Acc. No. U22416) were used. The forward primers used for the PCR amplification of various genomic isoforms were based on the respective promoter forms as described in table.3 (section 3.3.1). Three reverse primers designed based on the 3' end of the gene were:

1) **5' GGA TAC ATG CAT ACA TTG G 3' 2) 5'GGT TCT CTT ATT TAA TAC ATG 3'** and **3) 5'TGA GAA AAG GTT CTC TT 3'**

3.5. Bio-informatic analysis

3.5.1. Structural analysis of the proteins

The modeling of the proteins was done using the MODELLER software (Šali and Blundell, 1993). Various python scripts were used in the process of homology modelling. The first step is to generate an alignment between the query and the subject sequence. Using this alignment and the PDB structure of the subject sequence, modeller build the coordinates of the query sequences using the coordinate information available in the subject sequence taking the alignment as the reference. It is possible to generate several models of the query sequence based on loop modelling and side chain orientations and for each model a DOPE (**D**iscrete **O**ptimized **P**rotein **E**nergy, is a statistical potential used to assess homology models in protein structure prediction) score is generated. The model with the best DOPE score is usually picked up. In this case 50 models were generated for the query and subject sequences to align with. The modelled query protein and the subject protein is superimposed using PyMol, so as to view the position of the residues in the structures.

3.5.2. Hydropathy analysis of the deduced amino acid sequences of β-1,3-glucanases

The hydropathy analysis of the deduced amino acid sequence (ExPASy online software (http://web.expasy.org/translate/)) of six newly isolated β-1,3-glucanases were carried out using on-line tool Kyte-Doolittle Hydropathy plot (Kyte and Doolittle, 1982) and the Hopp-woods scale (Hoop and Woods, 1981) (http://www.vivo.colostate.edu/molkit/hydropathy/). Kyte-Doolittle hydropathy plots predict the hydrophobicity of the peptides whereas Hopp-Woods scale predicts the hydrophilicity of the peptides. The hydrophobic regions are usually the transmembrane regions and the hydrophilic regions form antigenic regions of the peptide. With Kyte-Doolittle plots, the regions with values above 0 are hydrophobic in character. Whereas with the Hopp

woods scale, values greater than 0 are hydrophilic and thus likely to be exposed on the surface of a folded protein. The region of maximal hydrophilicity is likely to be an antigenic site.

3.5.3. Signal P analysis of the deduced peptide sequence and Plant-mPLoc prediction for the target organelle for β-1,3-glucanases

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 (Jannick *et al.*, 2004). The prediction of signal peptides in the sequence was done by the online SignalP (version.4.0) analysis software (Petersen *et al.*, 2011). The method incorporates prediction of cleavage sites and a signal peptide/non-signal peptide prediction (http://www.cbs.dtu.dk/services/SignalP/). 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 Plant-mPLoc software prediction shows the target organelle to which the peptide is transported.

3.5.4. Predicton of glycosylation sites in the deduced peptide sequence of β-1,3-glucanases using the NetNGlyc software

N- glycosylation usually occurs in the asparagines residues which occur in the Asn-Xaa-Ser/Thr stretch (where Xaa is any amino acid except proline). The NetNGlyc online software (http://www.cbs.dtu.dk/services/NetNGlyc/) was used to predict the high potential asparagines to be glycosylated in a peptide sequence (Gupta *et al.*, 2004). Here it predicted the potential glycosylation sites residing in the deduced peptide sequence of β-1,3-glucanases. C-glycosylation of the aminoacids were predicted through the NetCGlyc software (http://www.cbs.dtu.dk/services/NetCGlyc/) which predicts the potential C-glycosylated amino acid residue (usually tryptophan) aiding in the anchorage of the protein to the memberanes (Julenius, 2007).

3.5.5. Prediction of DNA architecture using the Z-curves and Isochore plots of β-1,3-glucanases

The Z curve is a three-dimensional space curve of DNA/ RNA. The Z-curve (http://tubic.tju.edu.cn/zcurve/) is a novel method to map the nucleotide sequence into a folding curve in three dimensional space, the Z curve. This has been proposed based on the symmetry of the regular tetrahedron plots for both the sequences (Zhang and Zhang, 1994). For a given DNA sequence there is a unique Z curve corresponding to it and it contributes further to a new area to visualize and analyze the DNA sequences by a geometrical approach. Here an attempt was carried out to learn the variations in the DNA architecture of the different β-1,3-glucanases. CpG methylation reduces backbone flexibility and dynamics. The DNA stiffness also negatively influences the nucleosome formation. The CpG in the promoter region can adopt a Z-DNA conformation under negative superhelical stress, and this is greatly facilitated by its methylation (Runkel and Nordheim, 1986). The isochore plots (http://www.ebi.ac.uk/Tools/emboss/cpgplot/) show DNA architecture with reference to their 'GC' content. The high potential regions shown by the isochore peaks show the areas of high nucleosome forming potential also.

3.5.6. Identification of potential antigenic sites residing in the deduced peptide sequence of β-1,3-glucanases

The online EMBOSS-Antigenic software (http://www.cgi-bin/emboss/antigenic) had been used to identify the potential antigenic regions residing in the peptide sequence of β-1,3-glucanases. Antigenic predicts potential antigenic regions of a protein sequence, using the method of Kolaskar and Tongaonkar (Kolaskar & Tongaonkar, 1990). The algorithm is purely based on the analysis of data from experimentally determined antigenic sites on proteins. If Cys, Leu and Val residues on the surface of a protein, they are more likely to be a part of antigenic sites. This method makes use of physicochemical properties of amino acid residues and their frequencies of occurrence in experimentally known segmental epitopes.

3.6. Functional validation of the promoters

By constructing chimeric promoter:GUS gene fusion binary plasmid vectors, functional characterization of the isolated promoters of different forms of β-1,3- glucanase gene was carried out through *Agrobacterium* mediated transgenic approach. pCAMBIA 1381 Z T-DNA binary vector constructs were made with the promoter sequences isolated. The basic promoters isolated at different lengths as well as different deletions of the basic constructs (total 11 constructs) have been made upstream to the translation initiation codon, in such a way that all fragments contain core promoter sequence. The efficiency and tissue specificity of the promoter could be monitored in terms of GUS (marker) gene expression.

For construction of the desired binary plasmid vector, pCAMBIA1381 Z were, obtained from M/S CAMBIA Vectors, (CAMBIA - **C**entre for **A**pplication of **M**olecular **B**iology to **I**nternational **A**griculture, Canberra, Australia). The complete nucleotide sequences of these vectors are available in NCBI site accession number-AF 234306 (Centre for Application of Molecular Biology to International Agriculture, Canberra, Australia). This vector is exclusively designed for promoter analysis in plan.

3.6.1. Promoter : GUS fusion binary plasmid vector construction

The T-DNA of pCAMBIA 1381 Z T-DNA vector harbors a promoter less GUS A reporter gene (*uidA*) with catalase intron (from castor bean catalase gene) inside the coding sequence to ensure that the expression of glucuronidase activity is derived from eukaryotic cells and not from residual *A. tumefaciens* cells (Hajdukiewicz *et al.*, 1994). The vector has the antibiotic hygromycin resistance gene (*hpt II* - **h**ygromycin **p**hosphotransferase), under CaMV 35S promoter for plant selection and kanamycin resistance gene (*npt II*- **n**eomycin **p**hosphotransferase B for bacterial selection. The truncated Z_{α} is functional for blue white screening of clones in *E. coli* host strain $DH5_{\alpha}$.

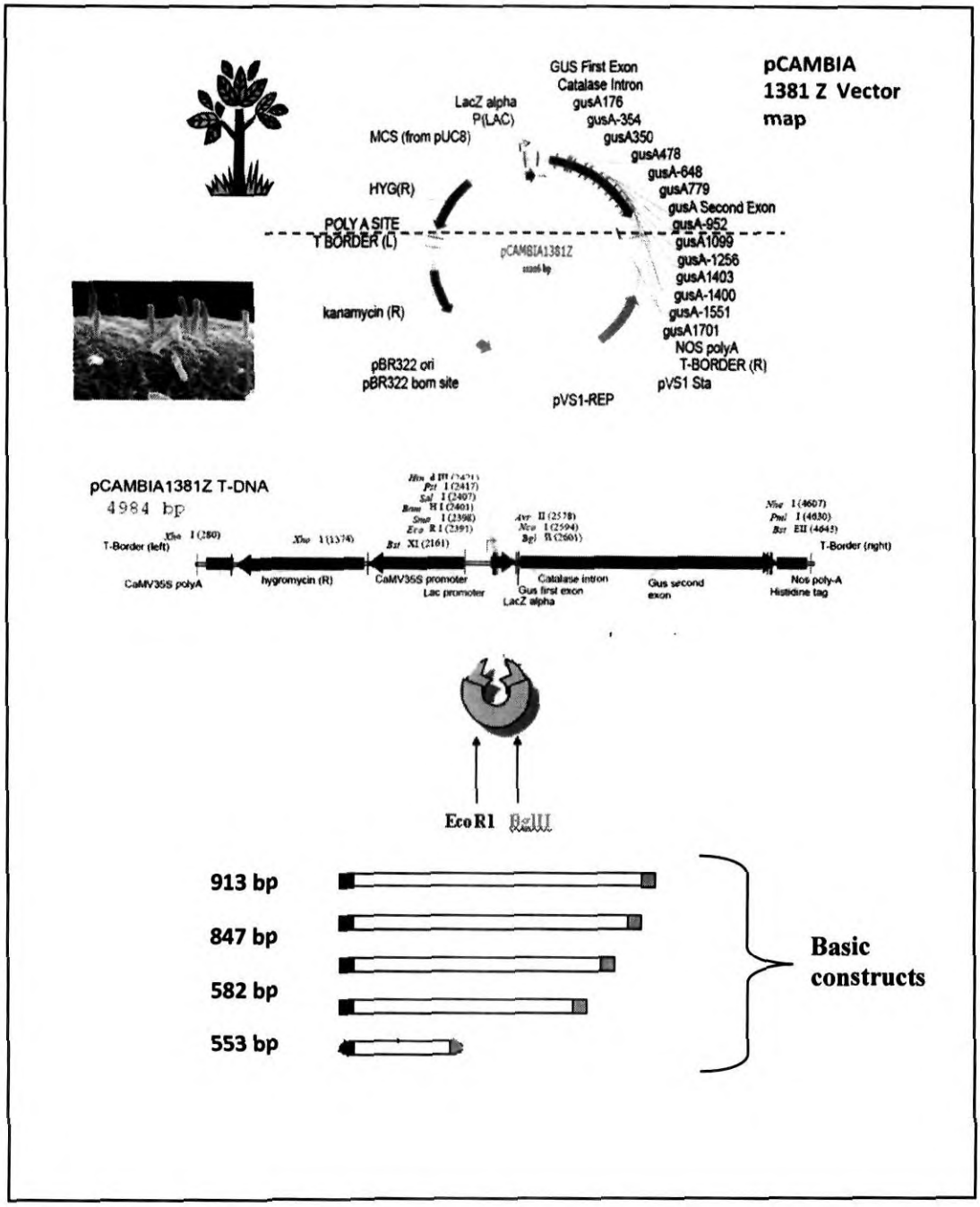


Fig.11. Schematic representation of the promoter: GUS gene fusion binary plasmid vector construction

3.6.1.1. PCR amplification of the promoter fragment (insert) from the Topo TA vector

The insert to be transformed to the pCAMBIA 1381Z TDNA vector was amplified from the cloned Topo TA vector (which was sequenced and

confirmed for the presence of the insert earlier). The PCR was performed using specific primers tagged with two separate restriction sites. Forward primer has been tagged with *EcoR* 1 site and reverse primer with *Bgl* II restriction enzyme site respectively. Primer sequences for the preparation of 11 binary constructs were synthesized. Total 11 binary vectors were developed which includes; five basic constructs with 913, 847, 582, 553 and 198 bps; the other six include two deletions of the 913 bp promoter with 550 and 200 bps (913_{550} and 913_{200}), two deletions of the 847 bp promoter with 550 and 200 bps (847_{550} and 847_{200}), a 200 bp deletion with 582 (582_{200}) and a 200 bp deletion with 553 bp (553_{200}) promoter. The details of the primer sequences used to amplify the deletion and non-deletion fragments of the promoter inserts is shown in table.4.

Table.4. List of primers used for the binary vector construction

Forward primers:

EcoR 1

5' GGA ATT CAG ATA TAT TCA GTT TC 3'	**- 582 bp promoter**
5' GGA ATT CGC TAT TAA GTA TGA TG 3'	**- 550 bp promoter**
5' GGA ATT CAT TTC TTC CAT TAA AT 3'	**- 198 bp promoter**
5' GGA ATT CTT AGA ATT AAA ATT TT 3'	**- 913 bp promoter**
5' GGA ATT CGC TCC CTA GGC TAT TA 3'	**- 847 bp promoter**

Forward primers for the amplification of deletion inserts:

5' G GA ATT C AG ATG CAA GGA GAG T 3'	**- 582_{200} bp**
5' G GA ATT C AA GAG CAC TAT ATT TAT G 3'	**- 550_{200} bp**
5' G GA ATT C AG AGC ACT ATA TTT TAG 3'	**- 910_{200}/ 847_{200}**
5' G GA ATT C CA TCC ATG TTT AAT AGA TC 3'	**- 910_{550}/ 847_{550}**

Reverse primers:

Bgl II

5' GAA GAT CTA CCA TTA AGA AGG ATG G	**- 579/550/847bp promoters**
5' GAA GAT CTA CCA TTA AGA AGG ATG G	**- 198 bp promoter**
5' GAA GAT CTA CCA TTA AGA AGG CGG G	**- 910 bp promoter**

3.6.1.2. Isolation of pCAMBIA 1381 Z TDNA vector from the DH5α glycerol stock

Alkaline lysis procedure for the large scale isolation of plasmid

i. An overnight grown 3 ml culture was taken and centrifuged at 6000 rpm for 5 min at 4°C and the supernatant was drained completely.

ii. The bacterial pellet was washed with 10 ml ice-cold STE buffer.

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

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

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

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

Solution II
0.2N NaOH, 1 % SDS

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

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

vi. Centrifuged at 6000 rpm for 15 min at 4 ^{0}C and the supernatant was saved.

vii. The supernatant was treated with 5 µl of RNase (10 mg/ml) and incubated at 37 ^{0}C for 1 hour.

viii. Supernatant was washed twice with an equal volume of chloroform : isoamyl alcohol and centrifuged at 10,000 rpm for 10 min.

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

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

xi. Pellet was washed in 70% ethanol, air-dried and dissolved in 100 µl of distilled water and stored at -20^0C.

xii. The plasmids isolated from DH5α were checked in 0.8% agarose gel.

3.6.1.3. Restriction enzyme digestion of the vector and the insert

PCR amplified promoter fragments and the vector (1µg each) were digested first with *Eco* RI (M/S Bangalore Genei, Bangalore) and the enzyme was heat inactivated at 65 ^{0}C for 20 minutes. The product was subjected to second digestion with the enzyme *Bgl* II (M/S New England Biolabs, UK). After second digestion, samples were purified by phenolic extraction followed by precipitation of DNA using 2 volumes of absolute ethanol.

3.6.1.4. Dephosphorylation of the vector using alkaline phosphatase

To reduce the vector background in the downstream cloning processes, the dephosphorylation of the vector had been carried out. Calf intestinal alkaline phosphatase (CIP) catalyzes the removal of 5'phosphate groups from the ribo and deoxyribo nucleoside triphosphates. Since CIP-treated fragments lack the 5' phosphoryl termini required by ligases, they cannot self ligate. For de-phosphorylation, the vector was suspended (0.5µg/10ul) in 1X buffer. Added 0.5U of CIP (M/S New England Biolabs, England) per microgram of vector DNA, incubated for 60 minutes at 37 ^{0}C and purified the DNA by phenol- extraction.

3.6.1.5. Ligation of the vector with insert and transformation

Ligation of the purified insert DNA and the dephosphorylated vector was carried out using the enzyme T4 DNA ligase (New England BioLabs, USA). Ligation of vector and inserts were carried out with an insert vector ratio of 1:10 using T_4-DNA ligase (New England Biolabs, USA). To this 1 µl of the T_4 DNA ligase enzyme was added and mixed well. The whole reaction mixture was made up to 20 µl with autoclaved double distilled water. All these were done inside a laminar air flow chamber. The reaction mixture was

incubated at 16 ^{0}C, overnight. For transformation, 2µl of the ligation mixture was added to the competent cells of DH5α cells prepared as described in section 3.3.2.1. The transformed cells was later plated on to LB agar medium with 50 µg/ml Kanamycin and incubated at 37 ^{0}C for 16 hours. To confirm transformation, colony PCR has been carried out using a single colony of transformed DH5α as the template and specific forward and reverse primers of the inserts, GUS (Forward primer 5' TAG AGA TAA CCT TCA CCC GG 3', Reverse primer 5' CGC GAA AAC TGT GGA ATT GA 3') and hygromycin gene (*hpt II* primers : Forward primer : 5' CGA TTG CGT CGC ATC GAC 3', Reverse primer: 5' CGT GCA CAG GGT GTC ACG 3'). Alkaline lysis procedure has been carried out to isolate the plasmid from the transformed colonies (section 3.6.1.2). The isolated plasmids have later been electrophoresed in 0.8 % agarose gel to check the quality.

3.6.1.6. Purification of recombinant plasmid DNA by Polyethylene glycol (PEG)

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

i. To 32 µl of plasmid DNA, 8 µl of 4M NaCl was added followed by 40 µl of 13% PEG 8000.
ii. After thorough mixing, the sample was incubated on ice for 20 min.
iii. The precipitated plasmid DNA was pelleted by centrifugation at 10,000 rpm for 10 min at 4 ^{0}C.
iv. The supernatant was discarded and the pellet was rinsed with 70% ethanol.
v. Pellet was air-dried and re-suspended in 20 µl of sterile double distilled H_2O and stored at –20 ^{0}C.

The sequencing of the recombinant binary vectors was done at M/S Macrogen, Korea to confirm the presence and orientation of the insert. The sequencing was done using the forward primer designed from the respective promoter regions.

3.6.2. *Agrobacterium* mediated genetic transformation

3.6.2.1. Transformation of *Agrobacterium tumefaciens* with the recombinant binary vectors

Agrobacterium tumefaciens strain EHA 105, an octopine type *vir* helper strain that harbors the disarmed Ach5 Ti plasmid has been used for the transformation experiments.

3.6.2.1.1. Competent cell preparation of the *Agrobacterium* strain EHA 105

The competent cells of *Agrobacterium tumefaciens* strain EHA 105 were prepared following the method reported by Jyothishwaran *et al.*, (2007). *Agrobacterium* strain was streaked out on a LB plate containing 20 µg/ ml rifampicin and grown at 28 ^{0}C overnight. A single colony was used to inoculate 3 ml of LB medium containing 20 µg/ ml rifampicin and grown for 48 hrs at 28 ^{0}C, with shaking at 160 rpm. A 50 ml of LB medium with 20 µg/ ml rifampicin was inoculated with 0.5 ml (1/100th volume) of the overnight culture and grown in an incubator- shaker at 28 ^{0}C at 160 rpm to obtain cell density of 0.3 at OD_{600}. The cultures were chilled once for 15 min and the cells were harvested by centrifugation at 3000 rpm for 5 min at 4 ^{0}C. Resuspended in 10 ml of sterile ice-cold 100 mM $MgCl_2$ solution, and incubated on ice for 1 h. Centrifuged and the resulting pellet was resuspended in sterile ice-cold 2 ml 20 mM $CaCl_2$, and incubated on ice for 6 h to yield the competent cell suspension. The suspension was dispensed into 1.5 ml tubes and stored at 80 ^{0}C.

3.6.2.1.2. Transformation of *Agrobacterium*

Transformation of the *Agrobacterium competent cells* was carried out through freeze - thaw method (Holsters *et al.*, 1978).

i. 100 µl of cell suspension was mixed with 0.5 µg plasmid DNA and frozen in liquid nitrogen.
ii. Competent cells were thawed at 37 ^{0}C for 20 min
iii. One ml medium (LB) supplemented with 1 µg/ml kanamycin was then added and incubated for 2 hr at 28 ^{0}C.
iv. 100 µl of the bacterial culture was plated on LB agar plate containing 50 µg/ml kanamycin and 30 µg/ml rifampicin.

v. Plates were incubated for two days at 28 ^{0}C.

vi. White colonies observed were selected for screening.

Along with this *A. tumefaciens* was also transformed with the original pCAMBIA 1381 T-DNA vector having promoter less GUS gene for using as negative control.

3.6.2.1.3. Screening of putatively transformed colonies of *Agrobacterium*

Colonies observed were screened through colony PCR to confirm the presence of binary vector using the respective promoter fragment specific primers at an annealing temperature of 55 ^{0}C (The other parameters followed were as reported in section. 3.1.4).

3.6.3. Genetic transformation of Tobacco leaf discs and *Hevea* callus

A. Media Composition : Media used was Murashige and Skooge's basal media (Murashige and Skooge, 1962).

1. **Co- cultivation medium** – MS supplemented with 1.0 mg/L BAP and 0.10 mg/L NAA.
2. **Regeneration and selection medium** – MS supplemented with 1.0 mg/L BAP and 0. 1 mg/L NAA.

The media supplemented with the hormones were autoclaved at 121°C for 15 min and cooled to ~ 50 ^{0}C, added hygromycin 30 mg/l and cefotaxime 300 mg/l. Further the media was poured into sterile petri plates and bottles.

Table. 5. The basic components in co-cultivation and selection/ regeneration medium for tobacco

Components	Co-cultivation medium (for 500 ml)	Selection medium (for 500 ml)
MS major	25.0 ml	25.0 ml
MS minor	2.5 ml	2.5 ml
MS vitamins	2.5 ml	2.5 ml
Fe-EDTA	0.01835 g	0.01835 g
Sucrose	10.0 g	10.0 g
Myoinositol	0.05 g	0.05 g
BA	2.5 ml (1.0 mg/l)	2.5 ml (1.0 mg/l)
NAA	250.0 µl (0.1 mg/l)	250.0 µl (0.1 mg/l)
Phytagel	1.0 g	1.0 g
Acetosyringone	20.0 mg	-
Proline	57.5 g	-
Betane hydrochloride	76.5 mg	-
Hygromycine	-	30.0 µg/µl
Cefotaxime	-	0.2 g

3.6.3.1. Development of explants

3.6.3.1.1. Tobacco leaf discs

Sterile tobacco (*Nicotiana tabaccum* L. Petite Havana strain) plants were developed from leaves obtained from glass house grown plants. Leaves were thoroughly washed and surface sterilized with 0.1% mercuric chloride for 3 min using Tween- 20 as surfactant. The explants were again treated with 70 % ethanol for 3 min. Further the explants were washed extensively with sterile distilled water. Water from the explants was removed using sterile blotting paper. Leaves were chopped into discs of suitable pieces and placed (right side up) on MS medium supplemented with 1.0 mg/l BAP and 0.10 mg/l NAA. After two weeks, shoots emerged directly from the explants. Shoots

were cut at the base and placed on to MS medium without any plant growth regulators. The leaf discs from the regenerated plantlets were directly used for *Agrobacterium* mediated genetic transformation.

3.6.3.1.2. Development of *Hevea* callus

Embryogenic calli derived from *Hevea* leaf were tried for infection with *Agrobacterium*. Calli were induced in leaf explants collected from glass house grown plants of clone RRII 105 (Kala *et al.*, 2006). Leaf segment after two days culture in ½ MS (Murashige and Skoog, 1962) liquid medium containing phytohormones BA (1 mg/l), GA_3 (1.5 mg/l) and NAA (0.5 mg/l) were cultured with the adaxial surface in contact with the callus induction medium. Callus induction medium contained MS salts with addition of calcium nitrate (850 mg/l) and supplemented with casein hydrolysate (1 mg/l), B_5 vitamins, sucrose (20 g/l) and phytohormones, 2,4-D (1.2 mg/l), BA (2.0 mg/l) and NAA (0.2 mg/l). The medium was solidified with 0.2% phytagel. The cultures were maintained in the dark. After 45 days culture, the calli formed were carefully excised from the explants and proliferated. These were subcultured in modified MS medium containing B_5 vitamins (Gamborg, 1968) and amino acids, organic supplements and phytohormones NAA (0.1 mg/l), kinetin (0.3 mg/l), BA (0.5 mg/l) and GA_3 (0.8 mg/l). Calli were kept under the same environmental conditions as during callus induction in the dark. The calli turned black after 50 days in the embryo induction medium and yellow embryogenic calli emerged from them. Embryogenic calli could be proliferated when sub-cultured in the same medium with reduced auxin and increased sucrose. Proliferated calli obtained was used for transient assay with *Agrobacterium*.

3.6.3.2. Preparation of *Agrobacterium* Culture for Transformation

The bacteria were streaked, incubated and grown for 48 h at 28 ^{0}C on a solid LB plate supplemented with 50 µg/ml kanamycin and 20 µg/ml

rifampicin. Single colony from the LB agar plate was inoculated into liquid LB medium and cultured overnight at 28 ^{0}C for 18-20 h in a rotary incubator till OD_{600} reached 1.3. The culture was centrifuged; pellet was re-suspended in MS liquid medium.

40-50 µl of the resuspended culture was added to 5 ml MGL medium pH 7.0 (Jones *et al.*, 2005), containing 50 mg/l rifampacin and 100 mg/l carbenicillin. This was kept in an incubated shaker overnight at 28 ^{0}C at 250 rpm.

MGL Medium : pH 7.0

Tryptone- 5g/l, Yeast extract- 2.5g/l, NaCl - 5g/l, Glutamic acid- 1.16g/l, KH_2PO_4 – 250 mg/l, $MgSO_4.7H_20$- 100 mg, Biotn 1 mg/l

2.5 ml of the overnight grown culture was transferred to 7.5 ml of TY medium (pH- 5.5) containing the antibiotics and 200 µm acetosyringone. The cultures were incubated overnight at 28 ^{0}C at 250 rpm.

TY MEDIUM : pH 5.5

Tryptone- 5g/l, Yeast extract- 3g/l, Acetosyringone- 40 mg/l, Kanamycin- 500 µl, Hygromycin- 300 µl, Rifampacin- 200 µl

Next day 1.5 ml of bacterial culture was diluted to 20 ml by adding TY medium (pH 5.5), containing 200 µm acetosyringone.The OD was measured against TY blank at 600 nm adjusted to optimum level (0.1- 0.2) for transformation.

3.6.3.3. *Agrobacterium* infiltration of the explants

i. Tobacco leaf bits and *Hevea* calli were immersed in bacterial suspension taken in petri dishes and kept in a vacuum desiccator connected to a vacuum pump. Vacuum was applied for 15 min at 40

psi to infiltrate *Agrobacterium* into callus. The whole setup was swirled gently to avoid trapping of air bubbles in and to promote infiltration.

ii. Leaf bits and callus were then dried by blotting with filter paper and kept in a petri dish containing sterile whatman filter paper, wetted by drenching with MS solution.

iii. The blotted leaf discs were placed with its adaxial side down on co-cultivation medium (table: 5) prepared and stored for 2 days in the dark at 23 ^{0}C. Similarly callus was also placed in the co-cultivation medium. After 48 hrs, the tobacco leaf discs and the *Hevea* callus were checked for the GUS activity (transient assay).

iv. After co-cultivation, leaf discs were washed with sterile distilled water thrice to remove excess bacterial residue.

v. Wet leaf discs were blotted dry and transferred with adaxial side up on regeneration medium MS-2 containing hygromycin (30 mg/l) as the selection agent and cefotaxime (300 mg/l) to prevent bacterial overgrowth.

vi. The regenerated shoots were removed and placed in culture tubes (17.5 cm length, 3.5 cm upper diameter, 5.5 cm bottom diameter) containing the regeneration medium for further shoot growth and rooting

vii. Petri dishes were sealed with Para film and transferred to 16 h day length at 22 ^{0}C until shoots regenerated.

3.6.3.4. Histochemical analysis of GUS Activity

Histochemical GUS activity was detected by staining with X-gluc (5-bromo-4-chloro-3-indoyl β-D-glucuronide).

X- gluc Solution
50 mM sodium phosphate buffer (pH-7.0), 10 mM EDTA, 0. 1% Triton-X-100, 0.5 mM potassium ferricyanide, 2 mM X-gluc

Transgenic tissues were placed in a solution of X-gluc and incubated at 37 ^{0}C overnight (Jefferson *et al.*, 1987) after 48 hrs. The enzyme β-glucuronidase contained in the transgenic tissues/ callus cleaves the substrate

X-gluc into glucuronic acid and indoxyl derivative. The indoxyl derivative dimerizes and is then oxidized to form insoluble, highly colored indigo dye (dichloro-di bromo indigo, ClBr indigo). The stained tissues were cleared of chlorophyll (in the case of tobacco leaves) by washing with 70% alcohol (v/v) and photographed.

3.7. Heterologous expression of β-1,3-glucanase gene isoforms and *in vitro* antifungal assay with the purified proteins

3.7.1. Yeast transformation

pPICZ A (non-secretory vector), is a 3.3 kb vector used to express recombinant proteins in yeast *Pichia pastoris*. The *Pichia* 'X- 33', is a wild type strain which utilizes methanol in the medium (Mut^+) has been used here. Recombinant proteins are expressed as fusions to a C-terminal peptide containing the *c-myc* epitope and a polyhistidine (6X His) tag. The vector allows high level, methanol inducible expression of the gene of interest in *Pichia*. pPICZ A (fig. 12) contains the following elements:

i. 5' fragment containing the *AOX 1* (Alcohol oxidase) promoter for methanol-induced expression of the gene of interest
ii. Zeocin resistance gene for selection in both *E. coli* and *Pichia*
iii. C-terminal peptide containing the *c-myc* epitope and a polyhistidine (6X His) tag for the detection and purification of a recombinant fusion protein
iv. The reading frame to facilitate in-frame cloning with the C-terminal peptide

Experimental Overview

Following are the basic steps involved in the cloning and expression of the gene of interest in pPICZ A yeast vector.

i. Propagated pPICZ A by transformation into a *rec*A, *end*A1 *E. coli* strain such as DH5α.

ii. Developed a cloning strategy and ligated the gene into pPICZ Avector in frame with the C-terminal tag.

iii. Transformed into *E. coli* and selected transformants on Low Salt LB plates containing 25 µg/ml Zeocin.

iv. Analyzed the transformants by sequencing to confirm in-frame fusion of the gene with the C-terminal tag.

v. Purified and linearized the recombinant plasmid for transformation into *Pichia pastoris*.

vi. Transformed *Pichia* strain and plate on to YPDS plates containing the appropriate concentration of Zeocin.

vii. Selected for Zeocin-resistant transformants.

viii. Optimized expression of the gene.

ix. Purified the fusion protein with His-tag purification column.

3.7.2. Isolation of the vector pPICZ A from DH5α cells

The non- secretory vector pPICZ A had been multiplied in DH5α cells after transformation. The Zeocin antibiotic (25µg/ml) was used as the selection marker here. Later the vector was isolated in large quantities from the transformed cells using the alkaline lysis method (section. 3.6.1.2).

3.7.3. Restriction digestion of the pPicZ A vector followed by de-phosphorylation

The vector was digested with the appropriate restriction enzymes as follows: First digestion with *Eco*R1 followed by 2nd digestion with *Kpn* 1. The first digestion was followed by heat inactivation of the enzymes and at 65 ^{0}C, 20'. After the second digestion, the fragments were purified from the solution by ethanol precipitation followed by 70 % alcohol wash, dried and dissolved in autoclaved Milli Q water. The vector had been de-phosphorylated using the calf intestinal alkaline phosphatase (CIP) (section.3.6.1.4) to prevent self ligations during the subsequent vector : insert ligation procedure.

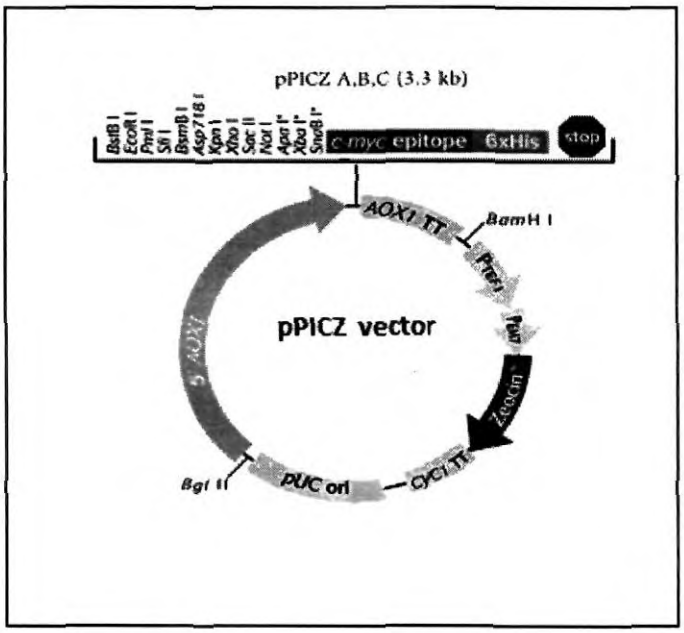


Fig: 12. Map of pPICZ vector

3.7.4. Amplification of the insert using restriction site tagged primers followed by enzyme digestion

The insert should contain an initiation ATG codon as part of a yeast consensus sequence (Romanos *et al.*, 1992) **[(G/A)NNATGG].** To express the gene as a recombinant fusion protein, it must be cloned in frame with the C-terminal peptide containing the *c-myc* epitope and the polyhistidine tag. For this the ORF inserted should not contain the stop codon.

The six inserts corresponding to six forms of β-1,3-glucanase have been amplified from the appropriate cloned TA vector (Strataclone vector) using the restriction enzyme site tagged primers for each form of the β-1,3-glucanase gene. The following primers were used to clone the gene forms in the suitable pPICZ A vector (synthesized at M/S Metabion, Germany). PCR was performed as described in section 3.1.4. Restriction digestion of the

nserts amplified from the cloned pGEM®- T Easy vector was done similar to hat of the pPIC Z vector (*EcoR*1 and *Kpn* I digestion).

The forward primers were tagged with *EcoR* 1 restriction site (GAA↓ TTC) and reverse primers with *Kpn* 1 site (GGT↓ ACC). An yeast Kozak consensus ' ACG' sequence has been provided before the start codon ATG. The primers were designed in such a way that while cloning the 'ATG' codon s in frame with the C-terminal peptide containing the *c-myc* epitope and the polyhistidine tag (table.6)

Table.6. List of primers used for the amplification of different β-1,3-glucanase isoforms for cloning in pPICZ A vector

For pPICZ A vector
Forward primers : (start codon underlined)

	EcoR I	Kozak consesus	
5' GGA ATT CAC G	AT G	GC TAT CTC CTC TAC 3'	- Gln 5, Gln 6, Gln 7
5' GGA ATT CAC G	AT G	GC TAT CTG CTC TTC 3'	- Gln 2
5' GGA ATT CAC G	AT G	GC TAT CTT CTC TTC 3'	- Gln 4
5' GGA ATT CAC G	AT G	GC CAT GCT TTC TTC 3'	- Gln 3

Reverse primers (without the stop codon)

Kpn I
5' GGG GTA CCA TAT CAC TCT TAA GGA C 3' - (for all five forms except Gln 4)
5' GGG GTA CCG TAA ATA TCA CTC 3' - (Gln 4 reverse)

3.7.5. Ligation of the vector and the insert followed by transformation to competent *E.coli*

Ligation of the digested insert and vector which was de-phosphorylated after the digestion had been carried out with T4 DNA ligase enzyme in a 20 µl reaction. An insert : vector ratio of 3:1 has been used for efficient ligation. The ligated mixture was later transformed into chemically competent DH5α cells for the multiplication. To screen the positive colonies, the transformed cells were plated on antibiotic plates containing the Zeocin (25 µg/ml) antibiotic. To identify the positive transformants, the transformed colonies obtained in the zeocin plates were subjected to colony PCR

(section.3.1.8) using the insert specific primers. From the positive colonies, recombinant plasmids have been isolated by the alkaline lysis method (section 3.6.1.2).

3.7.6. Sequencing of the recombinant plasmids

The sequencing of the recombinant plasmids representing each of the six forms of β-1,3-glucanases were done to confirm the presence and also to confirm the right orientation of the insert in the pPICZ A vector. For sequencing 5'AOX (forward) and 3'AOX primers (reverse) were used.

Forward: **5' GAC TGG TTC CAA TTG ACA AGC 3' - 5'AOX**

Reverse: **5' GCA AAT GGC ATT CTG ACA TCC 3' - 3' AOX**

3.7.7. Linearization of the recombinant plasmids

For integration of the cassette into the *Pichia* genome linearization of the vector is necessary. Here the enzyme chosen for the linearization is *Sac* I (M/S Bangalore Genei, Bangalore). About 5μg of each recombinant vectors (positive for the six inserts of β-1,3-glucanase) and insert-less vector (negative control) were treated with the *Sac* I enzyme (M/S Bangalore Genei, Bangalore) in appropriate buffering conditions. The reactions (20μl) were set up in separate tubes and incubated at 37 ^{0}C overnight. The enzyme cuts its single site available in the *5' AOX1* region to linearize the recombinant and the non-recombinant pPICZ vectors. Checked for the occurrence of the linearization event *via* agarose gel (1%) electrophoresis.

3.7.8. Purification of the linearized plasmid

After ensuring the complete linearization of the vectors, heat inactivated the reaction mixture at 65 ^{0}C for 20 minutes to stop the reaction. Extracted once with phenol/chloroform. The DNA in the supernatant was precipitated in presence 1/10 volume of 3M sodium acetate and 2.5 volumes of 100% ethanol. After the precipitation, centrifuged at 5,000 rpm for 3

minutes to pellet the DNA and washed the pellet with 80% ethanol. The pellet was air-dried, and resuspended in 10 µl sterile, de-ionized water.

3.7.9. Transformation of *Pichia pastoris* with the expression vector

The preparation of chemically competent *Pichia pastoris* strain 'X-33' and transformation has been done using the The *Pichia* EasyComp™ Kit (M/S Invitrogen, USA). X-33 is a wild-type *Pichia* strain that is useful for selection on Zeocin™ and large scale growth. The strain is methanol utilization positive (Mut^+ -Methanol utilization plus) so that 'X-33' can metabolize methanol as the sole carbon source. This will grow in YPD and in minimal media. As per the manufacturer's instructions, the competent cell preparation and the transformation was undertaken with X-33.

3.7.9.1. Preparation of competent *Pichia* and transformation

i. Streaked a YPD plate with the *Pichia pastoris* strain – X-33 and incubated at 30 ^{0}C for 2 days.

ii. Inoculated 10 ml of YPD with a single colony of the *Pichia* strain. Grown overnight at 30 ^{0}C in a shaking incubator (280 rpm).

iii. Diluted the cells from the overnight culture to an OD_{600} of 0.2 in 10 ml of YPD.

iv. Grown the cells at 30 ^{0}C in a shaking incubator until the OD_{600} is 0.8 (This will take approximately 5 hours).

YPD (+ Zeocin™) Yeast Extract Peptone Dextrose Medium (1 liter)

1% yeast extract, 2% peptone, 2% dextrose (glucose) and 2% agar.

Dissolved 10 g yeast extract and 20 g of peptone in 900 ml of water. Included 20 g of agar for making YPD plates. Autoclaved for 20 minutes at 121 ^{0}C for 15 minutes. Cooled the solution to ~60 ^{0}C and added 100 ml of filter sterilized 10X D (10X dextrose).

v. Pelleted the cells by centrifugation at 500 X g for 5 minutes at room temperature.

vi. Resuspended the cell pellet in 10 ml of Solution I (sorbitol solution containing ethylene glycol and DMSO).

vii. Immediately pelleted the cells by centrifugation at 500 X g for 5 minutes at room temperature. Discarded the supernatant.

viii. Resuspend the cell pellet in 1 ml of Solution I in the kit. The cells are now competent.

ix. Aliquoted 50 µl of competent cells into labeled 1.5 ml sterile screw-capped microcentrifuge tubes.

x. For immediate use, the cells kept at room temperature were taken directly for transformation. The remaining ones were frozen and wrapped in several layers of paper towels and placed in a –80 ^{0}C freezer for the future use.

xi. Added 5 µg of linearized *Pichia* expression vector DNA of each of the six different forms to the thawed tubes containing the competent cells.

xii. Added 1 ml of Solution II (PEG solution) (after warming at 37 ^{0}C) from the *Pichia* Easy Comp™ Kit (M/S Invitrogen, USA) to the DNA mixture and mixed by vortexing the tube.

xiii. Incubated the reactions for 1 hour at 30 ^{0}C in a water bath or incubator for 15 minutes (mix by vortexing)

xiv. Heat shocked the cells in a 42 ^{0}C heat block for 10 minutes.

xv. Divided the cells into 2 micro centrifuge tubes (approximately 525 µl per tube) and added 1 ml of YPD medium to each tube.

xvi. Incubated the cells at 30 ^{0}C for 1 hour to allow expression of Zeocin resistance.

xvii. Pelleted the cells by centrifugation at 3,000 X g for 5 minutes at room temperature.

xviii. Resuspended each tube of cells in 500 µl of Solution III supplied in the kit (after equilibration at room temperature) and combined the cells into one tube.

xix. Pelleted the cells by centrifugation at 3,000 X g for 5 minutes at room temperature. Discarded the supernatant.

xx. Resuspended the cell pellet in 100 to 150 µl of Solution III (Salt solution for washing and plating transformed cells).

xxi. Plated the entire transformation mixture in YPDS selection plates containing the antibiotic Zeocin (100 µg/ ml) using a sterile spreader. Incubated the plates for 3 to 10 days at 30 ^{0}C.

YPDS + Zeocin™ Agar

Yeast Extract Peptone Dextrose Medium (1 liter)

1% yeast extract, 2% peptone, 2% dextrose (glucose), 1M sorbitol
2% agar, 100 µg/ml Zeocin.
Dissolved 10 g yeast extract, 182.2 g sorbitol and 20 g of peptone in 900 ml of water. Added 20 g of agar. Autoclaved for 20 minutes at 121 ^{0}C. Added 100 ml of 10X D. Cooled the solution to ~60 ^{0}C and added 1.0 ml of 100 mg/ml Zeocin™.

3.7.9.2. Colony PCR to confirm the transformation in *Pichia*

A few *Pichia* colonies from each YPDS plates representing each of the six different forms of β–1,3-glucanases were selected after 96 hours and colony PCR was done to identify the positive clones. The confirmation of transformation was done by amplification of the colonies obtained. PCR was amplified with 5' AOX1 and 3' AOX 1 primers (section 3.7.6). Amplification was checked in 1.5 % agarose gel.

3.7.10. Confirmation of methanol utilization of *Pichia* by growing in MM plates

Though the X-33 strain used for the transformation is methanol utilization positive, a screening was done to check whether the gene for methanol utilization is functionally active in the transformed host cells. If the inserted gene fragment integrates into the native AOX gene, it will distrupt the native AOX gene making the host a non- methanol utilizing one (Mut^S). So the host in which the gene is intact can be methanol induced for the expression of the protein. They will grow on the MM plates containing methanol (Mut^+).

i. Using a sterile toothpick, picked up ten transformed colonies of *Pichia* for each of the six forms of the glucanase and inoculated in a regular pattern on an MM plate. Used a new toothpick for each transformant and continued until 10 transformants have been patched for each of the six *Pichia* clones.

ii. Incubated the plates at 30 ^{0}C for 2 days.

iii. After 2 days at 30 ^{0}C, the plates were observed for the growth of the *Pichia* clones. Mut$^+$ strains will grow normally on the plates, while Mut^S strains show little or no growth on the MM plate. Methanol was added to the growth medium daily to compensate for the loss due to evaporation and consumption. Everyday 100 µl of 100% methanol was supplemented to the lid and the plates were kept inverted.

Preparation of Minimal Methanol plates (MM plates) (1 liter).

Stock solutions:

i) 10X YNB (13.4% Yeast Nitrogen Base with Ammonium Sulfate without amino acids)

Dissolved 134 g of yeast nitrogen base (YNB) with ammonium sulfate and without amino acids in 1000 ml of water and filter sterilized. Heated the solution to dissolve YNB completely in water. Stored at 4 ^{0}C.

ii) 500X B (0.02% Biotin)

Dissolved 20 mg biotin in 100 ml of water and filter sterilized. Stored at 4 ^{0}C.

iii) 10X M (5% Methanol)

Mixed 5 ml of methanol with 95 ml of water. Filter sterilized and stored at 4 ^{0}C.

Preparation of MM Medium (1 L)

34% YNB, 4 X 10^{-5}% biotin and 0.5% methanol

For preparing the medium, autoclaved 800 ml of water for 20 minutes at 121 ^{0}C. Cooled the autoclaved water to 60 ^{0}C and added 100 ml of 10X YNB, 2 ml of 500X B, 100 ml of 10X M . For MM agar plates added 15 g agar to the water prior to autoclaving. After mixing, poured the plates immediately. MM plates were stored at 4 ^{0}C.

3.7.11. Southern hybridization

3.7.11.1. Genomic DNA isolation from the positive transformant and negative control of *Pichia pastoris*

The isolation of the genomic DNA from *Pichia pastoris* has been done following the protocol developed by Tomita *et al.*(2002). The procedure is as follows,

i. Inoculated 3 ml of the YPD medium (1% yeast extract, 2% peptone, 2% dextrose) (medium composition shown in section 3.7.9.1) with a single colony of *P. pastoris* transformants. Incubated overnight at 30 ^{0}C, with shaking (250 rpm).

ii. Pelleted 2 ml of an overnight culture of *P. pastoris* by microcentrifugation at 10,000 Xg for 1 min at room temperature and removed the medium.

iii. Added 400 µl of the lysis buffer (50 mM Tris-HCl pH 7.2, 50 mM EDTA, 3% SDS, 1% β-Mercaptoethanol) and vortexed until the mixture is homogeneous. Incubated at 65 ^{0}C for 1 h.

iv. Added an equal volume of phenol (50mM Tris-HCl (pH 8.0) saturated): chloroform (1:1) and vortexed for 20s. Microcentrifuged at 10,000 X g for 15 min at room temperature or until aqueous phase is clear.

v. Removed the aqueous phase containing DNA to a new tube without disturbing the interphase. Added 0.1 volume of 3M sodium acetate buffer (pH 5.2) to the aqueous phase followed by a 0.6 volume of isopropanol. Mixed gently by inversion for 2 min. Centrifuged at 10,000 X g for 15 min. Poured off the supernatant. Rinsed the pellet once with 70% ethanol and centrifuged again under the same conditions as above.

vi. Dried the pellet at room temperature for 15 min. Resuspended the pellet in 50 µl water with 50 mg/mL *RNase* A. The final concentration of total DNA should be in the range of 1 to 10 mg/ml.

3.7.11.2. Restriction digestion of *Pichia* genomic DNA

Southern blot analysis was conducted with genomic DNA isolated from transformed *Pichia pastoris* harbouring Gln 2. The isolated genomic DNA from the positive and negative control DNA (20 µg) was digested with *Bcl 1, Bam Hl, Ass 1,* enzymes. The *Pichia* genome transformed with the insertless vector was used as the negative control here.

The reaction mix (50 µl) was prepared as follows:

DNA	-	15 µl (20 µg)
Enzyme buffer	-	5.0 µl (1X)
Restriction enzyme	-	5.0 µl (50 U)
Distilled H_2O	-	25 µl

The digestion was continued overnight at 37 ^{0}C and the fragments were separated in 1% agarose gels.

3.7.11.3. Blotting

Blotting was carried out following the standard procedure developed by Southern (1975). DNA digests were loaded in 1.0 % agarose gel and electrophoresis was carried out at 40V for 5h. The gel was processed after electrophoresis as follows:

i. DNA in the gel was depurinated by soaking in a solution of 0.25 N HCl for 15 min and rinsed the gel twice with distilled water briefly.

ii. Denaturation of the DNA was carried out by treating the gel in denaturation solution for 25 min with gentle shaking followed by rinse with water

Denaturation Solution:
0.2M NaOH, 1.5M NaCl

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

Neutralization Solution:
1M Tris-HCl (pH-8.0), 1.5M NaCl

iv. DNA was transferred from the treated gel to nylon membrane (Hybond N$^+$, Amersham, UK) through capillary blotting method (Sambrook and Russell, 2001). The gel after neutralization was briefly washed in 10X SSC and kept ready.

20 X SSC:
3M NaCl, 0.3M Sodium citrate (pH.7.0)

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

vi. The surface of the platform was covered with whatman No. 3 filter paper presoaked in 10X SSC in such a way that the ends of the paper are immersed in the SSC. Three sheets of whatman No.1 filter paper trimmed to the same dimensions of the gel and presoaked in 10X SSC was placed on top of the platform. Air bubbles trapped any were removed by rolling the surface with a glass rod.

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

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

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

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

xiii. The membrane was fixed using a UV cross linker (Hoefer, USA) at 12000 J/cm^2.The membrane was covered with saran wrap and stored between ordinary filter papers in the refrigerator.

3.7.11.4. Preparation of labeled probes

The *Gln 2* gene was used as the probe for southern hybridization here. The probe amplified from pGEM–T® Easy vector through PCR using specific primers, were purified and radio labelled using 'Multiprime DNA labeling system' from M/S Amersham (UK) following manufacturer's instructions. It utilizes random hexanucleotides to prime DNA synthesis on denatured template DNA at numerous sites along its length. The procedure:

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

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

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

iv. 2.5 µl of random primer solution was added.

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

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

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

viii. Spun for few seconds and incubated at 37 ^{0}C for 30 min.

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

x. The labeled probe was purified by passing through a Sephadex G-50 column.

Preparation of sephadex G-50 column

Sephadex G-50 was added to dist.water to form a slurry (10 g of dry powder yields around 160 ml of slurry). Glass wool was placed at the bottom of a 1 ml column and 1 ml of the slurry was added without trapping air bubbles. The column was spun at 3000 rpm for 3 min in a swinging bucket rotor. More slurry was added until the sephadex tightly packed up to 1 ml level. The column was equilibrated first with STE buffer and then with distilled water.

xi. The column purified probe was denatured by boiling at 100 ^{0}C for 3 min and immediately chilled in ice. The purification helps to remove all the small nucleotides and other unincorporated nucleotides to avoid background signals in the blot.

3.7.11.5. Hybridization of the blot

Hybridization of the labeled probe to the nylon membrane was performed according to Sambrook and Russell (2001).

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

Pre-hybridization solution

6 X SSC, 5 X Denhardt's reagent, 0.5 % SDS

50 X Denhardt`s reagent

1.0 % BSA, 1.0 % Ficoll, 1 % polyvinylpyrrolidone

ii. The pre-hybridization was carried out at 65 ^{0}C for 1 h in a hybridization oven (M/S Amersham, UK) with rotary movement at very low speed.

iii. The pre-hybridization solution was poured out and hybridization solution (pre-hybridization solution containing denatured probe DNA labeled with α-^{32}P) was poured into the tube and then incubated with slow rotation for 12 – 16 h at 65 ^{0}C.

3.7.11.6. Washing the blot and autoradiography

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

Washing solution I
2 X SSC, 0.1 % SDS

ii. Then the blot was subjected to two high stringent washes at 65 ^{0}C for 30 min each with solution II.

Washing Solution II
0.1 X SSC, 0.5 % SDS

iii. The membrane was then floated briefly in 0.1 X SSC at room temperature, air-dried, wrapped in a cling film and placed and subjected to autoradiography with the Phosphor image analyzer FLA 5000 (M/S Fujifilm, Japan). The blot was exposed to the image plate BAS IP (MS 2025) (M/S Fujifilm, Japan) and kept sealed in the BAS cassette for 4h. The image plate was removed and read in the Phosphor image analyzer with red laser beam (635 nm). The presence of the gene insert in the *Pichia* genome was determined by visualizing the net intensity of band in the southern blot.

3.7.12. Recombinant Protein expression

Single colony from each of the *Pichia* isolates representing the six different forms of β-1,3-glucanase was inoculated to 10 ml of MGY in a 250 ml baffled flask.

MGY (Glycerol-complex Medium)
1% yeast extract, 2% peptone, 1.34% YNB, 4 × 10-5% biotin, 1% glycerol or 0.5% methanol Dissolved 10 g of yeast extract, 20 g peptone in 700 ml water. Autoclaved 20 minutes at 121 ^{0}C. Cooled to room temperature and added 100 ml 10X YNB, 2 ml 500X B, 100 ml 10X GY and mix well. Stored media at 4 ^{0}C.

i. Grown at 30 ^{0}C in a shaking incubator (300 rpm) until culture reached an OD_{600} = 4 (approximately 16–18 hours).

ii. Harvested these cells in the log phase by centrifuging at 3,000 X g for 5 minutes at room temperature.

iii. Decanted the supernatant and resuspended the cell pellets to an OD_{600} of 1.0 in MM medium to induce expression (approximately 50 ml) (MM Medium (liquid) - the medium composition is shown in section 3.7.10).

iv. Placed the culture in a 250 ml baffled flask covered with two layers of sterile cheese cloth and returned to incubator shaker to continue growth.

v. Added 100% methanol to a final concentration of 0.5% every 24 hours to maintain induction. Transferred 1 ml of the expression culture to a 1.5 ml micro-centrifuge tube at each of the time points (hours): 0, 24, 48, 72, and 96. Centrifuged in a microcentrifuge for 3 minutes at room temperature.

vi. The cells collected were washed with chilled PBS buffer, and re-suspended in 500 µl of chilled PBS buffer.

vii. 300 µl aliquots of re-suspended yeast cells were transferred to 1.5 ml polyethylene tubes.

viii. The tubes kept in ice were subjected to sonication for 30 seconds at 100% power for 3 times.

ix. The samples were centrifuged at 8000 rpm for 15 minutes to pellet debris.

x. The supernatant containing solubilized protein was collected for analysis *via* SDS PAGE. The protein was purified through His-spin trap column (M/S. GE-Health care, USA) which traps the his-tagged protein only. The trapped protein was later eluted out using elution buffer containing 500 mM imidazole.

xi. PAGE followed by western blot analysis was done to check for the induction of the purified proteins at different time intervals.

xii. The presence of the purified his tagged protein was confirmed through western blot analysis with anti his tag antibody (M/S KPL, USA) and the functional efficiency of the purified proteins was checked through activity staining in native gels following the protocol described by Pan *et al.*, (1989).

3.7.13. Western blotting and detection of purified proteins

Identity of the induced proteins was confirmed through western blot analysis using the 'His-Tag AP western reagent' kit (M/S KPL, USA). His-tagged proteins have a high affinity for nickel (Ni^{2+}) ions. Nickel-NTA

(nitrilotriacetic acid) conjugated to alkaline phosphatase (AP) will therefore bind directly to the His-tag, eliminating the need for primary and secondary antibodies. This results in assay simplification and reduced detection time. Direct detection with nickel-NTA also offers excellent sensitivity and low background in applications such as Western blotting. Nickel-NTA conjugates can detect proteins with a His-tag at either their N- or C-terminus.

The purified samples were run on 10% PAGE and transferred to PVDF memberane (M/S Amersham, USA) as per standard protocols (Sambrook *et al.*, 1989) using a western blot apparatus (M/S Bio-rad, USA). Blocked the membrane by completely immersing in 20 ml 1X Detector Block Solution (supplied with the kit) for 1 hour at room temperature with gentle agitation. Added His-Detector™ Nickel-AP conjugate. For the colorimetric assay, diluted His Detector Nickel-AP 1/1,500 directly into the 20 ml of 1X Detector Block Solution used to block the membrane. Incubated for one hour at room temperature with gentle agitation. Washed the membrane by immersing in 1X TBST, 3 times for 5 minutes each with gentle rocking. Incubated membrane in 10 ml BCIP/NBT and allowed to develop for 10 minutes. Rinsed the membrane for 20 seconds in reagent quality water to stop the reaction. Allowed the membrane to air dry.

3.7.14. Activity staining of the purified proteins

To check for the functional activity of the purified proteins, activity staining has been performed with the purified β-1,3-glucanase enzymes following the protocol described by Pan *et al.*(1989). The procedure describes the functional assay of β-1,3-glucanases directly on polyacrylamide gel electrophoresis (PAGE) by using 2,3,5-triphenyltetrazolium chloride. The reagent reacts with reducing sugars released by β-1,3-glucanases from the substrate laminarin.

भारतीय रबड़ अनुसंधान संस्थान
Rubber Research Institute of India

3.7.14. 1. Electrophoresis of protein samples

The polyacrylamide gel electrophoresis (PAGE) was carried out under native conditions as described by Laemmelli *et al.* (1970) using the Mini-

PROTEIN II Dual Slab Cell System (Bio-Rad, Hercules, CA). Native-PAGE was performed on the mini-slab gel with a 15% separating gel and a 4% stacking gel. About 15 µl of the purified enzymes for the six glucanases have been loaded to the clear wells in native polyacrylamide gels prepared. After electrophoresis the gel was washed three times with distilled water. Following equilibration in 0.5M-potassium acetate (pH 4.5), it was incubated in the same buffer, containing 1% laminarin (M/S Sigma-Aldrich), for 2 h at 37 ^{0}C. After washing the gel three times with distilled water it was heated in a boiling water bath with 1M NaOH, containing 0.3% (wt/vol) 2,3,5-triphenyl-tetrazolium chloride (M/S Sigma-Aldrich), until red bands appeared. The intensity of these bands will be approximately proportional to the applied activity and incubation time with laminarin. The stained gels were quickly transferred to 75% acetic acid to reduce background staining and to conserve the contrast.

3.7.15. Anti-fungal assay of purified recombinant proteins

The experiment was carried out under sterile conditions. PDA plates (90 mm) were prepared and a single growing mycelial plug of 4 mm diameter of the oomycete fungi *Phytophthora meadii* was inoculated at the center. To allow initial vegetative growth, the plates were incubated at 25 ± 2 ^{0}C for 24 h. At this time, sterile filter paper discs (9 mm in diameter) were laid on the agar surface and solutions to be tested were applied to the discs. Fifty microlitre of the purified protein solution which contains around 12 µg of recombinant protein were used for each disc. The plates were further incubated and observed for the appearance of inhibition zones and photographed.

3.8. Expression analysis of β-1,3-glucanase isoform Gln 4 through RT-PCR analysis.

3.8.1. RNA isolation from the leaf, callus and latex

3.8.1.1. Isolation of RNA from latex

i. RNA from the latex of rubber tree was extracted through a modified protocol of Kush *et al.*, (1990). All the reagents required were prepared

in DEPC (an *RNase* inhibitor) treated water. The protocol involved the following steps.

ii. Equal vol of latex was collected directly into a centrifuge tube under ice-cold conditions which contains 10 ml extraction buffer.

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

iii. The sample was centrifuged at 12,000 rpm for 30 min at 4 ^{0}C.

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

v. Treated with an equal vol of extraction buffer saturated phenol, mixed gently and centrifuged at 10,000 rpm for 10 min.

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

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

viii. RNA in the aqueous layer was precipitated by adding 1/3 vol of 8M LiCl. The precipitation continued overnight in –20 ^{0}C.

ix. RNA was pelleted by centrifugation at 10,000 rpm for 20 min. at 4 ^{0}C.

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

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

xii. The RNA was pelleted by centrifugation at 10,000 rpm for 10 min at 4 ^{0}C.

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

xiv. The quantity of RNA was checked using UV sphectrophotometer (section. 3.1.2.) and its quality and DNA contamination, if any, was checked in 1% agarose gels.

xv. The isolated RNA samples were stored in 3 vol of 100% ethanol at – 70 ^{0}C.

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

3.8.I.2. Isolation of RNA from leaf and callus samples

RNA was isolated from medium mature leaves/ callus of clone RRII 105 according to the procedure of Venkatachalam *et al.* (1999) with suitable modifications. All the reagents required are prepared in DEPC treated H_2O. The protocol involved the following steps:

i. One gram of leaf tissue was thoroughly cleaned and rinsed with DEPC treated H_2O and ground to a fine powder with liquid nitrogen in a motor and pestle.

ii. Ten ml extraction buffer was added and the homogenate was transferred to a centrifuge tube.

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

> **Extraction buffer:** 0.2M NaCl, 0.1M Tris-HCl (pH-8.5), 0.01M EDTA, 1.5% SDS, 0.1% β-mercaptoethanol (added freshly) and insoluble PVPP (added during homogenization).

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

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

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

vii. RNA was pelleted by centrifugation at 10,000 rpm for 10 min at 4 ^{0}C.

viii. The pellet was washed first with 2M LiCl followed by 70% ethanol, air-dried and dissolved in 1 ml DEPC treated sterile H_2O.

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

x. The precipitated RNA was pelleted at 10,000 rpm for 10 min at 4 ^{0}C and washed twice with 70% ethanol.

xi. Pellet was air-dried, re-suspended in 200 µl of autoclaved DEPC treated H_2O

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

xiii. The isolated RNA was used immediately for subsequent analysis or stored in 3 vol of ethanol at –80 ^{0}C.

3.8.2. First strand cDNA synthesis

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

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

ii. The tube was incubated for 5 min at 70 ^{0}C in a pre-heated block and immediately chilled in ice for 5 min.

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

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

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

vi. Annealing was done by incubating the reaction at 25 ^{0}C for 5 min.

vii. Primer extension was carried out at 42 ^{0}C for 1 h in a heated block.

viii. The reaction was stopped by inactivating the reverse transcriptase by keeping the tube at 70 ^{0}C for 15 min.

ix. The synthesised first strand cDNA was stored at –20 ^{0}C for subsequent PCR amplification.

3.8.3. RT-PCR

3.8.3.1. Design of gene specific primers

Based on the newly isolated β-1,3-glucanase isoform of Gln 4, forward and reverse primers were designed as follows; Forward - **5' CTC CTA AGA GGC CTG GCA G 3'**, Reverse **-5' CTG TTG CAT TGT AAT CAG CGG 3'.**

β-actin gene was used as the endogenous control and the primer sequences are

Forward: **5' GTT CCA GCC ATC ACT CAT TG 3'**

Reverse: **5' CTC CTT ACT CAT ACG GTC TGC 3'**

The synthesised primers (M/ S Metabion, Deutschland) were dissolved in TE buffer to get a concentration of 100 pmols/µl.

3.8.3.2. PCR amplification of the cDNA from different tissues

PCR has been carried out with 150 ng cDNA using the gene specific primers. RT-PCR has been carried out at 55 ^{0}C annealing temperature and 32 cycles. β-actin gene has been used as the endogenous control for normalization here.

3.9. Pathogen induced expression of β-1,3-glucanase gene isoform Gln 4

3.9.1. Plant materials used for the expression analysis

The *Phytophthora* tolerant clone RRII 105 and a highly susceptible clone RRIM 600 were selected for challenge with the pathogen and subsequent northern hybridization and RT-PCR analysis to study the induction pattern of β-glu gene. The plants and fungal culture used in this study were obtained from the Pathology Division, Rubber Research Institute of India.

3.9.2. Fungal culture and inoculation

The fungus used was a highly virulent isolate of *P.meadii*, which was maintained in PDA medium. PDA plates were inoculated with the stock culture and were incubated at 25 ^{0}C for two days in light. White oats medium was used for sporulation (Rajalakshmy and Joseph, 1986). The oats broth was inoculated with two-day old fresh fungal mycelium from the PDA plates and kept for two days under darkness. The mycelia were then collected in a mesh and washed four times with sterile distilled water in petri dishes. All the media components were removed and the mycelia were dispersed in water using forceps and it is then kept in light for 24 h for sporangial growth. The mycelia were observed under microscope to check the sporangial growth. The plate containing the sporangia were kept in refrigerator for 10 min and then kept in room temperature. This heat shock liberated the motile zoopspores. Light green leaves of 10 –12 days old were inoculated with this zoospore suspension

(10^6 zoospores/ml). After inoculation, the plants were covered with transparent polythene bags to maintain the required humidity and observed for the development of the symptoms.

Control plants were also maintained under similar conditions. Leaf samples were taken at different time intervals (24, 48, 72 and 96 hrs after inoculation) and processed for the RNA isolation and cDNA synthesis has been carried out as described in section 3.8.1.2. and 3.8.2.



Fig.13. Plants kept in controlled conditions after the fungal inoculation

3.9.3. PCR amplification of the Gln4 gene from cDNA

The cDNA prepared from the infected leaf samples of both the clones taken at different time intervals and the reverse transcriptase (RT) PCR has been carried out with 150 ng cDNA using the gene specific primers at 55 ^{0}C annealing temperature for 32 cycles. β-actin gene has been used as the endogenous control for normalization here also.

Chapter IV

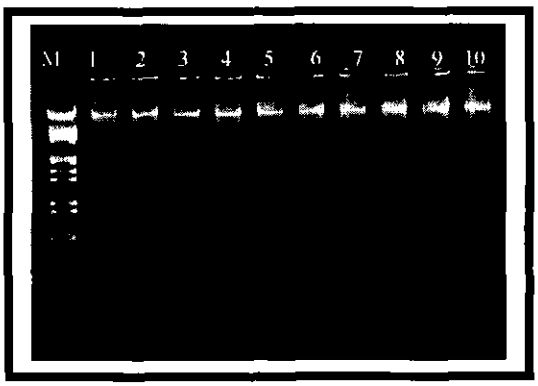
Results

4.1. Isolation, cloning and characterization of genomic DNA sequences coding for β-1,3-glucanase from different *Hevea* clones

The earlier studies held at Rubber Research Institute of India showed that the genomic sequence coding for β-1,3-glucanase is present in both tolerant and susceptible clones of *Hevea* towards abnormal leaf fall disease (ALF). In tolerant clones alone, a prolonged expression of β-1,3-glucanase beyond 48 h post – infection period (PIP) was observed on infection with the fungus. Nucleotide sequence variation of β-1,3-glucanase gene was observed between the Indian *Hevea* clone, RRII 105 (Acc. No. AY325498) and the Malaysian clone RRIM 600 (Acc. Nos. U22147, AJ133470, AF311749). Therefore, a preliminary attempt to understand the nucleotide sequence variations in the genomic sequence of β-1,3-glucanase among various *Hevea* clones with relative tolerance to abnormal leaf fall disease was made. For this, ten *Hevea* clones were selected based on their relative tolerance and susceptibility towards ALF. Among the relatively tolerant clones FX 516, RRII 105, TJIR 1, RRII 33, GL 1, RRII 414 and among the moderatively susceptible group PR107, PB 86 and RRIM 701 and finally from the highly susceptible group RRIM 600 was selected for the study.

4.1.1. Isolation of genomic DNA

Good quantity and pure quality genomic DNA without RNA contamination and no visible colouration due to presence of phenolics was isolated from leaves of all the clones selected (fig.14.) using the modified protocol of Doyle and Doyle (Doyle and Doyle,1990). The DNA isolated from all the clones were without shearing and showed a spectrophotometric value of (A 260-A 320)/(A 280 -A 320) value > 1.7.

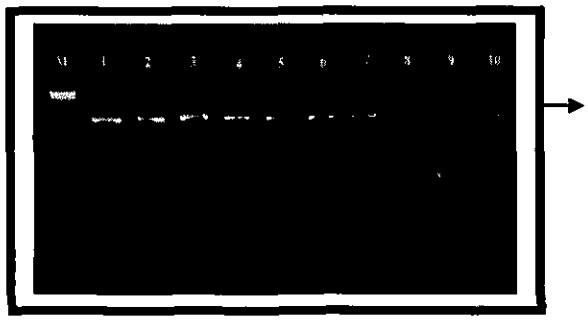


M- Marker
(λ DNA
*Eco*R1/*Hind* III
double digest)
1 - RRII 105
2 - FX 516
3 - TJIR 1
4 - RRII 33
5 - GL 1
6 - PB 86
7 - PR 107
8 - RRII 414
9 - RRIM 600
10 - RRIM 701

Fig. 14. Isolation of DNA from different *Hevea* clones

4.1.2 PCR amplification of β-1, 3-glucanase gene from the ten clones

When PCR was conducted using the forward and reverse primers designed based on a previously reported sequence from *Hevea brasiliensis* clone RRII 105 (Acc No. AY325498), under optimal conditions a single band of approximately 1.3 kb in size was amplified from the genomic DNA of all the ten clones selected (fig. 15). An annealing temperature of 55 ^{0}C as found to be optimum for the PCR amplification.



≅1.3 kb

M- Marker (λ DNA *Eco*R1/ *Hind* III double digest)
1 - RRII 105
2 - FX 516
3 - TJIR 1
4 - RRII 33
5 - GL1
6 - PB 86
7 - PR 107
8 - RRII 414
9 - RRIM 600
10 - RRIM 701

RRII 105

Fig.15. PCR amplification of β-1,3-glucanase gene from different *Hevea* clones

4.1.3. Cloning and sequence characterization of the amplified products

The PCR products were eluted from the gel and used for cloning. The fragments were cloned in TOPO TA® cloning vector (M/S Invitrogen, USA). The cloned mixture was later transformed to the chemically competent DH5α cells (supplied along with the kit). The selection of the transformants was carried out on the LB plates containing 50 μg/ml ampicillin and X-gal.

Transformation frequency was good as observed with the high number of colonies in the selection plates. The selected white colonies were subjected to colony PCR with an initial denaturation of 94 ^{0}C for 10 minutes. A blue non-recombinant colony was kept as the negative control. A 1.3 kb band was amplified from the positive colonies and no band was observed in the negative control which ruled out the possibility of non-specific amplification (fig.16).

Fig.16. Colony PCR of the transformed colonies using the β-1,3-glucanase gene specific primers



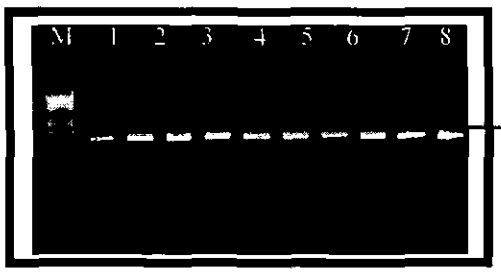
1 - RRII 105
2 - FX 516
3 - TJIR 1
4 - RRII 33
5 - GL1
6 - PB 86
7 - PR 107
8 - RRII 414
9 - RRIM 600
10 - RRIM 701
N - Negative control
M - Marker (λ DNA *Eco*R1/ *Hind* III double digest)

Recombinant plasmids were isolated in good concentration (15 µg/ 3µl) from the positive colonies (fig.17) using the Perfect prep Plasmid mini kit (M/S Eppendorff, USA). Before sequencing, the presence of inserts in all the recombinant plasmids were confirmed through PCR and all the samples showed good amplification as a single band product of approximately 1.3 kb (fig. 18).



Fig. 17. Recombinant plasmid vectors isolated from the positive colonies

M- Marker (λ DNA *Eco*R1/ *Hind* III double digest)
1 - RRII 105
2 - FX 516
3 - TJIR 1
4 - RRII 33
5 - GL1
6 - PB 86
7 - PR 107
8 - RRII 414
9 - RRIM 600
10 - RRIM 701
N - Plasmid without the insert



M- Marker (λ DNA *Eco*R1/ *Hind* III double digest)
1 - RRII 105
2 - FX 516
3 - TJIR 1
4 - RRII 33
5 - GL1
6 - PB 86
7 - PR 107
8 - RRII 414
9 - RRIM 600
10 - RRIM 701

Fig. 18. PCR amplification from the recombinant plasmids to show the presence of inserts in the vector

The sequencing was done using the universal M13 forward and reverse primers at M/S Macrogen, Korea. The nucleotide sequence analysis showed that all the sequences are single intronic and the intron starts from the +103 position from the TIC (transcription initiation codon). The intron splice junction 'GT' and 'AG' was conserved among the different sequences. It was interesting to note major variations in the intronic 'CT' repeat length among the various clones studied. Minor variations were also observed in the exonic sequences. The sequence alignment is shown in fig: 19.

Fig. 19. ClustalW multiple sequence alignment of *Hevea* β-1,3-glucanases from ten different clones

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105     GCTATCTCCTCTTCAACTTCAGGAACTAGTAGTTCCCTGCCCTCAAGAACTACTGTC 60
PR      GCTATCTCCTCTTCAACTTCAGGAACTAGTAGTTCCCTGCCCTCAAGAACTACTGTC 60
TJIR    GCTATCTCCTCTTCAACTTCAGGAACTAGTAGTTCCCTGCCCTCAAGAACTACTGTC 60
33      GCTATCTCCTCTTCAACTTCAGGAACTAGTAGTTCCCTGCCCTCAAGAACTACTGTC 60
701     GCTATCTCCTCTTCAACTTCAGGAACTAGTAGTTCCCTGCCCTCAAGAACTACTGTC 60
PB      GCTATCTCCTCTTCAACTTCAGGAACTAGTAGTTCCCTGCCCTCAAGAACTACTGTC 60
414     GCTATCTCCTCTTCAACTTCAGGAACTAGTAGTTCCCTGCCCTCAAGAACTACTGTC 60
GL      GCTATCTCCTCCTCAACTTCAGGAACTAGTAGTTCCTTGCCCTCAAGAACTACTGTC 60
600     GCTATCTCCTCTTCAACTTCAGGAACTAGTAGTTCCTTGCCCTCAAGAACTACTGTC 60
FX      GCTATCTCCTCTTCAACTTCAGGAACTAGTAGTTCCTTGCCCTCAAGAACTACTGTC 60
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105     ATGCTTCTTCTGATTTTCTTTACAGCAAGCCTTGGTATAACAGGTCTCTCTCTCTCTCTC 120
PR      ATGCTTCTTCTGATTTTCTTTACAGCAAGCCTTGGTATAACAGGTCTCTCTCTCTCTCTC 120
TJIR    ATGCTTCTTCTGATTTTCTTTACAGCAAGCCTTGGTATAACAGGTCTCTCTCTCTCTCTC 120
33      ATGCTTCTTCTGATTTTCTTTACAGCAAGCCTTGGTATAACAGGTCTCTCTCTCTCTCTC 120
701     ATGCTTCTTCTGATTTTCTTTACAGCAAGCCTTGGTATGACAGGTCTCTCTCTCTCTCTC 120
PB      ATGCTTCTTCTGATTTTCTTTACAGCAAGCCTTGGTATAACAGGTCTCTCTCTCTCTCTC 120
414     ATGCTTCTTCTGATTTTCTTTACAGCAAGCCTTGGTATAACAGGTCTCTCTCTCTCTCTC 120
GL      ATGCTTCTTCTGTTTTTCTTTACAGCAAGCGTTGGTATAACAGGTCTCTCTCTCTCTCTC 120
600     ATGCTTCTTCTGTTTTTCTTTACAGCAAGCGTTGGTATAACAGGTCTCTCTCTCTCTCTC 120
FX      ATGCTTCTTCTGATTTTCTTTACAGCAAGCCTTGGTATAACAGGTCTCTCTCTCTCTCTC 120
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105     TCTCTCTCTCTCTCTCTTTC--------CATCTATCTACTCTCATGTTAAAGTTGACGAT 172
PR      TCTCTCTCTCTCTCTCTCTC------CATCTATCTACTCTCATGTTAAAGTTGACGAT 174
TJIR    TCTCTCTCTCTCTCTCTCTCTCTCTCCATCTATCTACTCTCATGTTAAAGTTGACGAT 180
33      TCTCTCTCC--------------------ATCTATCTACTCTCATGTTAAAGTTGACGAT 160
701     TCTCT--CC--------------------ATCTATCTACTCTCATGTTAAAGTTGACGAT 158
PB      TCTCTCTCC--------------------ATCTATCTACTCTCATGTTAAAGTTGACGAT 160
414     TCTC----C--------------------ATCTATCTACTCTCATGTTAAAGTTGACGAT 156
GL      TCTCTCTCTC-------------------------------ATGTTAAAGTTGACGAT 147
600     TCTCTCTCTC-------------------------------ATGTTAAAGTTGACGAT 147
FX      TCTCTCTCTC--------------------TCTCTCT-CTCTCATGTTAAAGTTGACGAT 159
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105     GCTCTTTTTTTCTCTCCTTGTTCTAAAAGGTTTCAACTAATACCTGTATTTGGAATTTAG 232
PR      GCTCTTTTTTTCTCTCCTTGTTCTAAAAGGTTTCAACTAATACCTGTATTTGGAATTTAG 234
TJIR    GCTCTTTTTTTCTCTCCTTGTTCTAAAAGGTTTCAACTAATACCTGTATTTGGAATTTAG 240
33      GCTCTTTTTTTCTCTCCTTTTTCTAAAAGGTTTCAACTAATACCTGTATTTGGAATTTAG 220
701     GCTCTTTTTTTCTCTCCTTTTTCTAAAAGGTTTCAACTAATACCTGTATTTGGAATTTAG 218
PB      GCTCTTTTTTTCTCTCCTTTTTCTAAAAGGTTTCAACTAATACCTGTATTTGGAATTTAG 220
414     GCTCTTTTTTTCTCTCCTTTT-CAAAAAGGTTTCAACTAATACCTGTATTTGGAATTTAG 215
GL      GCTCTTTTTTTCTCTCCTTGTTCTAAAAGGTTTCAACTAATACCTGTATTTGGAATTTAG 207
600     GCTCTTTTTTTCTCTCCTTGTTCTAAAAGGTTTCAACTAATACCTGTACTTGGAATTTAG 207
FX      GCTCTTTTTTTCTCTCCTTTTTCTAAAAGGTTTCAACTAATATCTGTATTTGGAATTTAG 219
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105     ATGCCCAGGTAGGCGTTTGCTATGGAATGCAAGGCAACAACCTTCCATCTGTTTCGGAGG 292
PR      ATGCCCAGGTAGGCGTTTGCTATGGAATGCAAGGCAACAACCTTCCATCTGTTTCAGAGG 294
TJIR    ATGCCCAGGTAGGTGTTTGCTATGGAATGCAAGGCAACAACCTTCCATCTGTTTCAGAGG 300
33      ATGCCCAGGTAGGTGTTTGCTATGGAATGCAAGGCAACAACCTTCCATCTGTTTCAGAGG 280
701     ATGCCCAGGTAGGTGTTTGCTATGGAATGCAAGGCAACAACCTTCCATCTGTTTCAGAGG 278
PB      ATGCCCAGGTAGGTGTTTGCTATGGAATGCAAGGCAACAACCTTCCATCTGTTTCAGAGG 280
414     ATGCCCAGGTAGGTGTTTGCTATGGAATGCAAGGCAACAACCTTCCATCTGTTTCAGAGG 275
GL      ATGCCCAGGTAGGTGTTTGCTATGGAATGCAAGGCAACAACCTTCCACCTGTTTCAGAGG 267
600     ATGCCCAGGTAGGTGTTTGCTATGGAATGCAAGGCAACAACCTTCCACCTGTTTCAGAGG 267
FX      ATGCCCAGGTAGGTGTTTGCTATGGAATGCAAGGCAACAACCTTCCACCTGTTTCAGAGG 279
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105     TCATAGCTCTCTATAAACAATCTAACATCAAGAGAATGAGAATTTATGATCCAAATCGAG 352
PR      TCATAGCTCTCTATAAACAATCTAACATCAAGAGAATGAGAATTTATGATCCAAATCGAG 354
TJIR    TCATAGCTCTCTATAAAAAATCTAACATCAAGAGAATGAGAATTTATGATCCAAATCAAG 360
33      TCATAGCTCTCTATAAACAATCTAACATCAAGAGAATGAGAATTTATGATCCAAATCAAG 340
701     TCATAGCTCTCTATAAACAATCTAACATCAAGAGAATGAGAATTTATGATCCAAATCAAG 338
PB      TCATAGCTCTCTATAAACAATCTAACATCAAGAGAATGAGAATTTATGATCCAAATCAAG 340
414     TCATAGCTCTCTATAAACAATCTAACATCAAGAGAATGAGAATTTATGATCCAAATCAAG 334
GL      TCATAGCTCTCTATAAAAAATCTAACATCACGAGAATGAGAATTTATGATCCAAATCAAG 327
600     TCATAGCTCTCTATAAAAAATCTAACATCACGAGAATGAGAATTTATGATCCAAATCAAG 327
FX      TCATAGCTCTCTATAAAAAATCTAACATCACGAGAATGAGAATTTATGATCCAAATCGAG 339
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105     CAGTATTGGAAGCCCTTAGAGGCTCAAACATTGAACTCATACTAGGTGTTCCAAACTCAG 412
PR      CAGTATTGGAAGCCCTTAGAGGCTCAAACATTGAACTCATACTAGGTGTTCCAAACTCAG 414
TJIR    CAGTATTGGAAGCCCTTAGAGGCTCAAACATTGAACTCATACTAGGTGTTCCAAACTCAG 420
33      CAGTATTGGAAGCCCTTAGAGGCTCAAACATTGAACTCATACTAGGTGTTCCAAACTCAG 400
701     CAGTATTGGAAGCCCTTAGAGGCTCAAACATTGAACTCATACTAGGTGTTCCAAACTCAG 398
PB      CAGTATTGGAAGCCCTTAGAGGCTCAAACATTGAACTCATACTAGGTGTTCCAAACTCAG 400
414     CAGTATTGGAAGCCCTTAGAGGCTCAAACATTGAACTCATACTAGGTGTTCCAAACTCAG 394
GL      CAGTATTGGAAGCCCTTAGAGGCTCAAACATTGAACTCATACTAGGTGTTCCAAACTCAG 387
600     CAGTATTGGAAGCCCTTAGAGGCTCAAACATTGAACTCATACTAGGTGTTCCAAACTCAG 387
FX      CAGTATTGGAAGCCCTTAGAGGCTCAAACATTGAACTCATACTAGGTGTTCCAAACTCAG 399
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105     ATCTCCAAAGCCTTGCCAATCCTTCCAATGCAAACTCATGGGTACAAAAAAATGTTCGTG 472
PR      ATCTCCAAAGCCTTACCAATCCTTCCAATGCAAACTCATGGGTACAAAAAAATGTTCGTG 474
TJIR    ATCTCCAAAGCCTTACCAATCCTTCCAATGCAAACTCATGGGTACAAAAAAATGTTCGTG 480
33      ATCTCCAAAGCCTTACCAATCCTTCCAATGCAAACTCATGGGTACAAAAAAATGTTCGTG 460
701     ATCTCCAAAGCCTTACCAATCCTTCCAATGCAAACTCATGGGTACAAAAAAATGTTCGTG 458
PB      ATCTCCAAAGCCTTACCAATCCTTCCAATGCAAACTCATGGGTACAAAAAAATGTTCGTG 460
414     ATCTCCAAAGCCTTACCAATCCTTCCAATGCAAACTCATGGGAACAAAAAAATGTTCGTG 454
GL      ATCTCCAAAGCCTTACCAATCCTTCCAATGCAAAATCATGGGTACAAAAAAATGTTCGTG 447
600     ATCTCCAAAGCCTTACCAATCCTTCCAATGCAAAATCATGGGTACAAAAAAATGTTCGTG 447
FX      ATCTCCAAAGCCTTACCAATCCTTCCAATGCAAAATCATGGGTACAAAAAAATGTTCGTG 459
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105     GCTTCTGGTCAAGTGTCAGGTTCAGATATATAGCAGTTGGCAACGAAATTAGTCCTGTAA 532
PR      GCTTCTGGTCAAGTGTCAGGTTCAGATATATAGCAGTTGGCAACGAAATTAGTCCTGTAA 534
TJIR    GCTTCTGGTCAAGTGTCAGGTTCAGATATATAGCAGTTGGCAACGAAATTAGTCCTGTAA 540
33      GCTTCTGGTCAAGTGTCAGGTTCAGATATATAGCAGTTGGCAACGAAATTAGTCCTGTAA 520
701     GCTTCTGGTCAAGTGTCAGGTTCAGATATATAGCAGTTGGCAACGAAATTAGTCCTGTAA 518
PB      GCTTCTGGTCAAGTGTCAGGTTCAGATATATAGCAGTTGGCAACGAAATTAGTCCTGTAA 520
414     GCTTCTGGTCAAGTGTCAGGTTCAGATATATAGCAGTTGGCAACAAAATTAGTCCTGTAA 514
GL      GCTTCTGGTCAAGTGTCAGGTTCAGATATATAGCAGTTGGCAACGAAATTAGTCCTGTCA 507
600     GCTTCTGGTCAAGTGTCAGGTTCAGATATATAGCAGTTGGCAACGAAATTAGTCCTGTCA 507
FX      GCTTCTGGTCAAGTGTCAGGTTCAGATATATAGCAGTTGGCAATGAAATTAGTCCTGTAA 519
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105     ATGGAGGCACAGCTTAGTTGGCCCAATTTGTTTTGCCTGCCATGAGAAATATACATGATG 592
PR      ATGGAGGCACAGCTTGGTTGGCCCAATTTGTTTTGCCTGCCATGAGAAATATACATGATG 594
TJIR    ATGGAGGCACAGCTTGGTTGGCCCAATTTGTTTTGCCTGCCATGAGAAATATACATGATG 600
33      ATGGAGGCACAGCTTGGTTGGCCCAATTTGTTTTGCCTGCCATGAGAAATATACATGATG 580
701     ATGGAGGCACAGCTTGGTTGGCCCAATTTGTTTTGCCTGCCATGAGGAATATACATGATG 578
PB      ATGGAGGCACAGCTTGGTTGGCCCAATTTGTTTTGCCTGCCATGAGAAATATACATGATG 580
414     ATGAAGGCACAGCTTGGTTGGCCCAATTTGTTTTGCCTGCCATGACAAATATACATGATG 574
GL      ATAGAGGCACAGCTTGGTTGGCTCAATTTGTTTTGCCTGCCATGAGAAATATACATGATG 567
600     ATAGAGGCACAGCTTGGTTGGCTCAATTTGTTTTGCCTGCCATGAGAAATATACATGATG 567
FX      ATAGAGGCACAGCTTGGTTGGCTCAATTTGTTTTGCCTGCCATGAGAAATATACATGATG 582
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105     CTATAAGATCAGCTGGTCTTCAAGATAAAATCAAGGTCTCCACTGCAATTGACTTGACCC 652
PR      CTATAAGATCAGCTGGTCTTCAAGATAAAATCAAGGTCTCCACTGCAATTGACTTGACCC 654
TJIR    CTATAAGATCAGCTGGTCTACAAGATCAAATCAAGGTCTCCACTGCAATTGACTTGACCC 660
33      CTATAAGATCAGCTGGTCTTCAAGATCAAATCAAGGTCTCCACTGCAATTGACTTGACCC 640
701     CTATAAGATCAGCTGGTCTTCAAGATCAAATCAAGGTCTCCACTGCAATTGACTTGACCC 638
PB      CTATAAGATCAGCTGGTCTTCAAGATCAAATCAAGGTCTCCACTGCAATTGACTTGACCC 640
414     CTATAAGATCAGCTGGACTTCAAGATCAAATCAAGGTCTCCACTGCAATTGACTTGACCC 634
GL      CTATAAGATCAGCTGGTCTTCAAGATCAAATCAAGGTCTCCACTGCAATTGACTTGACCC 627
600     CTATAAGATCAGCTGGTCTTCAAGATCAAATCAAGGTCTCCACTGCAATTGACTTGACCC 627
FX      CTATAAGATCAGCTGGTCATCAAGATCGAATCAAGGTCTCCACTGCAATTGACTTGACCC 639
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105     TGGTAGGAAATTCCTACCCTCCTTCTGCAGGTGCTTTCAGGGATGATGTTAGATCATACT 712
PR      TGGTAGGAAATTCCTACCCTCCTTCTGCAGGTGCTTTCAGGGATGATGTTAGATCATACT 714
TJIR    TGGTAGGAAATTCCTACCCTCCTTCTGCAGGTGCTTTCAGGGATGATGTTAGATCATACT 720
33      TGGTAGGAAATTCCTACCCTCCTTCTGCAGGTGCTTTCAGGGATGATGTTAGATCATACT 700
701     TGGTAGGAAATTCCTACCCTCCTTCTGCAGGTGCTTTCAGGGATGATGTTAGATCATACT 698
PB      TGGTAGGAAATTCCTACCCTCCTTCTGCAGGTGCTTTCAGGGATGATGTTAGATCATACT 700
414     TGGTAGGAAATTCCTACCCTCCTTCTGCAGGTGCTTTCAGGGATGATGTTAGATCATACT 694
GL      TGGTAGGAAATTCCTACCCTCCTTCTGCAGGTGCTTTCAGGGATGATGTTAGATCATACT 687
600     TGGTAAGAAATTCCTACCCTCCTTCTGCAGGTGCTTTCAGGGATGATGTTAGATCATACT 687
FX      TGGTAGGAAATTCCTACCCTCCTTCTGCAGGTGCTTTCAGGGATGATGTTAGATCATACT 699
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105     TGGACCCAATTATTGGATTTCTATCCTCTAGCAGGTCACCTTTACTTGCCAATA--TTTA 770
PR      TGGACCCAATTATTGGATTTCTATCCTCTAGCAGGCCACCTTTACTTGCCAATA--TTTA 774
TJIR    TGGACCCAATTATTGGATTTCTATCCTCTAGCANGTCACCTTTACTTGCCAATA--TTTA 778
33      TGGACCCAATTATTGGATTTCTATCCTCTATCAGGTCACCTTTACTTGCCAATA--TTTA 758
701     TGGACCCAATTATTGGATTTCTATCCTCTATCAGGTCACCTTTACTTGCCAATA--TTTA 756
PB      TGGACCCAATTATTGGATTTCTATCCTCTATCAGGTCACCTTTACTTGCCAATA--TTTA 758
414     TGGACCCAATTATTGGATTTCTATCCTCTATCAGGTCACCTTTACTTGCCAATA--TTTA 752
GL      TGAACCCAATTATTAGATTTCTATCCTCTATCAGGTCACCTTTACTTGCCAATA--TTTA 745
600     TGAACCCAATTATTAGATTTCTATCCTCTATCAGGTCACCTTTACTTGCCAATA--TTTA 745
FX      TGAACCCAATTATTAGATTTCTATCCTCTATCANGTCACCTTTACTTGCCAATA--TTTA 757
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105     TCCT--TACTTTACTTATGCTTATAATCCAAGGGATATTTCCCTTCCCTATGCTTTGTTC 828
PR      TCCT--TACTTTACTTATGCTTATAATCCAAGGGATATTTCCCTTCCCTATGCTTTGTTC 832
TJIR    TCCT--TACTTTACTTATGCTTATAATCCAAGGGATATTTCCCTTCCCTATGCTTTGTTC 836
33      TCCT--TACTTTACTTATGCTGATAATCCAAGGGATATTTCCCTTCCCTATGCTTTGTTC 816
701     TCCT--TACTTTACTTATGCTGATAATCCAAGGGATATTTCCCTTCCCTATGCTTTGTTC 814
PB      TCCT--TACTTTACTTATGCTGATAATCCAAGGGATATTTCCCTTCCCTATGCTTTGTTC 816
414     TCCT--TACTTTACTTATGCTGATAATCCAAGGGATATTTCCCTTCCCTATGCTTTGTTC 810
GL      TCCT--TACTTTACTTATGCTGGTAATCCAAGGGATATTTCCCTTCCCTATGCTTTGTTC 803
600     TCCT--TACTTTACTTATGCTGGTAATCCAAGGGATATTTCCCTTCCCTATGCTTTGTTC 803
FX      TCCTCTTACTTTACTTATGCTGGTAATCCAAGGGATATTTCCCTTCCCTATGCTTTGTTC 817
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105     ACTTCACCATCAGTTGTTGTGTGGGATGGTCAGCGAGGTTATAAGAACCTTTTTGATGCA 888
PR      ACTTCACCATCAGTTGTTGTGTGGGATGGTCAGCGAGGTTATAAGAACCTTTTTGATGCA 892
TJIR    ACTTCACCATCAGTTGTTGTGTGGGATGGTCAGCGAGGTTATAAGAACCTTTTTGATGCA 896
33      ACTTCACCATCAGTTGTTGTGTGGGATGGTCAGCGAGGTTATAAGAACCTTTTTGATGCA 876
701     ACTTCACCATCAGTTGTTGTGTGGGATGGTCAGCGAGGTTATAAGAACCTTTTTGATGCA 874
PB      ACTTCACCATCAGTTGTTGTGTGGGATGGTCAGCGAGGTTATAAGAACCTTTTTGATGCA 876
414     ACTTCACCATCAGTTGTTGTGTGGGATGGTCAGCGAGGTTATAAGAACCTTTTTGATGCA 870
GL      ACTTCACCATCAGTTGTTGTGTGGGATGGTCAGCGAGGTTATAAGAACCTTTTTGATGCA 863
600     ACTTCACCATCAGTTGTTGTGTGGGATGGTCAGCGAGGTTATAAGAACCTTTTTGATGCA 863
FX      ACTTCACCATTCGTTGTTGTGTGGGATGGTCTGCGAGGTTATAAGAACCTTTTTGATGCA 877
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105     ACGTTGGATGCATTGTACTCTGCTCTTGAGAGGGCTAGTGGTGGTTCTCTGGAGGTGGTT 948
PR      ACGTTGGATGCATTGTACTCTGCTCTTGAGAGGGCTAGTGGTGGTTCTCTGGAGGTGGTT 952
TJIR    ACGTTGGATGCATTGTACTCTGCTCTTGAGAGGGCTAGTGGTGGTTCTCTGGAGGTGGTT 956
33      ACGTTGGATGCATTGTACTCTGCTCTTGAGAGGGCTAGTGGTGGTTCTCTGGAGGTGGTT 936
701     ACGTTGGATGCATTGTACTCTGCTCTTGAGAGGGCTAGTGGTGGTTCTCTGGAGGTGGTT 934
PB      ACGTTGGATGCATTGTACTCTGCTCTTGAGAGGGCTAGTGGTGGTTCTCTGGAGGTGGTT 936
414     ACGTTGGATGCATTGTACTCTGCTCTTGAGAGGGCTAGTGGTGGTTCTCTGGAGGTGGTT 930
GL      ACGTTGGATGCATTGTACTCTGCTCTTGAGAGGGCTAGTGGTGGTTCTCTGGAGGTGGTT 923
600     ACGTTGGATGCATTGTACTCTGCTCTTGAGAGGGCTAGTGGTGGTTCTCTGGAGGTGGTT 923
FX      ACTTTGGATGCATTGTACTCTGCTCTTGAGAGGGCTAGTGGTGGTTCTCTGGAGGTGGTT 937
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105     GTTTCGGAAAGTGGCTGGCCGTCAGCCGGAGCATTTGCTGCCACATTTGACAATGGGCGT 1008
PR      GTTTCGGAAAGTGGCTGGCCGTCTGCCGGAGCATTTGCTGCCACATTTGACAATGGGCGT 1012
TJIR    GTTTCGGAAAGTGGCTGGCCGTCTGCCGGAGCATTTGCTGCCACATTTGACAATGGGCGT 1016
33      GTTTCGGAAAGTGGCTGGCCGTCTGCCGGAGCATTTGCTGCCACATTTGACAATGGGCGT 996
701     GTTTCGGAAAGTGGCTGGCCGTCTGCCGGAGCATTTGCTGCCACATTTGACAATGGGCGT 994
PB      GTTTCGGAAAGTGGCTGGCCGTCTGCCGGAGCATTTGCTGCCACATTTGACAATGGGCGT 996
414     GTTTCGGAAAGTGGCTGGCCGTCTGCCGGAGCATTTGCTGCCACATTTGACAATGGGCGT 990
GL      GTTTCGGAAAGTGGCTGGCCGTCTGCCGGAGCATTTGCTGCCACATTTGACAATGGGCGT 983
600     GTTTCGGAAAGTGGCTGGCCGTCTGCCGGAGCATTTGCTGCCACATTTGACAATGGGCGT 983
FX      GTTTCGGAAAGTGGCTGGCCGTCTGCCGGAGCATTTGCTGCCACATTTGACAATGGGCGT 997
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105     ACTTATCTCTCAAATTTGATCCAACATGTTAAAGGAGGTACTCCTAAGAGGCCTAACAGA 1068
PR      ACTTATCTCTCAAATTTGATCCAACATGTTAAAGGAGGTACTCCTAAGAGGCCTAACAGA 1072
TJIR    ACTTATCTCTCAAATTTGATCCAACATGTTAAAGGAGGTACTCCTAAGAGGCCTAACAGA 1076
33      ACTTATCTCTCAAATTTGATCCAGCATGTTAAAGGAGGTACTCCTAAGAGGCCTAACAGA 1056
701     ACTTATCTCTCAAATTTGATCCAGCATGTTAAAGGAGGTACTCCTAAGAGGCCTAACAGA 1054
PB      ACTTATCTCTCAAATTTGATCCAGCATGTTAAAGGAGGTACTCCTAAGAGGCCTAACAGA 1056
414     ACTTATCTCTCAAATTTGATCCAGCATGTTAAAGGAGGTACTCCTAAGAGGCCTAACAGA 1050
GL      ACTTATCTCTCAAATTTGATCCAGCATGTTAAAAGAGGTACTCCTAAGAGGCCTAAAAGA 1043
600     ACTTATCTCTCAAATTTGATCCAGCATGTTAAAAGAGGTACTCCTAAGAGGCCTAAAAGA 1043
FX      ACTTATCTCTCAAATTTGATCGAGCATGTTAAAAGAGGTACTCCTAAGAGGCCTAACAGA 1057
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105     GCTATAGAGACTTACTTATTTGCCATGTTTGATGAAAATAAGAAGCAACCAGAGGTTGAG 1128
PR      GCTATAGAGACTTACTTATTTGCCATGTTTGATGAAAATAAGAAGCAACCAGAGGTTGAG 1132
TJIR    GCTATAGAGACTTACTTATTTGCCATGTTTGATGAAAATAAGAAGCAACCAGAGGTTGAG 1136
33      GCTATAGAGACTTACTTATTTGCCATGTTTGATGAAAATAAGAAGCAACCAGAGGTTGAG 1116
701     GCTATAGAGACTTACTTATTTGCCATGTTTGATGAAAATAAGAAGCAACCAGAGGTTGAG 1113
PB      GCTATAGAGACTTACTTATTTGCCATGTTTGATGAAAATAAGAAGCAACCAGAGGTTGAG 1116
414     GCTATAGAGACTTACTTATTTGCCATGTTTGATGAAAATAAGAAGCAACCAGAGGTTGAG 1110
GL      GCTATAGAGACTTACTTATTTGCCATGTTTGATGAAAATAAGAAGCAACCAGAGGTTGAG 1103
600     GCTATAGAGACTTACTTATTTGCCATGTTTGATGAAAATAAGAAGCAACCAGAGGTTGAG 1103
FX      GCTATAGAGACTTACTTATTTGCCATGTTTGATGAAAATAAGAAGCAACCAGAGGTTGAG 1117
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105     AAACACTTTGGACTTTTCTTTCCTGATAAACGGCCAAAATATAATCTCAATTTTGGTGCA 1188
PR      AAACACTTTGGACTTTTCTTTCCCGATAAACGGCCAAAATATAATCTCAATTTTGGTGCA 1192
TJIR    AAACACTTTGGACTTTTCTTTCCTGATAAACGGCCAAAATATAATCTCAATTTTGGTGCA 1196
33      AAACACTTTGGACTTTTCTTTCCTGATAAACGGCCAAAATATAATCTCAATTTTGGTGCA 1176
701     AAACACTTTGGACTTTTCTTTCCTGATAAACGGCCAAAATATAATCTCAATTTTGGTGCA 1173
PB      AAACACTTTGGACTTTTCTTTCCTGATAAACGGCCAAAATATAATCTCAATTTTGGTGCA 1176
414     AAACACTTTGGACTTTTCTTTCCTGATAAACGGCCAAAATATAATCTCAATTTTGGTGCA 1170
GL      AAACACTTTGGACTTTTCTTTCCTAATAAATGGCAAAAATATAATCTCAATTTTAGTGCA 1163
600     AAACACTTTGGACTTTTCTTTCCTAATAAATGGCAAAAATATAATCTCAATTTTAGTGCA 1163
FX      AAACACTTTGGACTTTTCTTTCCTAGCAAATGGCAAAAATATAATCTCAATTTTGGTGCA 1177
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105     GAAAAGAACTGGGATATTTCTACTGAACACAATGCAACAATACTCTTCCTTAAGAGTGAT 1248
PR      GAAAAGAACTGGGATATTTCTACTGAACACAATGCAACAATACTCTTCCTTAAGAGTGAT 1252
TJIR    GAAAAGAACTGGGATATTTCTACTGAACACAATGCAACGATACTTTTCCTTAAGAGTGAT 1256
33      GAAAAGAACTGGGATATTTCTACTGAACACGATGCAACAATACTTTTCCTTAAGAGTGAT 1236
701     GAAGAGAACTGGGATATTTCTACTGAACACGATGCAACAATACTTTTCCTTAAGAGTGAT 1233
PB      GAAAAGAACTGGGATATTTCTACTGAACACGATGCAACAATACTTTTCCTTAAGAGTGAT 1236
414     GAAAAGAACTGGGATATTTCTACTGAACACGATGCAACAATACTTTTCCTTAAGAGTGAT 1230
GL      GAAAAGAACTGGGATATTTCTACTGAACACAATGCAACAATACTTTTCCTTAAGAGTGAT 1223
600     GAAAAGAACTGGGATATTTCTACTGAACACAATGCAACAATACTTTTCCTTAAGAGTGAT 1223
FX      GAAAAGAACTGGAATATTTCTACTGAACACAATGCAACAGTACTTTTCCTTAAGAGTGAT 1237
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105     ATG[redacted] 1254
PR      ATG[redacted] 1256
TJIR    ATG[redacted] 1262
33      ATG[redacted] 1242
701     ATG[redacted] 1240
PB      ATG[redacted] 1242
414     ATG[redacted] 1237
GL      ATG[redacted] 1229
600     ATG[redacted] 1229
FX      ATG[redacted] 1243
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[redacted]

Introns

[redacted]

Upon sequencing it was found that the total number of nucleotides amplified from the start codon 'ATG' to the stop codon 'TGA' varied in the different clones. The number of nucleotides amplified in each clone ranged between 1229 – 1262 bps. The maximum number of nucleotides amplified was from TJIR 1 with 1262 base pairs and minimum number amplified was found to be with GL1 and RRIM 600 clones each of which is having 1229 base pairs in the genomic DNA. Presence of a 'CT' repeat was also observed in the intron of all the sequences obtained. The alignment showed remarkable variations in the number of 'CT' repeats within the intronic region thereby imparting a total gene length variation among the clones. Not much variations were found within the exonic region. Among the clones, <6% variation observed in the exon portion showed a conserved pattern of the sequence. The intron sequence started from the 103rd bp in all the clones and the 'GT' and 'AG' splice junctions are also seen conserved among the sequences. The details regarding the 'CT' length among the clones are shown in table.7.

The maximum number of non-coding nucleotides was found with the clone TJIR1 1 with 21 'CT' repeats. The minimum number of 104 nucleotides

was found with GL 1 and RRIM 600 clones. They are having 12 'CT' repeats each. The *Hevea* hybrid FX 516 also showed 12 'CT' repeats in the intron with a total number of 116 intronic nucleotides. The lowest number of 9 'CT' repeats was found with RRII 414 within the 112 intronic nucleotides. RRII 33 and PB 86 are similar with respect to their 11 Nos. of 'CT' repeats and 117 No. of total non-coding nucleotides.

Table. 7. Intron length and number of 'CT' repeats in the β-1,3-glucanase gene sequence in various *Hevea* clones

Sl no	Name of the clone	No: of nucleotides in the intron	No: of CT repeats
1	**RRII 105**	121	18
2	**PR 107**	131	18
3	**TJIR1**	137	21
4	**RRII 33**	117	11
5	**RRIM701**	115	10
6	**PB86**	117	11
7	**RRII414**	112	9
8	**GL1**	104	12
9	**RRIM600**	104	12
10	**FX516**	116	12

Thus when the intron sequences are excluded from the obtained sequences, the exon region alone contained 1125 nucleotides which exactly codes for 374 amino acids and a stop codon. This result is similar to the previously reported β-1,3-glucanase genes from *Hevea* which comprise 374 amino acids and the stop codon. In this preliminary study, the nucleotide variations observed could not be linked to tolerance/ susceptibility of the clones towards abnormal leaf fall disease.

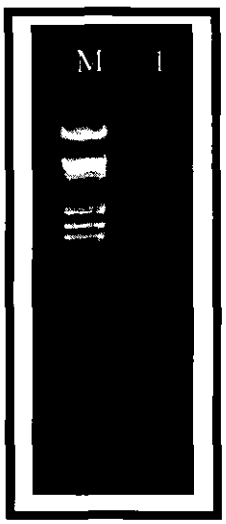
4.2. Characterization of 5' regulatory elements of β-1,3-glucanase gene through inverse PCR

Although a detailed study was done to characterize the β-1,3-glucanase genomic sequence from the clones with difference in tolerance level to ALF, the sequence variations could not be correlated with tolerance/ susceptibility of the clones towards ALF. Since the induction and regulation of all the genes are controlled by their corresponding promoters which harbours a variety of cis- elements, isolation of the promoter regions are essential to understand the

control of gene expression in full detail. Therefore, an attempt had been made to characterize the promoter regions of β-1,3-glucanase gene from the various clones, with reference to the earlier reported sequence (Acc. No. AY325498) through inverse PCR technique. Initially the work was focused to the *Hevea* clone RRII 105.

Good quality genomic DNA isolated from the clone RRII 105 was used for the inverse PCR experiment. The inverse PCR method includes restriction digestion of genomic DNA, self-ligation and further linearization with restriction endonuclease. This results in the formation of a linear sequence where the unknown sequence is flanked by the known sequences at the either end.

Target DNA was subjected to mild digestion using blunt end restriction endonucleases and products with small fragments to several kilobases were obtained. The enzyme *Ssp* I offered a good digestion suitable for inverse PCR experiment with an overnight incubation at 37 ^{0}C (fig.20).The enzyme is a six base pair blunt end cutter which recognizes the AAT ↓ATT palindromic sequence.



M- Marker (λ DNA *Eco*R1/ *Hind* III double digest)

1- *Ssp* 1 digested DNA

Fig. 20. DNA digested with *SSP* 1 restriction enzyme

The digested products were self circularized. More than 95% of the DNA fragments were expected to self-ligate under low concentrations (500 nm DNA) causing the phosphate backbone to reform with T_4 DNA ligase at 16°C. Further the target DNA is restricted with *Bgl* II endonuclease to get a linearized product. This generates a linear product with known terminal

sequences and unknown region in the middle suitable for PCR. This can now be used for PCR (polymerase chain reaction).

4.2.1. Inverse PCR amplification and sequence characterization of β-1,3-glucanase gene promoters

Standard PCR is conducted with primers complementary to the known flanking sequences of the linearised product mentioned above. The primers designed were specific for the coding sequence of the gene which gave four bands upon the standard PCR amplification. Forward primer had been taken from the downstream gene region prior to the restriction enzyme site AAT↓ATT and the reverse primer was taken from the upstream region so that the primers can amplify the unknown region which comes in between the primers. The resrriction enzyme site is an indicator of the beginning of the unknown site. After extensive optimizations of the PCR conditions, better results were obtained at an annealing temperature of 52 ^{0}C with 34 thermal cycles. The PCR products were visualized by ethidium bromide staining after migration of 20 µl samples on 1.2% agarose gels. In the initial attempt, four bands with approximately of 1.2, 0.8, 0.76 and 0.4 kb were obtained on inverse PCR (fig. 21). These four bands were observed in all the trials eventhough the PCR conditions were optimized to get a single band. Each band was eluted seperately using the GFX gel band purification aid (M/S Amersham, USA) and cloned using TOPO TA® vector (M/S Invitrogen, USA).

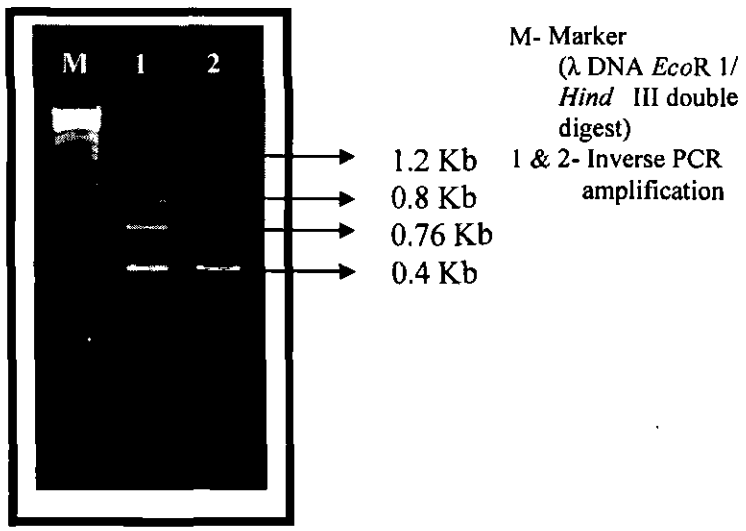
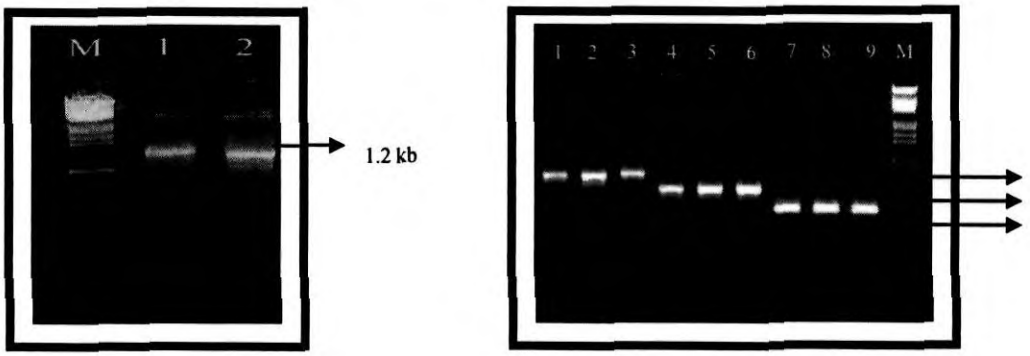


Fig. 21. Inverse PCR amplification of β-1,3-glucanase promoter

The purified inserts were cloned in TOPO TA® vector and later transformed to the chemically competent DH5α cells. The transformed bacterial cells were plated on to the selection plate containing the antibiotic ampicillin (50 μg/ml). The plates showed a fair number of transformed colonies which indicated that the transformation efficiency was good. The colonies harbouring the recombinant plasmids with different inserts (1.2, 0.8, 0.76 and 0.4 Kb) were selected through colony PCR (fig. 22 A&B).

Fig.22. A&B. Colony PCR showing the positive amplification of the four bands obtained through inverse PCR



A. 1.2 kb fragment

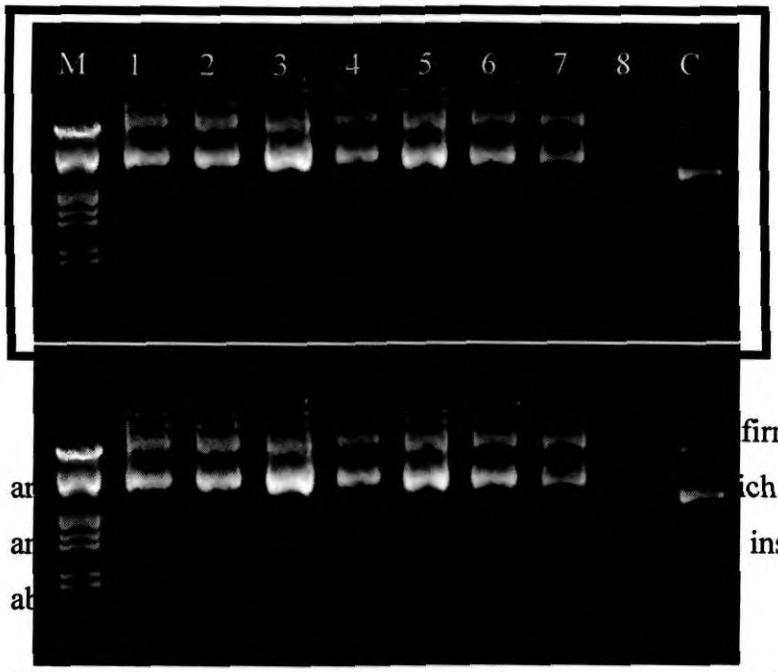
M-Marker : (λ DNA *EcoR*1/ *Hind* III double digest)
1 & 2 - 1.2 kb fragment

B. 0.8, 0.76 & 0.4 kb fragments

M-Marker : (λ DNA *EcoR*1/ *Hind* III double digest)
1-3 : 0.8 kb fragment
4-6 : 0.76 kb fragment
7-9 : 0.4 kb fragment

This confirmed the presence of cloned inserts in the transformed colonies. The recombinant plasmids with each of the four inserts were isolated from the corresponding positive colonies corresponding to each of the four inserts (fig. 23).

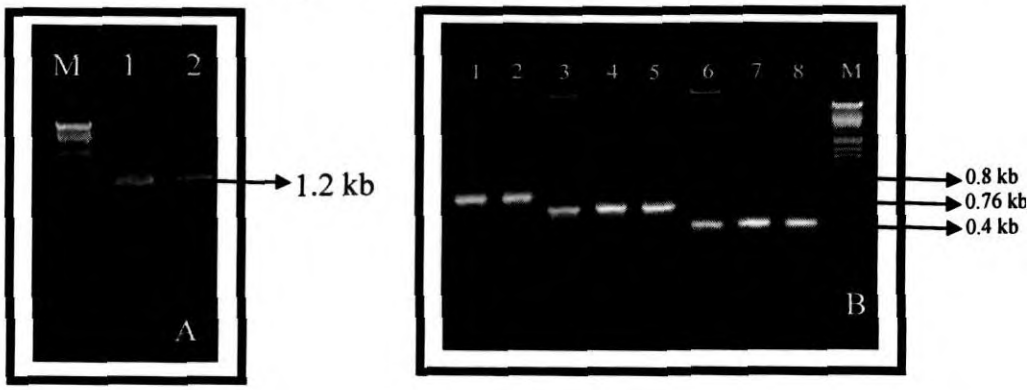
Fig. 23. Recombinant plasmids isolated from the positive colonies with different promoter fragments amplified



M - Marker (λ DNA *EcoR*1/ *Hind* III double digest)
1 & 2 - Plasmid harbouring the 1.2 kb insert
3 & 4 - 0.8 kb insert
5 & 6 - 0.76 kb insert
7 & 8 - 0.4 kb insert
C - Non-recombinan plasmid

firmed through PCR
ich again showed the
inserts as mentioned

Fig. 24. PCR of the recombinant plasmids with different promoter fragments amplified



A - 1.2 kb fragment

M- Marker (λ DNA *EcoR*1/ *Hind* III double digest)
1&2 : 1.2 kb fragment

B - 0.8, 0.76 & 0.4 kb fragments

1-2 : 0.8 kb fragment
3-5 : 0.76 kb fragment
6-8 : 0.4 kb fragment

The recombinant plasmids were sequenced at M/S Macrogen, Korea using M13 forward and reverse primers. The sequences from the four amplified bands revealed the existence of different promoter sequences of β-1,3-glucanase gene as determined from the known coding region of the gene.

The different bands amplified contained 913, 582, 553 and 198 nucleotides of the promoter region of β-1,3-glucanase gene upstream to the 'ATG' codon in the approximately 1.2, 0.8, 0.76 and 0.4 kb amplified products. The above PCR amplified products contain a portion of the known coding region of the gene at the 5' and 3' flanking regions. The nucleotide details of the 1.2 kb inverse PCR amplified band is given in fig. 25.

Fig. 25. The nucleotide sequence of the 1.2 kb inverse PCR product

TAGGAAATTCCTACCCTCCTTCTGCAGGTGCTTTCAGGGATGATGTTAGATCATACTTGG 60
ACCCAATTATTGGATTTCTATCCTCTATCAGGTCACCTTTACTTGCCAATATTTTTAGAA 120
TTAAAATTTTTAAATTTTTTGCATCTATAATAAAATTCCCATGATACCCTCAATTTATAA 180
TAATTTATATGCTCCCTAGGCTATTATTAAACCATAATTTGTTGGATTAGATTTATATTG 240
CATTCTTTTACGTATTTATATATTTATATTTTGATTTGTATTTTAATTATAAAACTTTTA 300
TTTTAAATTTTGTTATCTTATGATAAAATTTTAATAATTATATGATAGAGAATATACATT 360
ATCCACTGTATTAAATAACAATTTTTTTTTAAATTTTGGAAAATAAATAACTGGAAAATC 420
TCAAATTTTTTTTGAAATATCCCTGAATTAATAAAATAACATCCATGTTTAATAGATCCT 480
AAATTTCAATTTAAACGATAAAAATAATTATGTTATAAAGTGACAAATCTTATCCTTTTA 540
TCTTTTAAGGAGATAAGGAGGTGCAGAAAAAAGACTTAAACGTCTTCAACAGATAGAGAC 600
ATTCATTTATCCAACGAAAAATTGAAAGATGCCCAAGACATTTCAAACTAAATTAGCATT 660
AATTATCAGGATTATTAATTGGTAAAAAGTGCATTATTTTCAATAAAAGAAGTAAAATAA 720
TTTCACCGATTAGTTTTGAGAGATTGTTGTGCATTTTCGAAGCACATGTTTTCTTCCGAT 780
CAGACATTGGATCCACTGTTTCTTAATTATAATTATATATCTGTAAAGAGCACTATATTT 840
TAGTGCTCTAAATGCATGGTAGAATTTTTCTTAATTTTAAACCTCAGTTGAATTAAACAA 900
ACAAATAAACTCCAGCAACAGTATGCTCAAGAGTCCGCCTATAAAGCTTTGACATGGTAG 960
AAACCTAACACATATGCGTGGTCG**AAATTATAAGCAACTTTCTTCTAATTTCCCGCCTTC** 1020
TTAATGGCTATCTCCTCTTCAACTTCAGGAACTAGTAGTTCCCTGCCCTCAAGAACTACT 1080
GTCATGCTTCTTCTGATTTTCTTTACAGCAAGCCTGGTATAACAGG 1126

Total nucleotides - 1126
Upstream gene sequence - 110
Down stream gene sequence – 103
Promoter sequence : 913 bp.
5'UTR- **39**
ATG = TIC (start codon)
Forward primer
Reverse primer

Upon sequencing of the 1.2 kb band amplified through the inverse PCR, it contained 1126 nucleotides comprising a 913 bp promoter sequence (purple colour) including the 5'UTR and flanking known genomic portions of β-1,3-glucanase gene. As it is an inverse PCR product it contained a portion of the known genomic sequence at either side of the unknown or newly obtained promoter sequence. The flanking known coding region contained 213 nucleotides. Out of this, 110 nucleotides form one flanking region following the inverse PCR forward primer (24 mer) at the 5' end of the PCR product and

103 nucleotides form the other flanking region with the inverse PCR reverse primer (22 mer) at the 3' end of the PCR product. This is identified by the presence of the *Ssp*I enzyme recognition site AAT↓ ATT which is shown as underlined. The 5'UTR region comprises 39 nucleotides (shown in the green colour) starting from the 'AAATT' sequence (identified on alignment with the cDNA report by Chye *et al.*, 1996) extending upto the ATG initiation codon. The translation initiation codon 'ATG' is marked in blue. The forward and the reverse primers are highlighted in grey and yellow colour respectively. The BLASTn analysis of the obtained promoter sequence with the other reports showed that the 5'UTR region is showing a maximum identity of 97 % towards HGN1 mRNA reported from RRIM 600 (NCBI accession no: U22147) (fig. 26). Apart from very minor variations, the coding regions downstream to the newly obtained regulatory regions also get aligned to the reported sequences of β-1,3- glucanases in the GenBank, showing that the *promoter region belongs to β-1,3- glucanase gene itself.*

Fig. 26. BLASTn analysis of the 913 bp promoter showing the alignment of the 5'UTR region with that of the reported sequence -U22147

```
913 prom      AAATTATAAGCAACTTTCTTCTAATTTCCCGCCTTCTTA  913
              ||||||||||||||||||||||||||||| ||||||||
U22147      1 AAATTATAAGCAACTTTCTTCTAATTTCCCCCCTTCTTA  39
```

The alignment shows that out of the 39 UTR nucleotides in the 913 bp promoter, 38 no's are identical with the reported mRNA sequence (38/39 = 97% identity). It is interesting to observe that a portion of the promoter region is showing a 100 % similarity towards the Zebrafish (*Danio reiro*) DNA sequence from clone CH211-236H18 (NCBI Acc. No. CR385064) (fig. 27).

Fig. 27. BLASTn analysis of the 913 bp promoter showing the alignment of the 5'UTR region with that of the reported sequence from Zebrafish

```
>emb|CR385064.7|  Zebrafish DNA sequence from clone CH211-
236H18 in linkage group  5, complete sequence
Length=185245, Score = 52.8 bits (28),  Expect = 0.004
 Identities = 28/28 (100%), Gaps = 0/28 (0%), Strand=Plus/Minus
```

```
Query  263     AAATAACAATTTTTTTTTAAATTTTGGA  290
               ||||||||||||||||||||||||||||
Sbjct  171772  AAATAACAATTTTTTTTTAAATTTTGGA  171745
```

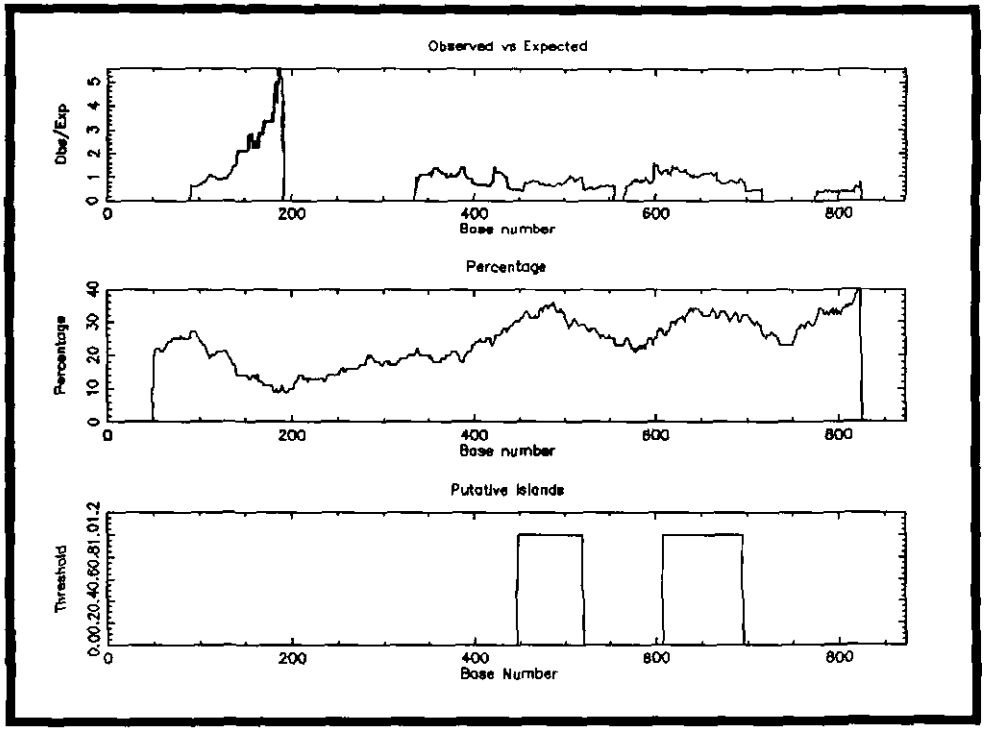
The promoter sequence is found to contain the essential cis-elements that is usually present in a biotic / abiotic stress related gene promoter. It contains the TATA, CAAT, GATA, WRKY, W box elements along with other complex regulatory regions. The details of the cis-elements and their frequency of occurence is presented in table. 8 (page no. 155)

The promoter region is rich in 'AT' base pairs similar to any other promoter. The 'GC' base pairs constitute only 24 %. The profiles of nucleosome potential identified through the RECON programme (Levitsky *et al*, 2004) showed the probability of nucleosome formation along the different promoter regions amplified. The program RECON has been used for constructing profiles of nucleosome potential, characterizing the probability of nucleosome formation along DNA sequences. Confidence level (CF) value is applied in case of the option 'Standardization by dispersion' is turned on. The CF value denotes the portion of correctly recognized sequences of the training set (i.e., within the interval [0, 1] of output range). The default CF value is set at 0.95 (reasonable values usually fall within the range 0.5 - 0.99). The higher CF allows to get more hits in the interval [0, 1]. Here the nucleosome packaging density was analyzed for the different functional types of glucanase gene promoters.

With the 913 bp promoter a very strong nucleosome forming potential was found in the region (5'- 3') between the nucleotides 85- 220, 399- 486 and also from 626 - 834 nucleotide (the value ranges between 0.5 - 0.9). The possibility of the existence of even more potential regions in the unidentified regions cannot be ruled out.

In the 913 bp promoter amplified, two CpG islands have been observed, first in the region 449-521 with about 32.88 % CG and second in the region 609 – 695 with 36.78 % CG (fig. 28)

Fig. 28. CpG island prediction of the 913 bp promoter using the EMBL-EBI – EMBOSS CpG island programme




```
Observed/Expected ratio > 0.60
     Percent C + Percent G > 30.00
     Length > 50

 Length 73 (449..521)
 Length 87 (609..695)
ID   913   BP.
DE   CpG Island report.
CC   Obs/Exp ratio > 0.60.
CC   % C + % G > 30.00.
CC   Length > 50.
FH   Key             Location/Qualifiers
FT   CpG island      449..521
FT                   /size=73
FT                   /Sum C+G=24
FT                   /Percent CG=32.88
FT                   /ObsExp=1.02
FT   CpG island      609..695
FT                   /size=87
FT                   /Sum C+G=32
FT                   /Percent CG=36.78
FT                   /ObsExp=1.02
FT   numislands      2
```

The details of the nucleotide sequence which showed approximately 0.8 kb band size is shown in fig.29.

The second amplified inverse PCR band (≈0.8 kb) showed approximately 0.8 kbp size and the nucleotide sequence revealed the presence of 793 nucleotides in it. As in the previous sequence the 5'UTR

sequence starts with 'AAATT' sequence and contain 37 nucleotides extending towards the ATG initiation codon. Out of these 793 nucleotides, 110 nucleotides from one flanking region comprising the inverse PCR forward primer (24 mer) and 103 nucleotides form the other flanking region with the inverse PCR reverse primer (22 mer). The unknown region start point is demarcated by the restriction enzyme site 'AATATT'. The amino acid start codon site ATG is marked in blue. The forward and the reverse primers are highlighted in grey and yellow colour respectively.

Fig. 29. Nucleotide sequence of the ≈0.8 kb inverse PCR product

TAGGAAATTCCTACCCTCCTTCTGCAGGTGCTTTCAGGGGTGATGTTAGATCATACTTGG 60
ACCCAATTATAGGATTCCTATCCTCTATTAGGTCACCTTTACTTGCCAATATTAGATATA 120
TTCAGTTTCATTCTTAAAGATATTATAAAAAAAATTAAAAAGATAAAAATTATTAAATTA 180
AAAAAATCTCAATTTTTTTTTTAAATATCTCTTAATAAAATAATACCCGTGATTATAAAT 240
CTTAGTTTTCAATTATTAGAGGATAAAAAATAATTATGTTGAAGAGTGAATTTAATCTTA 300
TTTTCTCAGTGTATTGTATTGCTGTGCTTGGACAAAATCTTATCCTTTATCTTTTAAGGA 360
GATTTCTAGGTGCAGAAATATGACTTTTAAGTCTTTAACAGATAGGGACATTCATCTGTC 420
CAACGAAAATTGAAAGATGGCTAAGACATTTTCCAAACTAAATTAATTGGCATTAATTGT 480
CATGATTGGTAAAGATGCAAGGAGAGTAATTGATAATTTTTAATTCAGTGATTGTTTAAA 540
ATTCCAATCTCAATTAAAGCCAAATAAATAAATTAATAAACTCCAGCAACAGTATGGAAA 600
AGGGTCCCTATATAAAGCATTGACATGGTAGTAACATAACACATATGCATGCCCCAAATT 660
AAGCTACTTTCTTCTAATTTCCCCCCTTCTTA**ATG**GCTATCTTCTCTTCAAATTCAGCAA 720
CTAGTAGTTCCTTGCCCTCAAGAACTACTTCCATACTTCTGGTTCTCTTTACAGCAAGCC 780
TTGGTGTAACAGG 793

Total: 793 bps
110 nucleotides upstream
101 nucleotides downstream
5'UTR - 37 bps
Promoter sequence: 582 no's
ATG = TIC
Forward primer
Reverse primer

The BLASTn analysis of the obtained promoter sequence showed that a small region in the promoter from the nucleotide no: 26-57 is getting aligned with a portion of the *Oryza sativa* japonica group genomic DNA BAC clone B1172G12 (NCBI Acc. No. AP006056) (fig. 30).

Fig. 30. BLASTn analysis of the 582 bp promoter showing 97% similarity with the reported subject sequence - AP006056

```
>dbj|AP006056.3| Oryza sativa Japonica Group genomic DNA,
chromosome 6, BAC clone:B1172G12
Length=171974
 Score = 52.8 bits (28),  Expect = 0.003
 Identities = 31/32 (97%), Gaps = 1/32 (3%)
 Strand=Plus/Minus

Query  26    AAAGATATTATAAAAAAAATTAAAAAGATAAA  57
             ||| ||||||||||||||||||||||||||
Sbjct  9547  AAA-ATATTATAAAAAAAATTAAAAAGATAAA  9517
```

The structure of this promoter fragment also shows typical characteristics of a biotic stress regulating promoter. The obtained promoter region is 'AT' rich with 26 % GC. It also contain the essential cis-elements including the TATA, CAAT, GATA, WRKY and W box elements along with other complex regulatory regions. The details of the cis-elements and their frequency of occurence is presented in table. 8 (page.no.155). The nucleosome binding potential recognized regions with a maximum nucleosome forming potential within a nucleotide range of 131-165, 225- 268, 294-412 bps (5'- 3' direction). Many of the regulatory regions like WRKY domain, some of the Dof core elements, E-boxes etc., occupy these regions. In this promoter no CpG islands were observed with the sense strand.

The third band amplified with 0.76 Kb in size was also cloned and sequenced. The details of this sequence is shown in fig.31. This sequence also belonged to the β-1,3-glucanase gene promoter as indicated by the similar downstream (102 bps) and upstream regions (110 bps) obtained from the known portions of β-1,3-glucanase gene. The sequence contained a promoter sequence of 553 nucleotides. This is also an 'AT' rich sequence with 26% 'GC'. It has a 5'UTR sequence of 39 bps starting with the 'AAATT' sequence upstream to the 'ATG' initiation codon. RECON software identified regions with high potential for nucleosome formation within the regions 81-144 bps and 163- 474 bps. The BLASTn analysis showed that the 5'UTR region shows 100% similarity with the reported β-1,3-glucanase mRNA sequence (Acc. No. U22147) (fig.32).

Fig. 31. Sequence of the 0.76 kb inverse PCR product

TAGGAAATTCCTACCCTCCTTCTGCAGGTGCTTTCAGGGATGATGTTAGATCATACTTGA 60
ACCCAATTATTAGATTTCTATCCTCTATCAGGTCACCTTTACTTGCCAATATTAAACTTA 120
TAAATATCCAAACTTATAAATTTATAATAATTGCTATTAATAAGTATGATGGTTAATTAT 180
ATGGTTAAAATGATCCACCAACACAAGAGTCAGGATTATTCTGGTCATTGGTCCATAAAT 240
TAACTATGACTTTGAACAAATTAGTAAATATTTTTAGAATTAAAATTTTCAAATTTTTTG 300
CATCTATAATATAATTCCCATGATACCCTCAATTTATAATAATATATATGCTGCCTAGGT 360
TATTATTAAATCATAATTTGTTGGATTAGATTTATATTGCATTCTTTTACATTGGATCCA 420
CTGTTTCTTAATTTAGAAAAATAACTATAATTATATAAGTGTAAAGAGCACTATATTTAT 480
GGTGCTCTAAATGCATGGTAGAATTTTTCTTAATTTTAAACCTCAGTTGAAATTAAATAA 540
ACAAATAAACTCCAGCAACAGTATGCTCAAGAGTCCGCCTATAAAGCATTGACATGGTAG 600
TAACCAAACGCATATGCGTGGTCGAAATTATAAGCAACTTTCTTCTAATTTCCCCCCTTC 660
TTA**ATG**GCTATCTGCTCTTCAACTTCAGGAACTAGTAGTTCCTTGCCCTCAAGAACTACT 720
GTCATGCTTCTTCTGTTTTTCTTTGCAGCAAGCCTGGATAACAGG 765

Total 765
Upstream known 110 bps
Downstream known 102 bps
5'UTR- 39bps
Promoter sequence : 553 bp
ATG = TIC

Forward primer
Reverse primer

Fig.32. BLASTn analysis of the 553 bp promoter.
The 5'UTR region is showing 100% similarity with the reported subject sequence- U22147

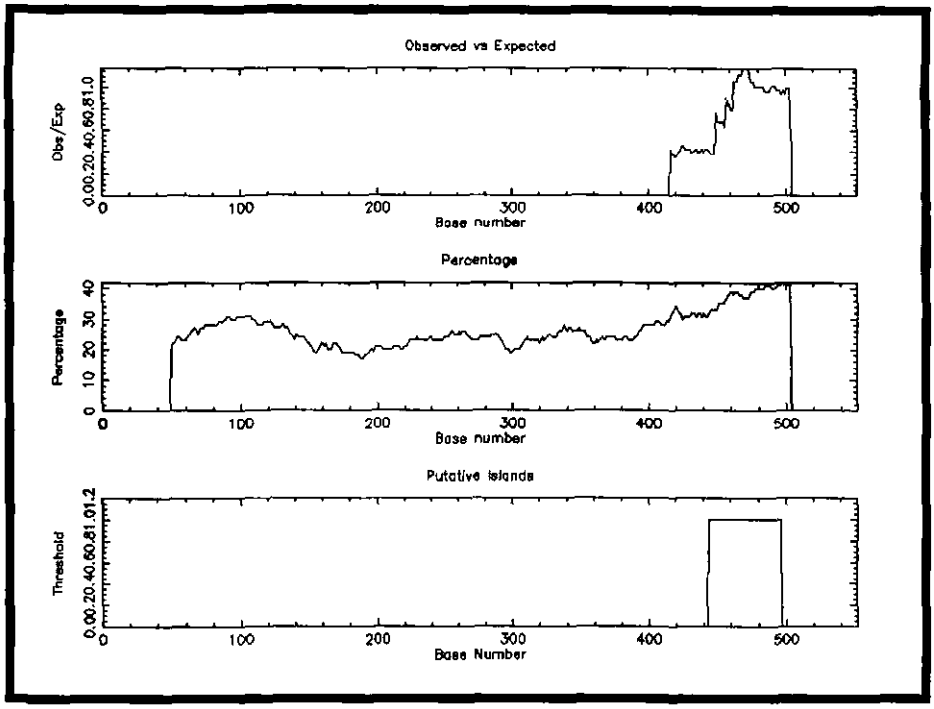
>gb|U22147.1|HBU22147 *Hevea brasiliensis* β-1,3-glucanase (HGN1) mRNA, complete cds
Length=1242

Score = 73.1 bits (39), Expect = 2e-09
Identities = 39/39 (100%), Gaps = 0/39 (0%)
Strand=Plus/Plus

Query 515 AAATTATAAGCAACTTTCTTCTAATTTCCCCCCTTCTTA 553
||||||||||||||||||||||||||||||||||||||||
Sbjct 1 AAATTATAAGCAACTTTCTTCTAATTTCCCCCCTTCTTA 39

The presence of the CpG islands showed a single CpG island (445..497) with a CG composition of 41.51% (fig: 33).

Fig. 33. CpG island present in the 553 bp promoter




```
ID     553 BP.
DE   CpG Island report.
CC   Obs/Exp ratio > 0.60.
CC   % C + % G > 30.00.
CC   Length > 50.
FH   Key              Location/Qualifiers
FT   CpG island       445..497
FT                    /size=53
FT                    /Sum C+G=22
FT                    /Percent CG=41.51
FT                    /ObsExp=0.44
FT   numislands       1
```

The details of the sequence which showed approximately 0.4 kb is shown in fig. 34. The 0.4 kb size band amplified was the smallest fragment amplified through inverse PCR. The sequencing result of the 0.4 kb band revealed the presence of a 412 bp band product harbouring a 198 bp promoter sequence of β-1,3-glucanase gene. The upstream and downstream elements of the known gene composed of 110 and 104 nucleotides respectively including the primer sequences. The 5'UTR region composed of 38 bps upstream to the the 'ATG' initiation codon. The promoter region is 'AT' rich with 30% 'GC' in the identified region. The promoter region contained the putative core elements. Since this promoter is having only 198 bps, it is clear that the frequency of occurrence of the core promoter sequences is also very less.

Fig. 34. Sequence of the 0.4 kb inverse PCR product

TAGGAAATTCCTACCCTCCTTCTGCAGGTGCTTTCAGGGATGATGTTAGATCATACTTGA 60
ACCCAATTATTAGATTTCTATCCTCTATCAGGTCACCTTTACTTGCCAATATTTCTTCCA 120
TTAAATAAATATATTAGTTTTAATTTGGCGATAGTTTAAATTCAAACCC**CAAT**TGACATT 180
AAATAAATAAATAAGCTCCAGAAATAGTATGCTAAGGAGTCCCTA**TATAA**AGCATAGACT 240
TGGTAGCAATGTAATGCATATGCATGCTCCAAATTAAGCTGCTCTCTTCTTAGGTTCCAT 300
CCTTCTTA**ATG**GCCATGCTTTCTTCAACTTCGAGAACTACTGGTTCCTTACTCTCAAGAA 360
CTCCTGTCATGCTTCTTCTGATTCTCTATGCAGCAAGCCTTGGTATAACAGG 412

Total: 412 nucleotides
Upstream 110 nucleotides
Downstream- 104 nucleotides
5'UTR- 38 bps
Promoter- 198 bp
ATG = TIC

Forward primer
Reverse primer

The BLASTn analysis of this promoter sequence showed high similarity towards the promoter region of the reported sequence of *Hevea* β-1,3- glucanase (Acc.No. AY325498) (fig. 35). The details of the consensus TATA box and the CAAT box motifs upstream of the ATG codon - TIC) are shown in the table. 8 (page no. 155).

Fig.35. BLASTn analysis of the 198 bp promoter sequence
The alignment shows very high homology towards the reported sequence of Hevea β-1,3-glucanase - AY325498)

```
>gb|AY325498.2|  Hevea brasiliensis beta-1,3-glucanase (Glu I)
gene, complete cds, Length=1468
 Score =  361 bits (195),  Expect = 1e-96, Identities = 197/198
(99%), Gaps = 0/198 (0%)

Query  1    ATTTCTTCCATTAAATAAATATATTAGTTTTAATTTGGCGATAGTTTAAATTCAAACCCC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  14   ATTTCTTCCATTAAATAAATATATTAGTTTTAATTTGGCGATAGTTTAAATTCAAACCCC  73

Query  61   AATTGACATTAAATAAATAAATAAGCTCCAGAAATAGTATGCTAAGGAGTCCCTATATAA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  74   AATTGACATTAAATAAATAAATAAGCTCCAGAAATAGTATGCTAAGGAGTCCCTATATAA  133

Query  121  AGCATAGACTTGGTAGCAATGTAATGCATATGCATGCTCCAAATTAAGCTGCTCTCTTCT  180
            |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  134  AGCATAGACTTGGTAGTAATGTAATGCATATGCATGCTCCAAATTAAGCTGCTCTCTTCT  193

Query  181  TAGGTTCCATCCTTCTTA  198
            ||||||||||||||||||
Sbjct  194  TAGGTTCCATCCTTCTTA  211
```

Although the CpG islands are absent in the short identified region, they may be present in the non-identified upstream regions of this promoter. The

nucleosome forming potential showed a dense region between the 81st and 119th nucleotide. The details of the consensus TATA box and the CAAT box motifs upstream of the 'ATG' codon - TIC) are shown in the table. 8 (page no. 155). The nucleotide comparison of the four β-1,3-glucanase gene promoter forms were made through Clustal W (fig.36).

Fig. 36. ClustalW multiple sequence alignment of the four promoter fragments obtained through inverse PCR

```
582         ------------------------------------------------------------
198         ------------------------------------------------------------
913         ATTTTTAGAATTAAAATTTTTAAATTTTTTGCATCTATAATAAAATTCCCATGATACCCT 60
553         ------------------------------------------------------------

582         ------------------------------------------------------------
198         ------------------------------------------------------------
913         CAATTTATAATAATTTATATGCTCCCTAGGCTATTATTAAACCATAATTTGTTGGATTAG 120
553         ------------------------------------------------------------

582         ------------------------------------------------------------
198         ------------------------------------------------------------
913         ATTTATATTGCATTCTTTTACGTATTTATATATTTATATTTTGATTTGTATTTTAATTAT 180
553         ------------------------------------------------------------

582         ------------------------------------------------------------
198         ------------------------------------------------------------
913         AAAACTTTTATTTTAAATTTTGTTATCTTATGATAAAATTTTAATAATTATATGATAGAG 240
553         ------------------------------------------------------------

582         ------------------------------------------------------------
198         ------------------------------------------------------------
913         AATATACATTATCCACTGTATTAAATAACAATTTTTTTTTAAATTTTGGAAAATAAATAA 300
553         ------------------------------------------------------------

582         ----------------------------------ATTAGATATATTCAGTTTCATTCTT 25          TTCAGTTTCATTCTT
198         ------------------------------------------------------------
913         CTGGAAAATCTCAAATTTTTTTTGAAATATCCCTGAATTAATAAAATAACATCCATGTTT 360
553         ------------------------------------------------------------ATT 3          ----------ATT      3

582         AAAGATATTATAAAAAAAATTAAAAAGATAAAAATTATTAAATTAAAAAAATCTCAATTT 85
198         ------------------------------------------------------------
913         AATAGATCCTAAATTTCAATTTAAACGATAAAAATAATTATGTTATAAAGTGACAAATCT 420
553         AAACTTATAAATATCCAAACTTATAAATTTATAATAATTGCTATTAATAAGTATGATGGT 63

582         TTTTTTTAAATATCTCTTAATAAAATAATACCCGTGATTATAAATCTTA-GTTTTCAAT  144
198         ------------------------------------------------------------
913         TATCCTTTTATCTTTTAAGGAGATAAGGAGGTGCAGAAAAAAGACTTAAACGTCTTCAAC 480
553         TAATT----ATATGGTTAAAATGATCCACCAACACAAGAGTCAGGATTATTCTGGTCATT 119

582         TATTAGAGGATAAAAAATAATTATGTTGAAGAGTGAATTTAATCTTATTTTCTCAGTGTA 204
198         ------------------------------------------------------------
913         AGATAGAGACATTCATTTATCCAACGAAAAATTGAAAGATGCCCAAGACATTTCAAACTA 540
553         GGTCCATAAATTAACTATGACTTTGAACAAATTAGTAAATATTTTTAGAATT--AAAATT
```

```
582         TTGTATTGCTGTGCTTGGACAAAATCTTATCCTTTATCTTTTAAGGAGATTTCTAGGTGC 264
198         ------------------------------------------------------------
913         AATTAGCATTAATTATCAGGATTATTAATTGGTAAAAAGTGCATTATTTTCAATAAAAGA 600
553         TTCAAATTTTTTGCATCTATAATATAATTCCCATGATACCCTCAATTTATAATAATATAT 177


582         AGAAATATGACTTTTAAGTCTTTAACAGATAGGGACATTCATCTGTCCAACGAAAATTGA 324
198         ------------------------------------------------------------
913         AGTAAAATAATTTCACCGATTAGTTTTGAGAGATTGTTGTGCATTTTCGAAGCACAT-GT 659
553         ATGCTGCCTAGGTTATTATTAAATCATAATTTGTTGGATTAGATTTATATTGCATTCTTT 237


582         AAGATGGCTAAGACATTTTCCAAACTAAATTAATTGGCATTAATTGTCATGATTGGTAAA 384
198         ------------------------------------------------------------ATT 3
913         TTTCTTCCGATCAGACATTGGATCCACTGTTTCTTAATTATAATTATAT--ATCTGTAAA 717
553         TACATTGGATCCACTGTTTCTTAATTTAGAAAAATAACTATAATTATAT--AAGTGTAAA 295
                                                                     *

582         GATGCAAGGAGAG-TAATTGATAATTTTTAATTCAGTGATTGTTTAAAATTCCAATCTCA 443
198         TCTTCCATTAAAT-AAATATATTAGTTTTAATTTGGCGATAGTTTAAA-TTCAAACCCCA 61
913         GAGCACTATATTT-TAGTGCTCTAAATGCATGGTAGAATTTTTCTTAATTTTAAACCTCA 776
553         GAGCACTATATTTATGGTGCTCTAAATGCATGGTAGAATTTTTCTTAATTTTAAACCTCA 355
                      *      *    *  *  *    *   *  * * ** **  ** * **

582         ATTAAAGCCAAATAAATAAATTAATAAACTCCAGCAACAGTATGGAAAAGGGTCCCTATA 503
198         ATTGACATTAAATAAATAAAT----AAGCTCCAGAAATAGTATGCTAAGGAGTCCCTATA 117
913         GTTGA-ATTAAACAAACAAAT----AAACTCCAGCAACAGTATGCTCAAGAGTCCGCCTA 831
553         GTTGAAATTAAATAAACAAAT----AAACTCCAGCAACAGTATGCTCAAGAGTCCGCCTA 411
             ** *    *** *** ****    ** ****** ** ******   * * ****   **

582         TAAAGCATTGACATGGTAGTAACATAACACATATGCATGCCCCAAATTA--AGCTACTTT 561
198         TAAAGCATAGACTTGGTAGCAATGTAATGCATATGCATGCTCCAAATTA--AGCTGCTCT 176
913         TAAAGCTTTGACATGGTAGAAACCTAACACATATGCGTGGTCGAAATTATAAGCAACTTT 891
553         TAAAGCATTGACATGGTAGTAACCAAACGCATATGCGTGGTCGAAATTATAAGCAACTTT 411
            ****** * *** ****** **   **  ******* **  * ******  ***  ** *

582         CTTCT-AATTTCCCCCCTTCTTA<b>ATGGCTATCTTCTCTTCAAATTCAGCAACTAGTAGTT</b> 620
198         CTTCTTAGGTTCCATCCTTCTTA<b>ATGGCCATGCTTTCTTCAACTTCGAGAACTACTGGTT</b> 236
913         CTTCT-AATTTCCCGCCTTCTTA<b>ATGGCTATCTCCTCTTCAACTTCAGGAACTAGTAGTT</b> 950
553         CTTCT-AATTTCCCCCCTTCTTA<b>ATGGCTATCTGCTCTTCAACTTCAGGAACTAGTAGTT</b> 470
            ***** *  ****  ************* **    ******* ***   ***** * ***

582         CCTTGCCCTCAAGAACTACTTCCATACTTC---TGGTTCTCTTTACAGCAAGCCTTGGTG 677
198         CCTTACTCTCAAGAACTCCTGTCATGCTTCTTCTGATTCTCTATGCAGCAAGCCTTGGTA 296
913         CCCTGCCCTCAAGAACTACTGTCATGCTTCTTCTGATTTTCTTTACAGCAAGCCT-GGTA 1009
553         CCTTGCCCTCAAGAACTACTGTCATGCTTCTTCTGTTTTTCTTTGCAGCAAGCCT--GGA 528
            ** * * ********** **  *** ****   ** ** *** * **********  *

582              TAACAGG 684
198              TAACAGG 303
913              TAACAGG 1016
553              TAACAGG 535
                 *******
```

Gene sequence
Promoter sequence
Initiation codon- ATG
Exon

Sequences (1:2) Aligned. Score: 71
Sequences (1:3) Aligned. Score: 50
Sequences (1:4) Aligned. Score: 68
Sequences (2:3) Aligned. Score: 33
Sequences (2:4) Aligned. Score: 74
Sequences (3:4) Aligned. Score: 68

1- 582 bp promoter
2- 198 bp promoter
3- 913 bp promoter
4- 553 bp promoter

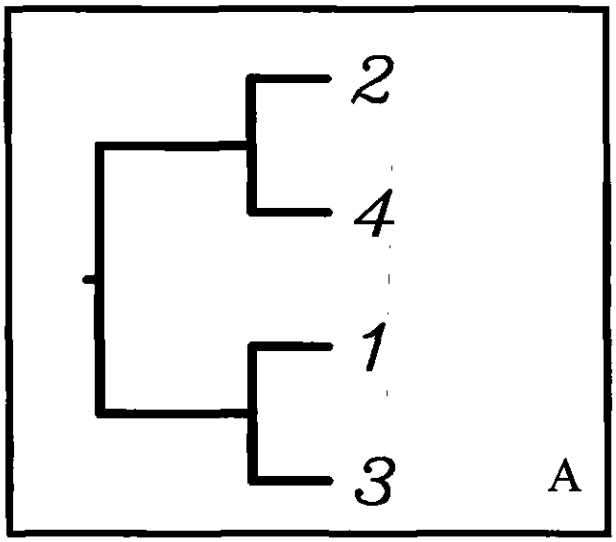
Upon alignment of the four different promoters, it was observed that the promoter regions at the distal end from the 'ATG' showed much nucleotide variations whereas the nucleotides towards the coding region is more homologous. Minor nucleotide variations were observed in the 5'UTR region with respect to the number of nucleotides. The known gene portions which flanked at either ends of the inverse PCR product showed significant

alignment among the four sequences. These similarities in the coding and 5'UTR regions showed that the four isolated fragments represent multiple forms of promoters that may regulate four different forms of β-1,3-glucanase gene. When each of the sequence is considered, the 582 bp sequence is showing more homology towards the sequence belonging to the 198 bp (score = 71) promoter. The sequence with the 913 bp promoter showed more homology towards the sequence with the 553 bp promoter (score 74).

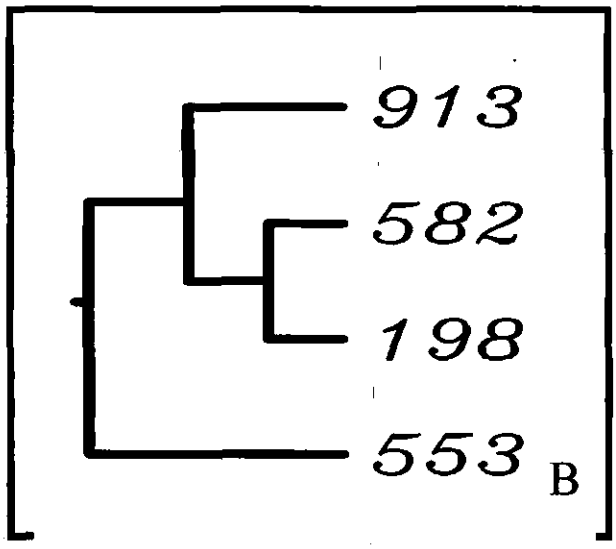
The phylogenetic tree analysis showed a difference in the clustering pattern of the promoters when the total length of the isolated promoters as well as the 198 bps (which represent the length of the smallest fragment) were considered. A 198 bp downstream region of each of the isolated promoter were taken first to construct the phylogenetic tree. This showed that the 913 and 553 bp promoter were clusterd together showing more similarity within the 198 bp region residing in both. Similarly the 582 and 198 bp promoter formed another cluster with this minimum 198 bp promoter length (fig.37. A).

The phylogenetic tree analysis with the total nucleotides of the isolated four promoters also showed another way of clustering. The 913 bp promoter showed more affinity towards 582 and 198 bp promoter. The promoter forms with 582 and 198 bps are clustered together showing more homology than with the 913 and the 553 bp promoters. The 913 and the 553 bp promoters is more aligned and clustered than the other two promoters (fig. 37. B).

Fig.37. Phylogenetic tree showing the way of clustering of the four different isolated promoters

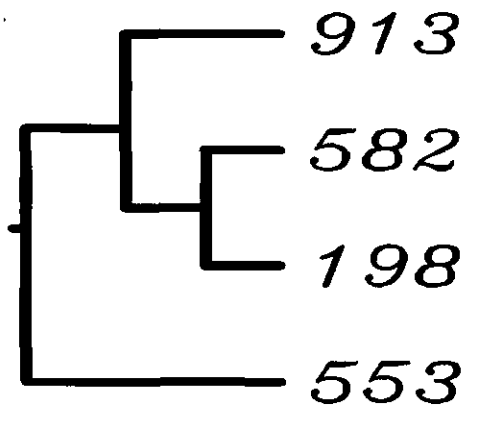


1 – 198 bps from the 913 bp promot
2 - 198 bps from the 582 bp promot
3- 198 bps from the 553 bp promote
4- 198 bps from the 198 bp promote



A - 3-glucanase gene within their

B - 1,3-glucanase gene with 913,



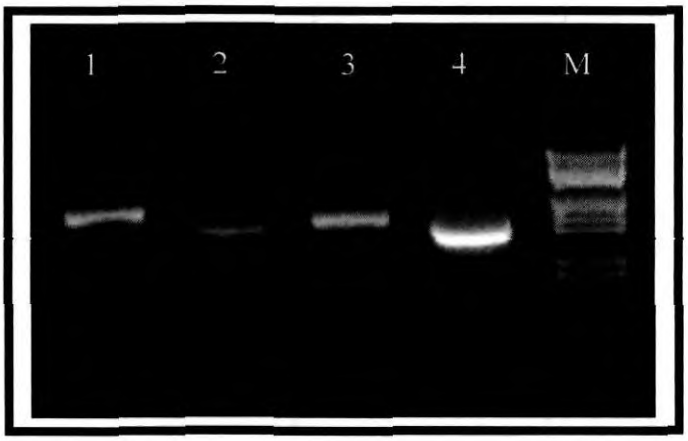
a vital part of the plant's defen elements residing within the prom cently been identified and inves trans-acting factors. Some of these as others are present only in plants, reflecting the fact that plants have developed a unique defence system

(Rushton and Somssich, 1998). This is the first report presenting the isolation of multiple promoter sequences of β-1,3-glucanases from a single clone of *Hevea brasiliensis*.

4.3. Cloning and sequence characterization of different β-1,3-glucanase promoter along with their coding DNA sequences

In the previous section, while attempting to characterize the promoter of β-1,3-glucanase gene, four different promoter sequences were obtained from a single *Hevea* clone RRII 105. This raised the curiosity to understand whether multiple forms of the coding region corresponding to different forms of β-1,3-glucanase gene promoter is also existing in a single clone of *Hevea*. To check this, attempts were made to clone and characterize different promoter forms along with their coding region.

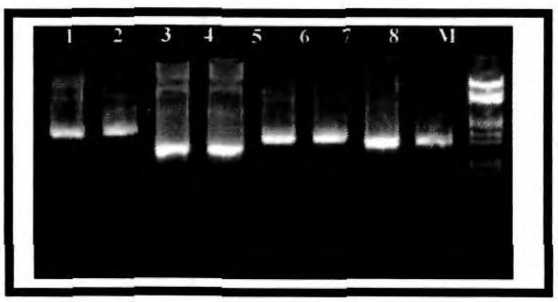
Genomic DNA isolated from the *Hevea* clone RRII 105 was used as the template for PCR amplification with specific primers. In this study good level of amplification was obtained with the forward primer specific to the different forms of β-1,3-glucanase gene promoter and reverse primer specific to the 3'end of CDS. The reverse primer was chosen from a region earlier detected to be a conserved portion of the genomic sequence including the stop codon of the sequence. Under optimal PCR conditions, that include concentrations of template DNA, dNTPs, *Taq* DNA polymerase and Mg^{2+}, approximately 1.3, 1.4, 1.5 and 1.8 kb single band products were amplified when 20 ng of genomic DNA was used as the template. The primers used were IPF1 + 1R , IPF2 + 1R and IPF4 + 1R (table.3) respectively. At an annealing temperature of 50°C, prominent amplification has been obtained with all the four different forms of the gene (fig.38). But one of the forms, which is having the 582 bp promoter region could not get amplified with the reverse primer -1R). So separate reverse primer (2R) was used for amplification of the promoter gene fusion of this fragment along with the forward primer IPF3. The reverse primer (2R) was designed within the exon region 286 bp upstream to the translation termination portion of the gene.



1- 1.8 kb (913 bp pro)
2- 1.3 kb (582 bp pro)
3- 1.5 kb (553 bp pro)
4- 1.4 kb (198 bp pro)
M- Marker ((λ DNA *EcoR*1/ *Hind* III double digest)

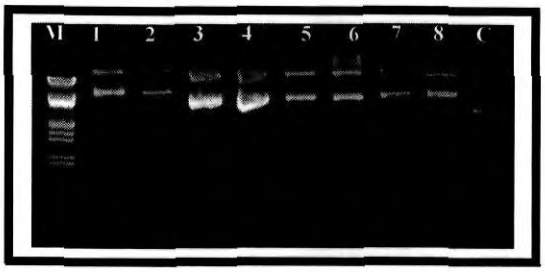
Fig.38. PCR amplification of the four different promoter forms along with the β-1,3-glucanase gene

The amplified fragments have been gel eluted and TA cloned in pGEM®-T easy vector (M/S Promega, USA). The recombinant plasmid was transformed into chemically competent *E.coli*- DH5α cells. Colony PCR done with each of the β-1,3-glucanase gene specific primers in the transformed colonies showed positive amplification confirming the presence of recombinant plasmids (fig. 39). Fragment sizes were analysed with the molecular weight marker and cross checked with the initial amplification of the different forms of the gene. The size of the different fragments were equal to the initial amplification obtained with the different forms of β-1,3-glucanase. Recombinant plasmids were isolated from the positive colonies (fig.40) and PCR was performed for the presence of inserts (fig.41). The plasmids representing each of the four different forms of β-1,3-glucanase were sequenced.



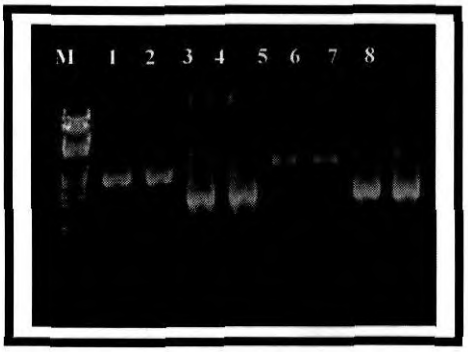
1& 2 - 1.8 kb
3& 4 - 1.3 kb
5& 6 - 1.5 kb
7& 8 - 1.4 kb
M - Marker (λ DNA *EcoR*1/ *Hind* III double digest)

Fig. 39. Colony PCR to show the amplification of inserts (promoter + gene fusion) corresponding to the different forms of β-1,3-glucanases



1& 2 - 1.8 kb inserted plasmid
3& 4 - 1.3 kb inserted plasmid
5& 6 - 1.5 kb inserted plasmid
7& 8 - 1.4 kb inserted plasmid
C - Control plasmid without insert
M - Marker (λ DNA *EcoR*1/ *Hind* III double digest)

Fig. 40. Recombinant plasmids isolated from the positive colonies



1& 2 - 1.8 kb
3& 4 - 1.3 kb
5& 6 - 1.5 kb
7& 8 - 1.4 kb
M - Marker (λ DNA *EcoR*1/ *Hind* III double digest)

Fig. 41. PCR of the recombinant plasmids

4.3.1. Sequence details of the coding DNA sequence of β-1,3-glucanase gene with the 913 bp promoter

The CDS (coding DNA sequence) corresponding to the 913 bp promoter is shown in fig.42 (NCBI Acc. No. JQ650525). The primers used for the amplification of the promoter with the coding gene portion is underlined and highlighted in blue colour. An amplicon with 1806 bps was obtained with the promoter specific forward and the gene specific reverse primer. The PCR product containing the amplified promoter-gene combination consisted of 564 bp promoter along with a 1242 bp gene ending with the stop codon 'TGA'. The intron region consists of 117 bp with 11 'CT' repeats. The start codon and the stop codon is highlighted in violet and red colour respectively. The coding region was found to be bi-exonic.

The amino acid sequence was deduced using the ExPASy -ProtParam (online) software programme. This predicted 374 amino acids and a stop codon (fig. 43).

Fig. 42. Nucleotide sequence of the coding region of β-1,3-glucanase following the 913 bp promoter

5′ TTTTTAGAATTAAAATTTTTAAATTTTTTGCATCTATAATAAAATTCCCATGATACCC
TCAATTTATAATAATTTATATGCTCCCTAGGCTATTATTAAATCATAATTTGTTGGATTA
GATTTATATTGCATTCTTTTACGTATTTATATATTTATATTTTGATTTGTATTTTAATTA
TAAAACTTTTATTTTAAATTTTGTTATCTTATGATAAAATTTTAATAATTATATGATAGA
GAATATACATTATCCACTGTATTAAATAACAATTTTTTTTTAAATTTTGGAAAATAAATA
ACTGGAAAATCTCAAATTTTTTTTTGAAATATCCCTGAATTAATAAAATAA**CATCCATGT**
TTAATAGATCCTAAATTTCAATTTAAACGATAAAAATAATTATGTTATAAAGTGACAAAT
CTTATCCTTTTATCTTTTAAGGAGATAAGGAGGTGCAGAAAAAAGACTTAAACGTCTTCA
ACAGATAGAGACATTCATTTATCCAACGAAAAATTGAAAGATGCCCAAGACATTTCAAAC
TAAATTAGCATTAATTATCAGGATTATTAATTGGTAAAGAGTGCATTATTTTCAATAAAA
GAAGTAAAATAATTTCACCGATTAGTTTGAGAGATTGTTGTGCATTTTCGAAGCACATGT
TTTCTTCCGATCAGACATTGGATCCACTGTTTCTTAATTATAATTATATATCTGTAAAGA
GCACTATATTTTAGTGCTCTAAATGCATGGTAGAATTTTTTTTAATTTTAAACCTCAGTT
GAAATTAAACAAACAAATAAACTCCAGCAACAGTATGCTCAAGAGTCCGCCTATAAAGCT
TTGACATGGTAGTAACCTAACACATATGCGTGGTCGAAATTATAAGCAACTTTCTTCTAA
TTTCCCGCCTTCTTA**ATG**GCTATCTCCTCTTCAACTTCAGGAACTAGTAGTTCCCTGCC
CTCAAGAACTACTGTCATGCTTCTTCTGATTTTCTTTACAGCAAGCCTTGGTATAACAGG
TCTCTCTCTCTCTCTCTCTCTCCATCTATCTACTCTCATGTTAAAGTTGACGATGCTC
TTTTTTTCCCTCCTTTTTCTAAAAGGTTTCAACTAATACCTGTATTTGGAATTTAGATGC
CCAGGTAGGTGTTTGCTATGGAATGCAAGGCAACAACCTTCCATCTGTTTCAGAGGTCAT
AGCTCTCTATAAACAATCTAACATCAAGAGAATGAGAATTTATGATCCAAATCAAGCAGT
ATTGGAAGCCCTTAGAGGCTCAAACATTGAACTCATACTAGGTGTTCCAAACTCAGATCT
CCAAAGCCTTACCAATCCTTCCAATGCAAACTCATGGGTACAAAAAAATGTTCGTGGGTT
CTGGTCAAGTGTCAGGATCAGATATATAGCAGTGGCAACGAAATTAGTCCCTGTAAATGG
AGGCACAGCTTGGTTGGCCCAATTTGTTTTGCCTGCCATGAGAAATATACATGATGCTAT
AAGATCAGCTGGTCTTCAAGATCAAATCAAGGTCTCCACTGCAATTGACTTGACCCTGGT
AGGAAATTCCTACCCTCCTTCTGCAGGTGCTTTCAGGGATGATGTTAGATCATACTTGGA
CCCAATTATTGGATTTCTATCCTCTATCAGGTCACCTTTACTTGCCAATATTTATCCTTA
CTTTACTTATGCTGATAATCCAAGGGATATTTCCCTTCCCTATGCTTTGTTCACTTCACC
ATCAGTTGTTGTGTGGGATGGTCAGCGAGGTTATAAGAACCTTTTTGATGCAACGTTGGA
TGCATTGTACTCTGCTCTTGAGAGGGCTAGTGGTGGTTCTCTGGAGGTGGTTGTTTCGGA
AAGTGGCTGGCCGTCTGCCGGAGCATTTGCTGCCACATTTGACAATGGGCGTACTTATCT
CTCAAATTTGATCCAGCATGTTAAAGGAGGTACTCCTAAGAGGCCTAACAGAGCTATAGA
GACTTACTTATTTGCCATGTTTGATGAAAATAAGAAGCAACCAGAGGTTGAGAAACACTC
TGGACTTTTCTTTCCTGATAAACGGCCAAAATATAATCTCAATTTTGGTGCAGAAAAGAA
CTGGGATATTTCTACTGAACACGATGCAACAATACTTTTC**CTTAAGAGTGATATG**TGA
3′

Total region amplified – 1806 bps
Promoter – 913 (amplified portion – 564)
5'UTR- 39 bps
Gene- 1242 bps (from ATG - stop codon)
Introns- 117 bps
Start codon
Stop codon
Primer sequences underlined

Fig. 43. Amino acid sequence deduced from the coding DNA sequence of β-1,3-glucanase having the 913 bp promoter

```
        10         20         30         40         50         60         70
         |          |          |          |          |          |          |
MAISSSTSGTSSSLPSRTTVMLLLIFFTASLGITDAQVGVCYGMQGNNLPSVSEVIALYKQSNIKRMRIY
DPNQAVLEALRGSNIELILGVPNSDLQSLTNPSNANSWVQKNVRGFWSSVRIRYIAVATKLVPVNGGTAW
LAQFVLPAMRNIHDAIRSAGLQDQIKVSTAIDLTLVGNSYPPSAGAFRDDVRSYLDPIIGFLSSIRSPLL
ANIYPYFTYADNPRDISLPYALFTSPSVVVWDGQRGYKNLFDATLDALYSALERASGGSLEVVVSESGWP
SAGAFAATFDNGRTYLSNLIQHVKGGTPKRPNRAIETYLFAMFDENKKQPEVEKHSGLFFPDKRPKYNLN
FGAEKNWDISTEHDATILFLKSDM
```

Number of amino acids: 374
Molecular weight: 41071.6
Theoretical pI: 8.45
Total number of negatively charged residues (Asp + Glu): 32
Total number of positively charged residues (Arg + Lys): 34

* *Red colour shows the hydrophobic and positively charged basic amino acids arginine (R) and lysine (K)*

The size of the predicted amino acid is approximately 41 kDa with an isoelectric point of 8.45 which shows that it is a basic protein. There are 32 negatively charged residues and 34 positively charged residues.

The conserved domain identification with the NCBI database showed that there is no 100 % amino acid sequence similarity, although similar domains were observed for the peptide sequence of other members in the glycosyl hydrosylase family 17 like *Hordeum vulgare, Solanum lycopersicum, Nicotiana tabaccum, Pisum sativum, H.vulgare* G III and *H.vulgare* G IV. It showed the presence of similar domains in different species of plants as well as in the yeast *Candida albicans* (fig. 44). Different isozymes of β-1,3-glucanase has been reported from other crops also from a single host (as in *H.vulgare*).

The BLASTp analysis of this petide sequence showed 90-98 % similarity of the peptide with other reported forms of β-1,3-glucanases from *H.brasiliensis*. It showed a 94 % homology with the Chain A-crystal structure of a native endo β-1,3-glucanase (Hev B 2), reported by Fuentes- silva *et al.* (2009) (PDB ID: 3EM5A). With the acidic β-1,3-glucanase reported from *Pisum sativum* (Acc.No. AAB24398) it is showing 62% similarity and 58% similarity with the basic β-1,3-glucanase from *N.tabaccum* (Acc.No. ACF93731). It is also showing 63 % similaty with acidic glucanase from *Medicago sativa* (Acc.No. ABY47902) and *Glycine max* (AE154334).

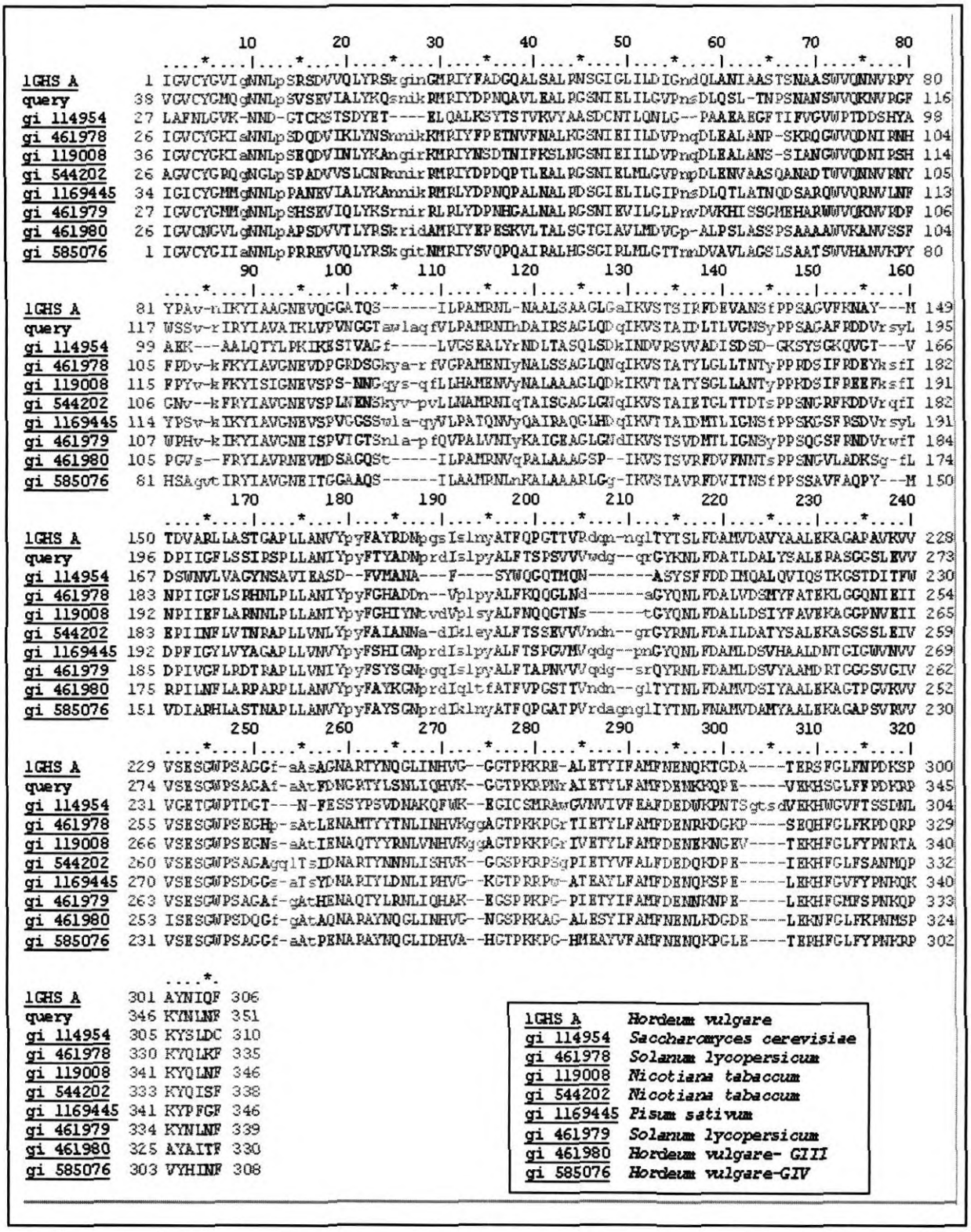


Fig.44. Conserved domain search results of amino acid sequence deduced from the coding DNA sequence of of β-1,3-glucanase having the 913 bp promoter with other reports (Glycosyl hydrolases family 17) in the database

In the attempts to get the promoter-gene combination of β-1,3-glucanase with the 913 bp promoter, with one of the forward primer (IPF5) (designed from the 5' region of the promoter) in combination with the 1R reverse primer, (designed from the downstream end of the coding DNA sequence including the stop codon); a new form of glucanse was obtained in PCR at an annealing temperature of 50 ^{0}C. The fragment showed a molecular size of approximately 2 kb (fig.45). Further the insert was amplified using the proof reading enzyme XT-5 *Taq* DNA polymerase also inorder to confirm the identity of this newly obtained β-1,3- glucanase. The amplified band was purified and cloned in Zero-blunt Topo vector (M/S Invitrogen, USA). The colony PCR showed positive for the presence of the insert (fig.46). The recombinant plasmids (fig. 47) were isolated and sequenced to confirm the presence of the inserts.

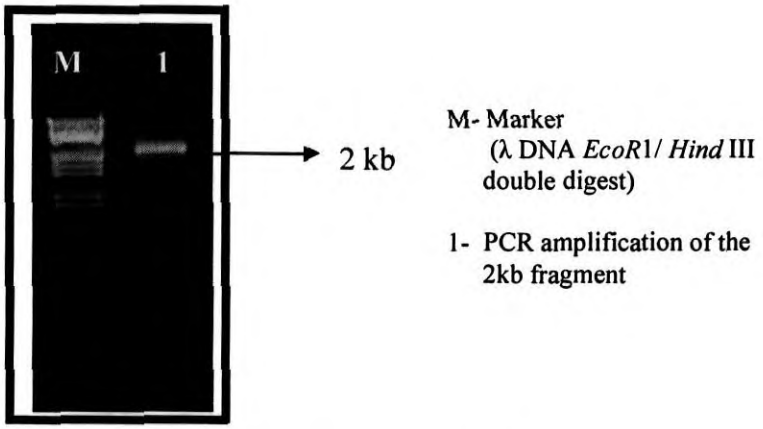


Fig. 45. Amplification of the 2 kb fragment containing the β-1,3-glucanase gene and the promoter

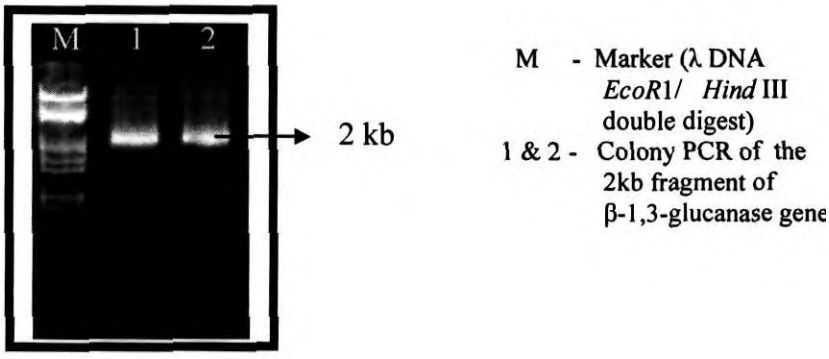


Fig. 46. Colony PCR of the 2kb fragment

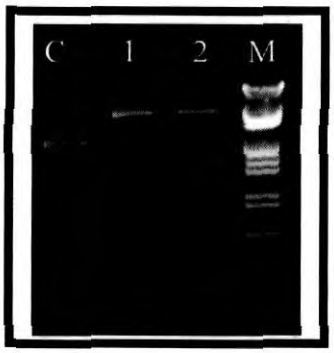


Fig. 47. Recombinant plasmids isolated from the positive colony

C - Non-inserted plasmid
1 & 2 - Recombinant plasmids
M - M (λ DNA *EcoR*1/ *Hind* III double digest)

4.3.1.1. Sequence details of the coding DNA sequence of β-1,3-glucanase gene with the 847 bp promoter

The total amplicon size of the isolated sequence contains 2103 nucleotides (NCBI Acc. No. JQ650524). The promoter region is composed of 847 bps which includes the 38 bp 5'UTR region. The coding DNA is composed of 1256 bps comprising 131 intronic nucleotides. There are 18 'CT' repeats within the intron sequence. Since the promoter sequence is showing significant similarity with that of the 913 bp promoter, an alignment of these two sequences with the report (AY325498) had been done to check for the presence of the variations if any (fig. 49).

Fig. 48. The gene sequence of of β-1,3-glucanase with the 847 bp promoter

5′ GCTCCCTAGGCTATTATTAAATCATAATTTGTTGGATTAGATTTATATTGCATTCTTT
TACATATTTATATATTTATATTTTGATTCGTATTTTAATTAGAAACTTTTATTTTAAATT
TTGTTATCTTATGATAAAATTTTAATAATTATATGATAGAGAATATACATTATCCACTGT
ATTAAATAACAATTTTTTTTAATTTTGGGAAAATAAATCACTGGAAAATCTCAAATTTTT
TTTGAAATATCCCTTAATTAATAAAATAACATCCATGTTTAATAGATCCTAAATTTGAAT
TTAAACGATAAAAATAATTATGTTATAAAGTGACAAATCTTATCCTTTTATCTTTTAAGG
AGATATGGGGGGGCAGAAAAAAGACTTAAAAGTCTTCAACAGATAGAGACATTCATTTAT
CCAACGAAAAATTGAAAGATGCCCAAGACATTTCAAACTAAATTAGCATTAATTATCAGG
ATTATTAATTGGTAAAGAGTGCATTATTTTCAATAAAAGAAGTAAAATAATTTCACCGAT
TAGTTTTGAGAGATTGTTGTGCATTTTCAAAGCACATGTTTTCTTCCCATCAGACATTGG
ATCCACTTTTTCTTAATTTAGAAAAATAACTATAACTATATATCTGTAAAGAGCACTATA
TTTTAGTGCTCTAAATGCATGGTTGAATTTTTCTTAATTTTAAACCTGATCAGTTGAAAT
TAAACAAACAAATAAACTCCAGCAACAGTATTCTCAAGAGTCCGCCTATAAAGCTTTGAC
ATGGTAGTAACCTAACACATATGCGTGGTCGAAATTATAAGCAACTTTCTTCTATTTCCC
CCCTTCTTA
ATGGCTATCTCCTCTTCAACTTCAGGAACTAGTAGTTCCCTGCCCTCAAGAACTACTGT
CATGCTTCTTCTGATTTTCTTTACAGCAAGCCTTGGTATAACAGGTCTCTCTCTCTCTCT
CTCTCTCTCTCTCTCTCTCTCTCCATCTATCTACTCTCATGTTAAAGTTGACGATGCTCT
TTTTTTCTCTCCTTGTTCTAAAAGGTTTCAACTAATACCTGTATTTGGAATTTAGATGCC
CAGGTAGGCGTTTGCTATGGAATGCAAGGCAACAACCTTCCATCTGTTTCAGAGGTCATA
GCTCTCTATAAACAATCTAACATCAAGAGAATGAGAATTTATGATCCAAATCGAGCAGTA
TTGGAAGCCCTTAGAGGCTCAAACATTGAACTCATACTAGGTGTTCCAAACTCAGATCTC
CAAAGCCTTACCAATCCTTCCAATGCAAACTCATGGGTACAAAAAAATGTTCGTGGCTTC
TGGTCAAGTGTCAGGTTCAGATATATAGCAGTTGGCAACGAAATTAGTCCTGTAAATGGA
GGCACAGCTTGGTTGGCCCAATTTGTTTTGCCTGCCATGAGAAATATACATGATGCTATA
AGATCAGCTGGTCTTCAAGATAAAATCAAGGTCTCCACTGCAATTGACTTGACCCTGGTA
GGAAATTCCTACCCTCCTTCTGCAGGTGCTTTCAGGGATGATGTTAGATCATACTTGGAC
CCAATTATTGGATTTCTATCCTCTAGCAGGTCACCTTTACTTGCCAATATTTATCCTTAC
TTTACTTATGCTTATAATCCAAGGGATATTTCCCTTCCCTATGCTTTGTTCACTTCACCA
TCAGTTGTTGTGTGGGATGGTCAGCGAGGTTATAAGAACCTTTTTGATGCAACGTTGGAT
GCATTGTACTCTGCTCTTGAGAGGGCTAGTGGTGGTTCTCTGGAGGTGGTTGTTTCGGAA
AGTGGCTGGCCGTCTGCCGGAGCATTTGCTGCCACATTTGACAATGGGCGTACTTATCTC
TCAAATTTGATCCAACATGTTAAAGGAGGTACTCCTAAGAGGCCTAACAGAGCTATAGAG
ACTTACTTATTTGCCATGTTTGATGAAAATAAGAAGCAACCAGAGGTTGAGAAACACTTT
GGACTTTTCTTTCCTGATAAACGGCCAAAATATAATCTCAATTTTGGTGCAGAAAAGAAC
TGGGATATTTCTACTGAACACAATGCAACAATACTTTTC CTTAAGAGTGATATG**TGA**
3′

Total region amplified: 2103 bps
Promoter – 847 bps
5' UTR- 38 bps
Gene -1256 bps (from ATG - stop codon)
Introns - 131 bps
Primer sequences are underlined
Start codon (ATG)
Stop codon

Fig. 49. Comparison of the 913 bp and 847 bp promoter sequences

**The green colour in the promoter region indicates the variation shown by the Gln 5 promoter sequence with respect to the Gln 6 promoter. The start codon is highlighted in magenta colour*

```
847         ----------------------------------------------------------
913         TTTTTAGAATTAAAATTTTTAAATTTTTTGCATCTATAATAAAATTCCCATGATACCCTC
Rep         ----------------------------------------------------------

847         ------------------GCTCCCTAGGCTATTATTAAATCATAATTTGTTGGATTAGA
913         AATTTATAATAATTTATATGCTCCCTAGGCTATTATTAAATCATAATTTGTTGGATTAGA
Rep         ----------------------------------------------------------

847         TTTATATTGCATTCTTTTACATATTTATATATTTATATTTTGATTCGTATTTTAATTA#G
913         TTTATATTGCATTCTTTTACGTATTTATATATTTATATTTTGATTTGTATTTTAATTATA
Rep         ----------------------------------------------------------

847         AAACTTTTATTTTAAATTTTGTTATCTTATGATAAAATTTTAATAATTATATGATAGAGA
913         AAACTTTTATTTTAAATTTTGTTATCTTATGATAAAATTTTAATAATTATATGATAGAGA
Rep         ----------------------------------------------------------

847         ATATACATTATCCACTGTATTAAATAACAATTTTTTTTT-AATTTTGGGAAAATAAATGAC
913         ATATACATTATCCACTGTATTAAATAACAATTTTTTTTTTAAATTTTGGAAAATAAATAAC
Rep         ----------------------------------------------------------

847         TGGAAAATCTCAAATTTTTTTT#GAAATATCCCT#AATTAATAAAATAACATCCATGTTT
913         TGGAAAATCTCAAATTTTTTTTTGAAATATCCCTGAATTAATAAAATAACATCCATGTTT
Rep         ----------------------------------------------------------

847         AATAGATCCTAAATTTGAATTTAAACGATAAAAATAATTATGTTATAAAGTGACAAATCT
913         AATAGATCCTAAATTTCAATTTAAACGATAAAAATAATTATGTTATAAAGTGACAAATCT
Rep         ----------------------------------------------------------

847         TATCCTTTTATCTTTTAAGGAGATA#GGGGGGCAGAAAAAAGACTTAAAAGTCTTCAAC
913         TATCCTTTTATCTTTTAAGGAGATAAGGAGGTGCAGAAAAAAGACTTAAACGTCTTCAAC
Rep         ----------------------------------------------------------

847         AGATAGAGACATTCATTTATCCAACGAAAAATTGAAAGATGCCCAAGACATTTCAAACTA
913         AGATAGAGACATTCATTTATCCAACGAAAAATTGAAAGATGCCCAAGACATTTCAAACTA
Rep         ----------------------------------------------------------

847         AATTAGCATTAATTATCAGGATTATTAATTGGTAAAGAGTGCATTATTTTCAATAAAAGA
913         AATTAGCATTAATTATCAGGATTATTAATTGGTAAAGAGTGCATTATTTTCAATAAAAGA
Rep         ----------------------------------------------------------

847         AGTAAAATAATTTCACCGATTAGTTT#GAGAGATTGTTGTGCATTTTC#AAGCACATGTT
913         AGTAAAATAATTTCACCGATTAGTTT-GAGAGATTGTTGTGCATTTTCGAAGCACATGTT
Rep         ----------------------------------------------------------

847         TTCTTCCCATCAGACATTGGATCCACTTTTTCTTAATTTAGAAAAATAACTATAACTATA
913         TTCTTCCGATCAGACATTGGATCCACTGTTTCTTAATT----------ATAATTATA
Rep         ----------------------------------------------CGCCCGGGCAGGTA
                                                           **

847         TATCTGTAAAGAGCACTATATTTTAGTGCTCTAAATGCATGGTTGAATTTTTGTTAATTT
913         TATCTGTAAAGAGCACTATATTTTAGTGCTCTAAATGCATGGTAGAATTTTTTTTAATTT
Rep         TTTCTTCCATTAAATAAATATATTAGT--TTTAATTTGGCGATAG-------TTTAAATT
            * ***   *  *     **** *****  * *** *    * * *        **** **
```

```
847         TAAACCTGATCAGTTGAAATTAAACAAACAAATAAACTCCAGCAACAGTATTCTCAAGAG
913         TAAACCT---CAGTTGAAATTAAACAAACAAATAAACTCCAGCAACAGTATGCTCAAGAG
Rep         CAAACCC---CAATTGACATTAAATAAATAAATAAGCTCCAGAAATAGTATGCTAAGGAG
             *****    ** **** ****** *** ****** ****** ** ***** ** * ***

847         TCCGCCTATAAAGCTTTGACATGGTAGTAACCTAACACATATGCGTGGTCGAAATTATAA
913         TCCGCCTATAAAGCTTTGACATGGTAGTAACCTAACACATATGCGTGGTCGAAATTATAA
Rep         TCCCTATATAAAGCATAGACTTGGTAGTAATGTAATGCATATGCATGCTCCAAATTA--A
            ***   ******** * *** *********  ***  ******* ** ** ******  *

847         GCAACTTTCTTCT-AATTTCCCGCCTTCTTA[redacted]GCTATCTCCTCTTCAACTTCAGGAAC
913         GCAACTTTCTTCT-AATTTCCCGCCTTCTTA[redacted]GCTATCTCCTCTTCAACTTCAGGAAC
Rep         GCTGCTCTCTTCTTAGGTTCCATCCTTCTTA[redacted]GCTATCTCCTCTTCAACTTCAGGAAC
            **  ** ****** *  ****  ************************************

847         TAGTAGTTCCCTGCCCTCAAGAACTACTGTCATGCTTCTTCTGATTTTCTTTACAGCAAG
913         TAGTAGTTCCCTGCCCTCAAGAACTACTGTCATGCTTCTTCTGATTTTCTTTACAGCAAG
Rep         TAGTAGTTCCCTGCCCTCAAGAACTACTGTCATGCTTCTTCTGATTTTCTTTACAGCAAG
            ************************************************************

847         CCTTGGTATAACAGGTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCCATCTAT
913         CCTTGGTATAACAGGTCTCTCTCTCTCTCTCTC--------------TCTCCATCTAT
Rep         CCTTGGTATAACAGGTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCCATCTAT
            ************************************              ***********


847         CTACTCTCATGTTAAAGTTGACGATGCTCTTTTTTTCTCTCCTTGTTCTAAAAGGTTTCA
913         CTACTCTCATGTTAAAGTTGACGATGCTCTTTTTTTCCCTCCTTTTTCTAAAAGGTTTCA
Rep         CTACTCTCATGTTAAAGTTGACGATGCTCTTTTTTTCTCTCCTTGTTCTAAAAGGTTTCA
            ************************************ ****** ***************

847         ACTAATACCTGTATTT-GGAATTTAGATGCCCAGGTAGGCGTTTGCTATGGAATGCAAGG
913         ACTAATACCTGTATTT-GGAATTTAGATGCCCAGGTAGGTGTTTGCTATGGAATGCAAGG
Rep         ACTAATACCTGTATTTAGGAATTCAGATGCCCAGGTAGGTGTTTGCTATGGAATGCAAGG
            **************** ****** *************** ********************

847         CAACAACCTTCCATCTGTTTCAGAGGTCATAGCTCTCTATAAACAATCTAACATCAAGAG
913         CAACAACCTTCCATCTGTTTCAGAGGTCATAGCTCTCTATAAACAATCTAACATCAAGAG
Rep         CAACAACCTTCCACCTGTTTCAGAGGTCATAGCTCTCTATAAACAATCTAACATCAAGAG
            ************* ************************************************

847         AATGAGAATTTATGATCCAAATCGAGCAGTATTGGAAGCCCTTAGAGGCTCAAACATTGA
913         AATGAGAATTTATGATCCAAATCAAGCAGTATTGGAAGCCCTTAGAGGCTCAAACATTGA
Rep         AATGAGAATTTATGATCCAAATCGAGCAGTATTGGAAGCCCTTAGAGGCTCAAACATTGA
            ********************** ***********************************

847         ACTCATACTAGGTGTTCCAAACTCAGATCTCCAAAGCCTTACCAATCCTTCCAATGCAAA
913         ACTCATACTAGGTGTTCCAAACTCAGATCTCCAAAGCCTTACCAATCCTTCCAATGCAAA
Rep         ACTCATACTAGGTGTTCCAAACTCAGATCTCCAAAGCCTTACCAATCCTTCCAATGCAAA
            ************************************************************

847         CTCATGGGTACAAAAAAATGTTCGTGGCTTCTGGTCAAGTGTCAGGTTCAGATATATAGC
913         CTCATGGGTACAAAAAAATGTTCGTGGGTTCTGGTCAAGTGTCAGGATCAGATATATAGC
Rep         CTCATGGGTACAAAAAAATGTTCGTGGCTTCTGGTCAAGTGTCCTGTTCAGATATATAGC
            *************************** ***************  * *************

847         AGTTGGCAACGAAATTAGTCC-TGTAAATGGAGGCACAGCTTGGTTGGCCCAATTTGTTT
913         AGT-GGCAACGAAATTAGTCCCTGTAAATGGAGGCACAGCTTGGTTGGCCCAATTTGTTT
Rep         AGTTGGCAACGAAATTAGTCC-TGTCAATGGAGGCACAGCTTGGTTGGCTCAATTTGTTT
            *** ***************** *** ************************ **********

847         TGCCTGCCATGAGAAATATACATGATGCTATAAGATCAGCTGGTCTTCAAGATAAAATCA
913         TGCCTGCCATGAGAAATATACATGATGCTATAAGATCAGCTGGTCTTCAAGATCAAATCA
Rep         TGCCTGCCATGAGAAATATACATGATGCTATAAGATCAGCTGGTCTTCAAGATCAAATCA
            ************************************************** ******


847         AGGTCTCCACTGCAATTGACTTGACCCTGGTAGGAAATTCCTACCCTCCTTCTGCAGGTG
913         AGGTCTCCACTGCAATTGACTTGACCCTGGTAGGAAATTCCTACCCTCCTTCTGCAGGTG
Rep         AGGTCTCCACTGCAATTGACTTGACCCTGGTAGGAAATTCCTACCCTCCTTCTGCAGGTG
            ************************************************************


847         CTTTCAGGGATGATGTTAGATCATACTTGGACCCAATTATTGGATTTCTATCCTCTAGCA
913         CTTTCAGGGATGATGTTAGATCATACTTGGACCCAATTATTGGATTTCTATCCTCTATCA
Rep         CTTTCAGGGATGATGTTAGATCATACTTGGACCCAATTATTGGATTTCTATCCTCTATCA
            ************************************************************ **
```

```
847             GGTCACCTTTACTTGCCAATATTTATCCTTACTTTACTTATGCTTATAATCCAAGGGATA
913             GGTCACCTTTACTTGCCAATATTTATCCTTACTTTACTTATGCTGATAATCCAAGGGATA
Rep             GGTCACCTTTACTTGCCAATATTTATCCTTACTTTACTTATGCTGGTAATCCAAGGGATA
                ****************************************  **************

847             TTTCCCTTCCCTATGCTTTGTTCACTTCACCATCAGTTGTTGTGTGGGATGGTCAGCGAG
913             TTTCCCTTCCCTATGCTTTGTTCACTTCACCATCAGTTGTTGTGTGGGATGGTCAGCGAG
Rep             TTTCCCTTCCCTATGCTTTGTTCACTTCACCATCAGTTGTTGTGTGGGATGGTCAGCGAG
                ************************************************************

847             GTTATAAGAACCTTTTTGATGCAACGTTGGATGCATTGTACTCTGCTCTTGAGAGGGCTA
913             GTTATAAGAACCTTTTTGATGCAACGTTGGATGCATTGTACTCTGCTCTTGAGAGGGCTA
Rep             GTTATAAGAACCTTTTTGATGCAACGTTGGATGCATTGTACTCTGCTCTTGAGAGGGCTA
                ************************************************************

847             GTGGTGGTTCTCTGGAGGTGGTTGTTTCGGAAAGTGGCTGGCCGTCTGCCGGAGCATTTG
913             GTGGTGGTTCTCTGGAGGTGGTTGTTTCGGAAAGTGGCTGGCCGTCTGCCGGAGCATTTG
Rep             GTGGTGGTTCTCTGGAGGTGGTTGTTTCGGAAAGTGGCTGGCCGTCTGCCGGAGCATTTG
                ************************************************************

847             CTGCCACATTTGACAATGGGCGTACTTATCTCTCAAATTTGATCCAACATGTTAAAGGAG
913             CTGCCACATTTGACAATGGGCGTACTTATCTCTCAAATTTGATCCAGCATGTTAAAGGAG
Rep             CTGCCACATTTGACAATGGGCGTACTTATCTCTCAAATTTGATCCAGCATGTTAAAGGAG
                ********************************************* ************


847             GTACTCCTAAGAGGCCTAACAGAGCTATAGAGACTTACTTATTTGCCATGTTTGATGAAA
913             GTACTCCTAAGAGGCCTAACAGAGCTATAGAGACTTACTTATTTGCCATGTTTGATGAAA
Rep             GTACTCCTAAGAGGCCTAACAGAGCTATAGAGACTTACTTATTTGCCATGTTTGATGAAA
                ************************************************************

847             ATAAGAAGCAACCAGAGGTTGAGAAACACTTTGGACTTTTCTTTCCTGATAAACGGCCAA
913             ATAAGAAGCAACCAGAGGTTGAGAAACACTCTGGACTTTTCTTTCCTGATAAACGGCCAA
Rep             ATAAGAAGCAACCAGAGGTTGAGAAACACTTTGGACTTTTCTTTCCTGATAAACGGCCAA
                ****************************** ******************************

847             AATATAATCTCAATTTTGGTGCAGAAAAGAACTGGGATATTTCTACTGAACACAATGCAA
913             AATATAATCTCAATTTTGGTGCAGAAAAGAACTGGGATATTTCTACTGAACACGATGCAA
Rep             AATATAATCTCAATTTTGGTGCAGAAAAGAACTGGGATATTTCTACTGAACACAATGCAA
                ************************************************** ******

847             CAATACTTTTCCTTAAGAGTGATATG[redacted]
913             CAATACTTTTCCTTAAGAGTGATATG[redacted]
Rep             CAATACTTTTCCTTAAGAGTGATATG[redacted]
                ****************************
```

Differences found in the 847 bp promoter with that of the 913 bp promoter
[redacted]

```
Sequences (1:2) Aligned. Score:  92        1- with 847 bp promoter
Sequences (1:3) Aligned. Score:  96        2- with 913 bp promoter
Sequences (2:3) Aligned. Score:  91        3- Rep
```

The alignment score of the coding DNA sequence alone:

```
Sequences (1:2) Aligned. Score:  99        1- coding DNA with 847 bp promot
Sequences (1:3) Aligned. Score:  98        2- Report (Acc. No. AY325498)
Sequences (2:3) Aligned. Score:  98        3- coding DNA with 913 bp promot
```

The promoter sequence (847 bps) upon alignment showed more similarity with that of the 913 bp (alignment score - 96) promoter except for some short deletions present in the 913 bp promoter. But the downstream coding DNA sequence showed more similarity towards the reported

nucleotide sequence of β-1,3-glucanase from our laboratory by Thanseem *et al.*, 2003 (NCBI Accession No. AY325498) (alignment score - 99) than with the gene sequence having the 913 bp promoter.

The characteristic cis-acting element is shown in table. 8 (page no. 155). The cis-acting elements found in the promoter with 913 bp is also present in the 847 bp promoter sequence. It is observed that the frequency of occurrence of the Dof core element responsible for the signal responsive or the tissue specific gene expression is more in the 847 bp promoter (16 Nos.) whereas in the 913 bp promoter it is only 13 in the isolated region. Likewise the I box core for light regulated/ tissue specific expression is found to be less in 847 bp promoter (10 Nos.) whereas it is more in the 913 bp promoter (13 Nos.). The cell type specific GT1 consensus is occurring 19 times in the 847 bp promoter whereas it is found only 17 times in the 913 bp promoter. These findings suggest that there may be differences in the gene expression of these two forms of β-1,3-glucanase in the tissues and also upon induction.

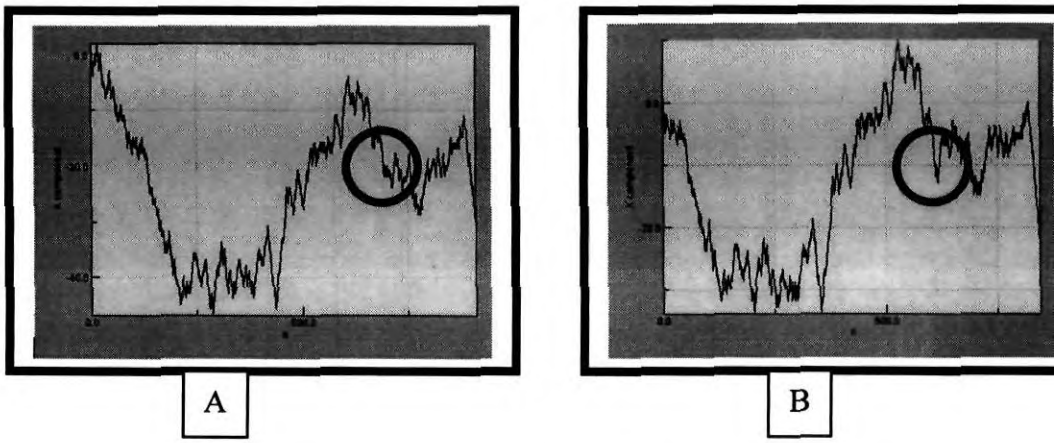
The nucleosome forming potential of the 847 bp promoter was found to be at regions: 81-136 bps, 584-608 bps, and 658-768 bps. It showed an increasing trend towards the coding DNA sequence of the gene (towards the ATG). CpG islands were found to be absent in the sequence. This feature is found interesting as the most similar isolated promoter sequence having the 913 bp is showing two CpG islands between the 449- 521th nucleotide and 609- 695th nucleotide.

Z-curve analysis

For a given DNA sequence there is a unique Z curve corresponding to it and it contributes further to visualize and analyze the DNA sequence by a geometrical approach. The symmetry, the periodicity, the local motif, and the distribution of bases of the DNA sequences are reflected by the rich folding structures of the Z curves. On the contrary, the DNA sequence can be uniquely determined by the given Z curve from their counterparts.

CpG methylation reduces backbone flexibility and dynamics. The DNA stiffness also negatively influences the nucleosome formation. The CpG in the promoter region can adopt a Z-DNA conformation under negative superhelical stress, and this is greatly facilitated by its methylation (Runkel and Nordheim, 1986). Fig A and B shows the difference of the Z- curve of the promoter sequences of both the 913 bp promoter (fig. 50. A) and that of the 847 bp promoter (fig. 50. B).

Fig. 50. Z - Curve plots for the sequences of 913 bp promoter (A) and 847 bp promoter (B)



The curves show strong –ve peaks in the 847 bp promoter than with the 913 bp promoter which is indicative of the difference in the DNA architecture of the portion. The region inside the violet circle is the area which is showing the difference in the peak. The predicted sequence of the amino acid from the coding DNA sequence having the 847 bp promoter using the ExPASy –ProtParam online tool is shown in the fig. 51.

Fig. 51. Predicted amino acid sequence from the coding DNA sequence of β-1,3-glucanase having the 847 bp promoter

```
        10         20         30         40         50         60         70
         |          |          |          |          |          |          |
MAISSSTSGTSSSLPSRTTVMLLLIFFTASLGITDAQVGVCYGMQGNNLPSVSEVIALYKQSNIKRMRIY
DPNRAVLEALRGSNIELILGVPNSDLQSLTNPSNANSWVQKNVRGFWSSVRFRYIAVGNEISPVNGGTAW
LAQFVLPAMRNIHDAIRSAGLQDKIKVSTAIDLTLVGNSYPPSAGAFRDDVRSYLDPIIGFLSSSRSPLL
ANIYPYFTYAYNPRDISLPYALFTSPSVVVWDGQRGYKNLFDATLDALYSALERASGGSLEVVVSESGWP
SAGAFAATFDNGRTYLSNLIQHVKGGTPKRPNRAIETYLFAMFDENKKQPEVEKHFGLFFPDKRPKYNLN
FGAEKNWDISTEHNATILFLKSDM
```

```
Number of amino acids: 374
Molecular weight: 41202.7 Da
Theoretical pI: 8.95
Total number of negatively charged residues (Asp + Glu): 31
Total number of positively charged residues (Arg + Lys): 35
```

**The red colour shows the basic amino acids arginine (R) and lysine (K) which are also apolar and hydrophobic.*

The predicted amino acid sequence shows the presence of 374 amino acids which contributes a molecular size of 41 kDa. The theoretical iso-electric point is found to be 8.95. There are 31 negatively charged amino acid residues (polar, hydrophilic) and 35 positively charged amino acid residues (apolar, hydrophobic).

4.3.2. Sequence details of the coding DNA sequence of β-1,3-glucanase gene with the 582 bp promoter

An amplicon with 1356 bps was obtained with the promoter specific forward (IPF2) and the gene specific reverse primer (1R). Here the gene could not be amplified using the reverse primer designed from the conserved region which is used to obtain the full length gene sequence with the stop codon in the other forms. Therefore, an internal primer (2R) was designed with which a gene portion of 968 bps was obtained apart from the promoter region. The sequence of 1356 bps obtained is shown in fig. 52.

The forward and the reverse primer used for the promoter-gene combination is shown as underlined and highlighted in blue colour. The amplified promoter region contained 388 bp (from the 582 bp originally amplified) promoter including the 37 bp 5'UTR region together with a 968 bp gene containing two exons and one intron. The intron region consists of 110 bp with 16 'CT' repeats. The start codon is highlighted in violet colour.

Fig. 52. Partial coding DNA sequence of β-1,3-glucanase following the 582 bp promoter

5′ ATTAGATATATTCAGTTTCATTCTTAAAGATATTATAAAAAAAATTAAAAAGATAAAA
ATTATTAAATTAAAAAAATCTCAATTTTTTTTTTAAATATCTCTTAATAAAATAATACCC
GTGATTATAAATCTTAGTTTTCAATTATTAGAGGATAAAAAATAATTATGTTGAAGAGTG
AATTTAATCTTATTTT**CTCAGTGTATTGTATTGCTGTGC**TTGGACAAAATCTTATCCTTT
ATCTTTTAAGGAGATTTCTAGGTGCAGAAATATGACTTTTAAGTCTTTAACAGATAGGGA
CATTCATCTGTCCAACGAAAATTGAAAGATGGCTAAGACATTTTCCAAACTAAATTAATT
GGCATTAATTGTCATGATTGGTAAAGATGCAAGGAGAGTAATTGATAATTTTTAATTCAG
TGATTGTTTAAAATTCCAATCTCAATTAAAGCCAAATAAATAAATTAATAAACTCCAGCA
ACAGTATGGAAAAGGGTCCCTATATAAAGCATTGACATGGTAGTAACATAACACATATGC
ATGCCCCAAATTAAGCTACTTTCTTCTAATTTCCCCCCTTCTTA**ATG**GCTATCTTCTCTT
CAAATTCAGCAACTAGTAGTTCCTTGCCCTCAAGAACTACTTCCATACTTCTGGTTCTCT
TTATAGCAAGCCTTAGTATAACAGGTCTCTCTCTCTCTCTCTCTCTCTCTCTACTCTC
ATGTTAAAGTTGATAATGCATTTTTTTTTTCTTGTTCTAAAAGGTTTCAACTAATATCTG
TATTTGGAATTCAGATGCCCAGGTAGGTGTTTGCTATGGAATGCTAGGCAACAACCTTCC
ACCTGCTTCACAGGTTATCTCTCTCTATAAACAAGCGAACATTAAGAGAATGAGAATTTA
TGATCCAAATCAAGCAGCATTGCAAGCCCTTAGAGGCTCAAACATTCAACTCATGCTTGG
TGTTCCAAACTCAGATCTCCAAAGCCTTACCAATCCTTCTAATGCAAACTCATGGATACA
AAGAAATGTTCGAGCCTTCTGGCCAAGTGTCAGATTCAGATATATAGCAGTTGGAAACGA
AATTAGTCCAGTGAATGGAGGCACAGCTTCCTTGGCTAAATTTGTTTTGCCTGCCATGAG
AAATATATATAATGCAATAAGATCAGCTGGTCTTCAGGATCAAATAAAGGTCTCTACCGC
AATTGACATGACCCTGATAGGAAATTCATACCCTCCTTCTGCAGGTGCTTTCAGGGGTGA
TGTTAGATCATACTTGGACCCAATTATAGGATTCCTATCCTCTATTAGGTCACCTTTACT
TGCCAATATTTATCCTTACTTTAGTTATGCTGGTAATCCAAGGGATATTTCCCTTCCCTA
TGCTTTGTTCACTTCACCATCAATAGTTGTATGGGATGGTCAGCGAGGTTATAAGAACCT
TTTCGATGCAATGTTGGATGCATTGTACTCTGCTCTTGAGAGGGCTGGTGGTGGTTCTCT
GGAGGTAGTTGTGTCGGAAAGTGGCTGGCC**GTCCGCCGGAGCTTTTGCAGCC** 3′

Total region amplified- 1356 bps
Promoter 388 bps
5' UTR- 37 bps
Gene - 968 bps (from ATG- stop codon)
Start codon
Introns- 110 bps
CT- 16 no's
Primer sequences underlined.

4.3.3. Sequence details of the CDS sequence of β-1,3-glucanase following the 553 bp promoter

The sequence with the 553 bp promoter is registered in the database with the the NCBI Acc. No. GU123623. An amplicon with 1521 bps was obtained with the promoter specific forward and the gene specific reverse primer which contains the 'TGA' stop codon also (fig. 53). The region underlined and highlighted in blue colour is the forward and the reverse primer used for the amplification of the promoter and the coding gene portion. The amplified region of the promoter-gene combination contained 253 bp

promoter including the 39 bp 5'UTR region together with a 1229 bp gene comprising two exons and one intron. The intron region consists of 104 bp with 12 'CT' repeats. The start codon and the stop codon is highlighted in violet and red colour respectively.

5' ATTAAACTTATAAATATCCAAACTTATAAATTTATAATAATTGCTATTAATAAGTATG
ATGGTTAATTATATGGTTAAAATGATCCACCAACACAAGAGTCAGGATTATTCTGGTCAT
TGGTCCATAAATTAACTATGACTTTGAACAAATTAGTAAATATTTTTAGAATTAAAATTT
TCAAATTTTTTGCATCTATAATATAATTCCCATGATACCCTCAATTTATAATAATATATA
TGCTGCCTAGGTTATTATTAAAT**CATAATTTGTTGGATTAG**ATTTATATTGCATTCTTTT
ACATTGGATCCACTGTTTCTTAATTTAGAAAAATAACTATAATTATATAAGTGTAAAGAG
CACTATATTTATGGTGCTCTAAATGCATGGTAGAATTTTTCTTAATTTTAAACCTCAGTT
GAAATTAAATAAACAAATAAACTCCAGCAACAGTATGCTCAAGAGTCCGCCTATAAAGCA
TTGACATGGTAGTAACCAAACGCATATGCGTGGTCG
AAATTATAAGCAACTTTCTTCTAATTTCCCCCCTTCTTA
ATGGCTATCTGCTCTTCAACTTCAGGAACTAGTAGTTCCTTGCCCTCAAGAACTACTGT
CATGCTTCTTCTGTTTTTCTTTACAGCAAGCGTTGGTATAACAG
GTCTCTCTCTCTCTCTCTCTCTCTCATGTTAAAGTTGACGATGCTCTTTTTTTCTCTC
CTTGTTCTAAAAGGTTTCAACTAATACCTGTATTTGGAATTTAGATGCCCAGGTAGGTGT
TTGCTATGGAATGCAAGGCAACAACCTTCCACCTGTTTCAGAGGTCATAGCTCTCTATAA
AAAATCTAACATCACGAGAATGAGAATTTATGATCCAAATCAAGCAGTATTGGAAGCCCT
TAGAGGCTCAAACATTGAACTCATACTAGGTGTTCCAAACTCAGATCTCCAAAGCCTTAC
CAATCCTTCCAATGCAAAATCATGGGTACAAAAAAATGTTCGTGGCTTCTGGTCAAGTGT
CAGGTTCAGGTATATAGCAGTTGGCAACGAAATTAGTCCTGTCAATAGAGGCACAGCTTG
GTTGGCTCAATTTGTTTTGCCTGCCATGAGAAATATACATGATGCTATAAGATCAGCTGG
TCTTCAAGATCAAATCAAGGTCTCCACTGCAATTGACTTGACCCTGGTAGGAAATTCCTA
CCCTCCTTCTGCAGGTGCTTTCAGGGATGATGTTAGATCATACTTGAACCCAATTATTAG
ATTTCTATCTTCTATCAGGTCACCTTTACTTGCCAATATTTATCCTTACTTTACTTATGC
TGGTAATCCAAGGGATATTTCCCTTCCCTATGCTTTGTTCACTTCACCATCAGTTGTTGT
GTGGGATGGTCAGCGAGGTTATAAGAACCTTTTTGATGCAACGTTGGATGCATTGTACTC
TGCTCTTGAGAGGGCTAGTGGTGGTTCTCTGGAGGTGGTTGTTTCGGAAAGTGGCTGGCC
GTCTGCCGGAGCATTTGCTGCCACATTTGACAATGGGCGTACTTATCTCTCAAATTTGAT
CCAGCATGTTAAAAGAGGTACTCCTAAGAGGCCTAAAAGAGCTATAGAGACTTACTTATT
TGCCATGTTTGATGAAAATAAGAAGCAACCAGAGGTTGAGAAACACTTTGGACTTTTCTT
TCCTAATAAATGGCAAAAATATAATCTCAATTTTAGTGCAGAAAAGAACTGGGATATTTC
TACTGAACACAATGCAACAATACTTTTC CTTAAGAGTGATATG**TGA** 3'

Gene - 1229 bps
Introns- 104 bps
UTR - 39 bps
Promoter – 253 (amplified within the 553 bp promoter)
Total region amplified – 1521 bps
Primer sequences has been underlined.
Start codon
Stop codon

Fig. 53. The coding DNA sequence of β-1,3-glucanase following the 553 bp promoter

Upon analysis with the ExPASy- ProtParam tool, a total of 374 amino acids was obtained upon translating the sequence belonging to the 553 bp promoter. It showed the highest basicity upon evaluation with an isoelectric pH of 9.48. The total number of negatively charged amino acid residues is 29 and positively charged residues are 37. The predicted molecular weight is 41.5 kDa. The details of the deduced amino acid sequence is shown in fig. 54.

Fig. 54. The deduced amino acid sequence of the coding DNA belonging to the 553 bp promoter

```
        10         20         30         40         50         60         70
         |          |          |          |          |          |          |
MAICSSTSGTSSSLPSRTTVMLLLFFFTASVGITDAQVGVCYGMQGNNLPPVSEVIALYKKSNITRMRIY
DPNQAVLEALRGSNIELILGVPNSDLQSLTNPSNAKSWVQKNVRGFWSSVRFRYIAVGNEISPVNRGTAW
LAQFVLPAMRNIHDAIRSAGLQDQIKVSTAIDLTLVGNSYPPSAGAFRDDVRSYLNPIIRFLSSIRSPLL
ANIYPYFTYAGNPRDISLPYALFTSPSVVVWDGQRGYKNLFDATLDALYSALERASGGSLEVVVSESGWP
SAGAFAATFDNGRTYLSNLIQHVKRGTPKRPKRAIETYLFAMFDENKKQPEVEKHFGLFFPNKWQKYNLN
FSAEKNWDISTEHNATILFLKSDM

Number of amino acids: 374
Molecular weight: 41528.3
Theoretical pI: 9.34
Total number of negatively charged residues (Asp + Glu): 29
Total number of positively charged residues (Arg + Lys): 37
```

* *Red olour indicates the positively charged basic amino acids arginine (R) and lysine (K).*

The BLASTp analysis of this sequence showed 85- 99% similarity with the other reported forms of amino acid sequences from *Hevea brasiliensis*. It is interesting to note that with 3EM5A sequence (chain A, crystal structure of a native endo-β-1,3-glucanase) reported from *Hevea*, the sequence is showing 100 % similarity. The other similarities found are as: 77 % with a basic vacuolar isoform of β-1,3-glucanase *Vitis vinifera* (Acc. No. XP002277169). 65% with an acidic glucanase from *Medicago sativa* (ABY47902). 65% with another acidic glucanase from *Medicago sativa* (AAV66071). 68% similarity with a β-1,3-glucanase regulator from *N. plumbaginifolia* (AAA34078). 62% similarity with β-1,3-glucanase from *Prunus persica* (AAL30426). 62% similarity with β-1,3-glucanase from *Vitis vinifera* (CAB91554). 63% similarity with another β-1,3-glucanase from *Vitis riparia* (ACD45060). 59% similarity with a basic β-1,3-glucanase from *N. tabaccum* (ACF93731.1).

4.3.4. Sequence characterization of glucanase with the 198 bp promoter

The sequence with the 198 bp promoter is submitted in NCBI with the Acc. No. GU191335. Here an amplicon with 1402 bps was obtained with the promoter specific forward and the gene specific reverse primer containing the stop codon (fig. 55). The region underlined and highlighted in blue colour is the forward and the reverse primer used for the amplification of the promoter–coding gene combination. An amplicon with 1806 bps was obtained with the promoter specific forward and the gene specific reverse primer. The amplified gene promoter fusion region of the Gln 3 sequence contained 173 bp promoter including the 38 bp 5'UTR region together with a 1229 bp gene containg two exons and one intron. The intron region consists of 102 bp with 10 CT repeats. The start codon and the stop codon is highlighted in violet and red colour respectively.

The deduced amino acid sequence of the coding DNA belonging to 198 bp promoter showed 374 amino acids with a stop codon. Fig.56. shows the predicted amino acid sequence of the glucanase gene with the 198 bp promoter. The theoretical molecular weight of the predicted amino acid is approximately 41 kDa with an isoelectric point of 8.75 indicating that it is also a basic peptide. There are 32 negatively charged residues and 35 positively charged residues.

Fig. 55. The CDS following the 198 bp promoter

5′ ATTTCTTCCATTAAATAAATATATTAGTTTTAATTTAGCGATAGTTTAAATTCAAACC
CCAATTGACATTAAATAAATAAATAAGCTCCAGAAATAGTATGCTAAGGAGTCCCTATAT
AAAGCATAGACTTGGGAGTAATGTAATGCATATGCATGCTCCAAATTAAGCTGCTCTCTT
CTTAGGTTCCATCCTTCTTA**ATG**GCCATGCTTTCTTCAACTTCGAGAACTACTGGTTCC
TTACTCTCAAGAACTCCTGTCATGCTTCTTCTGATTCTCTATATAGCAAGCCTTGGTATA
ACAGGTGTCTCTCTCTCTCTATCTATCTACTCTCATGTTAAAGTTGACCATGCTTTTTTT
CTTCCTCGTTCTGAAGGGTTTCAACTAATACCTGTATTTGGAATTCAGATGCCCAGGTAG
GTGTTTGCTATGGAAAGCTAGGCAACAACCTTCCACCTGCTTCAGAGGTCATAGCTCTCT
ATAAACAATCTAACATCAAGAGAATGAGAATTTATGATCCAAATCAAGAAGTCTTGCAAG
CCCTTAGAGGCTCAAACATTGAACTCATACTAGGTGTTCCAAACTCAGATCTCCAAAGCC
TTACCAATCCTTCCAATGCAAACTCATGGGTACAAAAAAATGTTCGTGACTTCTGGTCAA
GTGTCAGGCTCAGGTATATAGCAGTCGGCAACGAAATTAGTCCTGTAAATGGAGGCACAG
CTTGGTTGGCTCAATTTGTTTTGCCTGCCATGAGAAATATACATGATGCTATAAGATCAG
CTGGTCTTCAAGATCAAATCAAGGTCTCTACTGCGATTGACTTGACCCTGATGGGAAATA
CCTACCCTCCTTCTGCAGGTGCTTTCAGGGATGATGTTAGATCATATTTGGACCCAATTA
TTGGATTCCTATCCTCTATCAGGTCACCTTTACTTGCCAATATTTATCCTTACTTTACTT
ATGCTGGTAATCCAAGGGATATTTCCCTTCCCTATGCTTTGTTCACTTCACCATCAGTTG
TTGTGTGGGATGGTCAGCGAGGTTATAAGAACCTTTTTGATGCAACGTTGGATGCATTGT
ACTCTGCTCTTGAGAGGGCTAGTGGTGGTTCTCTGGAGGTGGTTGTTTCGGAAAGTGGCT
GGCCGTCTGCCGGAGCATTTGCTGCCACATTTGACAATGGGCGTACTTATCTCTCAAATT
TGATCCAGCATGTCAAAGGAGGTACTCCTAAGAGGCCTGACAGAGCTATAGAGACTTACT
TATTTGCCATGTTTGATGAAAATCAGAAGCAACCAGAGGTTGAGAAACACTTTGGACTTT
TCTTTCCTGATAAACGGCCAAAATATAATCTCAATTTTAGTGCAAAAAAGAACTGGGATA
TTTCTACTGAACACAATGCAACAGTACTTTTCCTTAAGAGTGATATG**TGA** 3′

Total region amplified: 1402 bps
Promoter- 198 bps
5'UTR- 38 bps
Gene- 1229 bps (from ATG- stop codon)
Introns- 102 bps
CT - 10 no's
Start codon
Stop codon

Fig. 56. Amino acid sequence of glucanase gene with the 198 bp promoter.

```
        10         20         30         40         50         60         70
         |          |          |          |          |          |          |
MAMLSSTSRTTGSLLSRTPVMLLLILYIASLGITDAQVGVCYGKLGNNLPPASEVIALYKQSNIKRMRIY
DPNQEVLQALRGSNIELILGVPNSDLQSLTNPSNANSWVQKNVRDFWSSVRLRYIAVGNEISPVNGGTAW
LAQFVLPAMRNIHDAIRSAGLQDQIKVSTAIDLTLMGNTYPPSAGAFRDDVRSYLDPIIGFLSSIRSPLL
ANIYPYFTYAGNPRDISLPYALFTSPSVVVWDGQRGYKNLFDATLDALYSALERASGGSLEVVVSESGWP
SAGAFAATFDNGRTYLSNLIQHVKGGTPKRPDRAIETYLFAMFDENQKQPEVEKHFGLFFPDKRPKYNLN
FSAKKNWDISTEHNATVLFLKSDM
```

Number of amino acids: 374
Molecular weight: 41335.1
Theoretical pI: 8.75
Total number of negatively charged residues (Asp + Glu): 32
Total number of positively charged residues (Arg + Lys): 35

* *Red colour indicates the positively charged basic amino acids arginine (R) and lysine(K)*

The BLASTp analysis shows 86- 93% amino acid sequence similarity with other reported forms of β-1,3-glucanase amino acid sequences from *Hevea brasiliensis*. With 3EM5A sequence (chain A, crystal structure of a native endo-β-1,3-glucanase) reported from *Hevea*, the sequence is showing only 93 % similarity. Similarities found with the other sequences are;
77 % with a basic vacuolar isoform of β-1,3-glucanase from *Vitis vinifera* (Acc. No. XP002277169). 65% with an acidic glucanase from *Medicago sativa* (ABY47902).64% with another acidic glucanase from *Medicago sativa* (AB41551). 63% similarity with an acidic β-1,3-glucanase from *Glycine max (*AE154334). 69% similarity with a β-1,3-glucanase regulator from *N. plumbaginifolia* (AAA34078). 62% similarity with β-1,3-glucanase from *Vitis vinifera* (CAB91554). 62% similarity with a basic β-1,3-glucanase from *N. tabaccum* (ACF93731). 62% similarity with another basic β-1,3-glucanase from *N. tabaccum* (AAA63541). 63% similarity with β-1,3-glucanase from *Pisum sativum* (AAB24398).

In this study so far five new isoforms of β-1,3-glucanase gene have been characterized from a single *Hevea* clone RRII 105 in addition to the earlier reported one which was designated as Gln 1. Accordigly the newly obtained forms of β-1,3-glucanases have been designated as follows. The Nos. have been given according to the order in which the cloned sequences were obtained. Here onwards, the gene and promoter sequences will be named accordingly.

Reported sequence from RRII - Gln 1 (NCBI Acc. No. AY325498)
Coding DNA with the 553 bp promoter - Gln 2 (Acc. No. GU123623)
Coding DNA with the 198 bp promoter - Gln 3 (Acc. No. GU191335)
Coding DNA with the 582 bp promoter - Gln 4 (Acc. No. JN251022)
Coding DNA with the 847 bp promoter - Gln 5 (Acc. No. JQ650524)
Coding DNA with the 913 bp promoter - Gln 6 (Acc. No. JQ65052)

Putative *cis*-element/consensus	Function/ response	No. of elements found in the promoter containing					
		913 bp	847 bp	582 bp	553 bp	198 bp	Consensus sequence
Amy Box 1	Amylase box- responsible for gibberellic acid induced expression	1	1	1	-	-	TAACARA
CAAT Box		9	7	13	6	3	CAAT
DOF core	Signal responsive and /or tissue specific gene expression	13	16	12	5	1	AAAG
E Box	Plant pathogen interaction	6	6	4	4	4	CANNTG
GATA Box	Light responsive element	13	13	9	2	1	GATA
GT1 consensus	Cell type specific	17	19	11	4	-	GRWAAW
I Box core	Light regulated & tissue specific expression	13	10	7	-	-	GATAAGR
Myb core	Regulatory roles in developmental processes and defense responses in plants	4	3	2	2	-	CNGTTR
Myb consensus	Regulatory roles defense responses and dehydration stress in plants	2	1	-	1	-	YAACKG
Myb ST1	Dehydration stress	4	4	2	1	-	GGATA
Myc consensus	Dehydration induction	6	6	4	4	4	CANNTG
Root Motiff	Expression in root	6	7	4	7	2	ATATT
TATA box		9		8	12	5	TATAA
W box (TGACY) associated with WRKY (Zn finger)	Stress / pathogen defense regulated Expression	1	1	3	3	1	TTGAC
WRKY	Stress / pathogen induced expression	2	2	3	4	1	TGAC

Table.8. The putative cis-acting elements and their frequencies of occurrence in the isolated promoter regions of different forms of β-1,3- glucanase from the clone RRII 105

4.3.5. Isolation of the new form of β-1,3-glucanse from the tolerant *Hevea* interspecific hybrid - FX 516 – (Gln7)

A new form of β-1,3-glucanase was identified from the *Hevea* clone – FX 516 (an inter specific hybrid- *H. brasiliensis* X *H. benthamiana*). This new form had been obtained while attempting to amplify the Gln 6 from different *Hevea* clones relatively tolerant and susceptible to ALF. The clone FX 516 is tolerant towards ALF. Upon visualization of the PCR products, it was found that along with the expected 2 kb band, another band of approximately 1.4 kb in size had also been amplified with FX-516. But in RRII 105 and RRIM 600 only the 2kb single band was amplified (fig. 57). This new form has been designated as Gln 7.

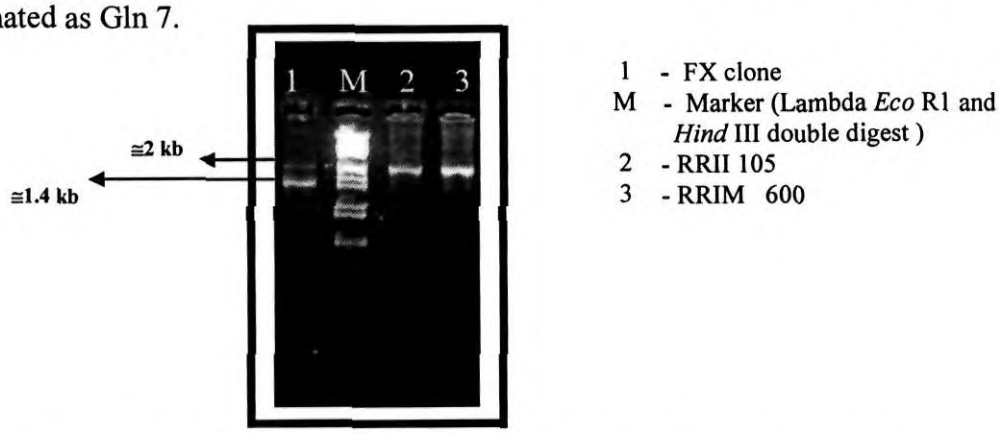


Fig. 57. PCR amplification of a new form of β-1,3-glucanase from the FX -516 hybrid of *Hevea*

The new fragment (≅1.4 kb) amplified along with the 2kb fragment corresponding to the Gln 6 fragment was cloned in pGEM®-T Easy vector. Plasmids isolated from the positively transformed recombinant colonies were confirmed through colony PCR (fig. 58 & 59). Recombinant plasmids isolated from the positive colonies of DH5α cells were sequenced. The sequence obtained is shown in fig. 60.

Upon analysis of the sequence it was found that the promoter region comprises 145 nucleotides (currently designated as Gln 7), showing a peculiar pattern of alignment with the Gln 6 promoter. Some of the inner regions of the Gln 6 promoter is found to be absent in the FX 516 - glucanase promoter. The alignment with the Gln 6 promoter is shown in the fig.62. No regions in the promoter sequence showed the potential for the nucleosome formation.

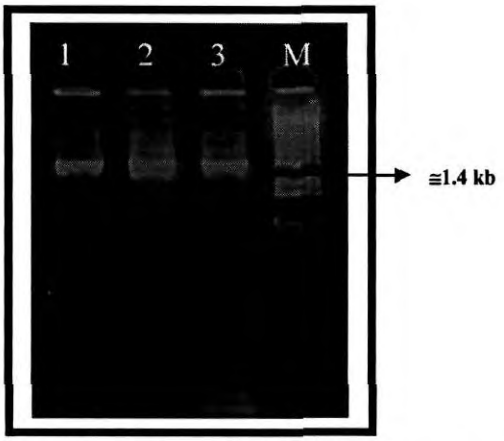


Fig. 58. Colony PCR showing positive amplification

1 -3 : 1.4 kb amplification
M - Marker

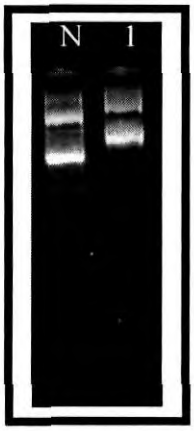


Fig. 59. Recombinant plasmid isolated from the positive colony

N - Non- recombinant plasmid without insert.
1 - Recombinant plasmid with the insert

5′ CATCCATGTTTAATAGATCATAAATTTCAATTTAAACGATAAAAATAATTATGTTATAAAGT
GACAAATCTTATCCTTCATCTTTTAAGGACATATGGAGGTGTATAAATTATAAGCAACTTTCTT
CTAATTTCCCCCCTTCTTAATGGCTATCTCCTCTTCAACTTCAGGAACTAGTAGTTCCTTGCCC
TCAAGAACTACTGTCATGCTTCTTCTGATTTTCTTTACAGCAAGCCTTGGTATAACAGGTCTCT
CTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTTTCTCATGTTAAAGTTGACGATGCTCTTTTTT
TCTCTCCTTGTTCTAAAAGGTTTCAACTAATACCTGTATTTGGAATTTAGATGCCCAGGTAGGT
GTTTGCTATGGAATGCAAGGCAACAACCTTCCACCTGTTTCAGAGGTCATAGCTCTCTATAAAA
AATCTAACATCACGAGAATGAGAATTTATGATCCAAATCGAGCAGTATTGGAAGCCCTTAGAGG
CTCAAACATTGAACTCATACTAGGTGTTCCAAACTCAGATCTCCAAAGCCTTACCAATCCTTCC
AATGCAAAATCATGGGTACAAAAAAATGTTCGTGGCTTCTGGTCAAGTGTCAGGTTCAGATATA
TAGCAGTTGGCAATGAAATTAGTCCTGTAAATAGAGGCACAGCTTGGTTGGCTCAGTTTGTTTT
GCCTGCCATGAGAAATATACATGATGCTATAAGATCAGCTGGTCTTCAAGATCAAATCAAGGTC
TCCACTGCAATTGACTTGACCCTGGTTGGAAATTCCTACCCTCCTTCTGCAGGTGCTTTCAGGG
ATGATGTTAGATCATACTTGAACCCAATTATTAGATTTCTATCCTCTATCAGGTCACCTTTACT
TGCCAATATTTATCCTTACTTTACTTATGCTGGTAATCCAAGGGATATTTCCCTTCCCTATGCT
TTGTTCACTTCACCATTCGTTGTTGTGTGGGATGGTCTGCGAGGTTATAAGAACCTTTTTGATG
CAACGTTGGATGCATTGTACTCTGCTCTTGAGAGGGCTAGTGGTGGTTCTCTGGAGGTGGTTGT
TTTGGAAAGTGGCTGGCCGTCTGCCGGAGCATTTGCTGCCACATTTGACAATGGGCGTACTTAT
CTCTCAAATTTGATCCAGCATGTTAAAAGAGGTACTCCTAAGAGGCCTAACAGAGCTATAGAGA
CTTACTTATTTGCCATGTTTGATGAAAATAAGAAGCAACCAGAGGTTGAGAAACACTTTGGACT
TTTCTTTCCTGGTAAACGGCAAAAATATAATCTCAATTTTGGTGCAGAAAAGAACTGGAATATT
TCTACCGAACACAATGCAACAATACTTTTCCTTAAGAGTGATATGTGA 3′

Total region amplified: 1390 bps
Promoter- 145
5'UTR- 39
Gene-1245 (from ATG- stop codon)
Intron-120
CT repeats- 19
Start codon
Stop codon
Prrimer sequences are underlined

Fig. 60. Sequence of β-1,3-glucanase obtained from the hybrid of *Hevea*: FX- 516 (designated as Gln 7)

The Z curve plotter showed the difference in the DNA architecture occurring in the glucanase promoter from FX 516 (fig. 61. A) and that of the Gln 6 promoter (fig. 61.B). The picture reveals the difference in the DNA architecture due to the deletion of the inner fragment of the promoter region found in the Gln 6 promoter.

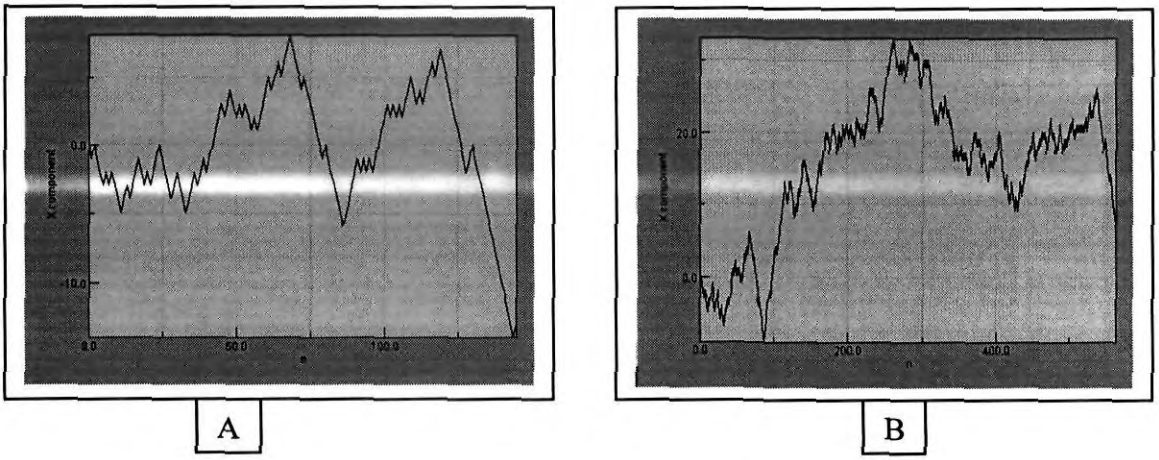


Fig. 61. The difference in the Z-curve pattern of the promoter sequences in FX (A) and Gln 6 (B)

Fig. 62. shows the alignment of the promoter sequences of the 913 and 847 bp promoter sequences (from *Hevea* clone RRII 105) and 145 bp promoter (from FX-516) of β-1,3-glucanase gene.

Fig. 62. ClustalW multiple sequence alignment of the promoter sequences of Gln 5 , Gln 6 and Gln 7 (FX 516)

```
913             CATCCATGTTTAATAGATCCTAAATTTCAATTTAAACGATAAAAATAATTATGTTATAAA 60
847             CATCCATGTTTAATAGATCCTAAATTTGAATTTAAACGATAAAAATAATTATGTTATAAA 60
FX              CATCCATGTTTAATAGATCATAAATTTCAATTTAAACGATAAAAATAATTATGTTATAAA 60
                ********************* ******* ********************************

913             GTGACAAATCTTATCCTTTTATCTTTTAAGGAGATAAGGAGGTGCAGAAAAAAGACTTAA 120
847             GTGACAAATCTTATCCTTTTATCTTTTAAGGAGATATGGGGGGGCAGAAAAAAGACTTAA 120
FX              GTGACAAATCTTATCCTTC-ATCTTTTAAGGACATATGGAGG------------------- 101
                ******************  ************ *** ** **

913             ACGTCTTCAACAGATAGAGACATTCATTTATCCAACGAAAAATTGAAAGATGCCCAAGAC 180
847             AAGTCTTCAACAGATAGAGACATTCATTTATCCAACGAAAAATTGAAAGATGCCCAAGAC 180
FX              ------------------------------------------------------------

913             ATTTCAAACTAAATTAGCATTAATTATCAGGATTATTAATTGGTAAAAAGTGCATTATTT 240
847             ATTTCAAACTAAATTAGCATTAATTATCAGGATTATTAATTGGTAAAGAGTGCATTATTT 240
FX              ------------------------------------------------------------

913             TCAATAAAAGAAGTAAAATAATTTCACCGATTAGTTTTGAGAGATTGTTGTGCATTTTCG 300
847             TCAATAAAAGAAGTAAAATAATTTCACCGATTAGTTTTGAGAGATTGTTGTGCATTTTCA 300
FX              ----------------------------------------------------TGTAT----- 106
                                                                     ** **
```

```
913             AAGCACATGTTTTCTTCCGATCAGACATTGGATCCACTGTTTCTTAATT---------- 349
847             AAGCACATGTTTTCTTCCCATCAGACATTGGATCCACTTTTTCTTAATTTAGAAAAATAA 360
FX              ------------------------------------------------------------


913             --ATAATTATATATCTGTAAAGAGCACTATATTTTAGTGCTCTAAATGCATGGTAGAATT 407
847             CTATAACTATATATCTGTAAAGAGCACTATATTTTAGTGCTCTAAATGCATGGTTGAATT 420
FX              ------------------------------------------------------------


913             TTTCTTAATTTTAAACCT---CAGTTGAA-TTAAACAAACAAATAAACTCCAGCAACAGT 463
847             TTTCTTAATTTTAAACCTGATCAGTTGAAATTAAACAAACAAATAAACTCCAGCAACAGT 480
FX              ------------------------------------------------------------


913             ATGCTCAAGAGTCCGCCTATAAAGCTTTGACATGGTAGAAACCTAACACATATGCGTGGT 523
847             ATTCTCAAGAGTCCGCCTATAAAGCTTTGACATGGTAGTAACCTAACACATATGCGTGGT 540
FX              ------------------------------------------------------------


913             CGAAATTATAAGCAACTTTCTTCTAATTTCCCGCCTTCTTAATG 567
847             CGAAATTATAAGCAACTTTCTTCTA-TTTCCCCCCTTCTTAATG 583
FX              --AAATTATAAGCAACTTTCTTCTAATTTCCCCCCTTCTTAATG 148
                ********************** ****** **********
```

Sequence 1: 913
Sequence 2: 847
Sequence 3: FX
Sequences (1:2) Aligned. Score: 97
Sequences (1:3) Aligned. Score: 96
Sequences (2:3) Aligned. Score: 95

Upon alignment (alignment upto 567 bps of Gln 6 promoter) it is seen that the Gln 7 (from FX 516) promoter sequence is more or less homologous towards the promoter sequences of Gln5 (847 bps) and Gln 6 (913 bp promoter). But it is slightly more homologous towards the 913 bp promoter as indicated by the alignment score of 95 rather than 93 with the 847 bp promoter of Gln 5. It is clear from the alignment that the region between the - 43 to – 471 bp found in the 913 bp promoter is absent in the FX glucanase promoter except for the presence of a TGAT sequence between the -231 to -235 bps. This made the FX promoter a shorter one. Therefore, some of the major cis-elements found in the Gln6 promoter is found to be absent in the FX glucanase promoter (Gln 7). The frequency of occurrence of some of the major cis-elements in this promoter will also be less compared to that of the Gln 5 and 6 promoters. No CpG islands have been found in the 145 bp sequence identified in the FX 516 (Gln 7) glucanase promoter. The region which is not present in the FX gene promoter and was present in the Gln6 promoter (453- 874 bps) was found to have two CpG islands. The promoter region of Gln 7 contains some of the important cis-elements like Dof core (3), E-box (2), GATA box (2), GT1 consensus (2), I- box (3), Myb consensus (2), TATA box (2), WRKY element (1). The WRKY occurs within the +62 bp promoter region. No W box

some of the important cis-elements like Dof core (3), E-box (2), GATA box (2), GT1 consensus (2), I- box (3), Myb consensus (2), TATA box (2), WRKY element (1). The WRKY occurs within the +62 bp promoter region. No W box is present in the isolated promoter region of FX. It is also interesting to see that the isolated promoter regions are not having any nucleosome potential. The region does not contribute to the formation of any nucleosomes.

The translated gene sequence of Gln 7 comprises 374 amino acids with a molecular weight of approximately 41 kDa (fig. 63). There are 28 acidic amino acids and 38 basic amino acids. This is having an isoelectric point of 9.57.

Fig. 63. Amino acid sequence of Gln 7

```
         10        20        30        40        50        60        70
         |         |         |         |         |         |         |
MAISSSTSGTSSSLPSRTTVMLLLIFFTASLGITDAQVGVCYGMQGNNLPPVSEVIALYKKSNITRMRIY
DPNRAVLEALRGSNIELILGVPNSDLQSLTNPSNAKSWVQKNVRGFWSSVRFRYIAVGNEISPVNRGTAW
LAQFVLPAMRNIHDAIRSAGLQDQIKVSTAIDLTLVGNSYPPSAGAFRDDVRSYLNPIIRFLSSIRSPLL
ANIYPYFTYAGNPRDISLPYALFTSPFVVVWDGLRGYKNLFDATLDALYSALERASGGSLEVVVLESGWP
SAGAFAATFDNGRTYLSNLIQHVKRGTPKRPNRAIETYLFAMFDENKKQPEVEKHFGLFFPGKRQKYNLN
FGAEKNWNISTEHNATILFLKSDM
```

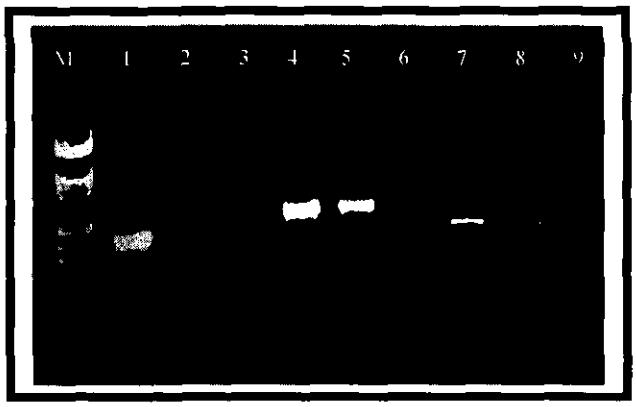
Number of amino acids: 374
Molecular weight: 41459.3 Da
Theoretical pI: 9.57
Total number of negatively charged residues (Asp + Glu): 28
Total number of positively charged residues (Arg + Lys): 38
* *The red colour indicates the apolar, hydrophobic basic amino acids arginine (R) and lysine (K).*

4.4. Amplification of the 3'UTR regions of the different forms of β-1,3-glucanase

The 3'untranslated regions (3'UTRs) are considered as an important post-transcriptional regulatory region of mRNAs. The 3' UTR of an mRNA also have an equally important role as the 5'UTR in regulating post – transcriptional gene expression. This region comprises a set of gene sequence on the 3' end of mRNA which is not translated into protein. 3' UTRs usually contain sequences that regulate translation efficiency, mRNA stability, and polyadenylation signals. In addition to the poly (A) tail, which enhances

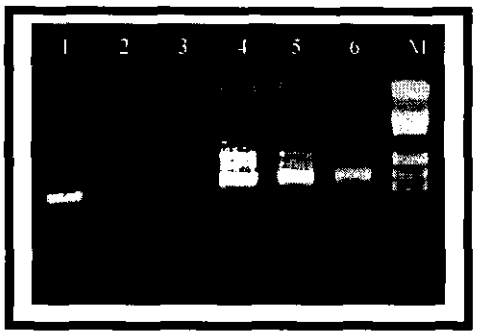
translation (Munroe & Jacobson, 1990), there are other sequences within the 3' UTR that regulate mRNA stability or translational efficiency, or both.

Keeping these points in mind, attempts have been made to identify the 3' UTR regions of the previously identified five different forms of β-1,3-glucanases (Gln2, 3, 4, 5, & 6) from the single *Hevea* clone RRII 105 through PCR . For the specific amplification, promoter specific forward and the 3' UTR specific reverse primers have been used for the PCR. Three reverse primers have been used to amplify all the forms (designated as '1','2', and '3'). The primer designing has been done based on a reported 3'UTR sequence of an mRNA of β-1,3-glucanase (Acc. No: U22147) for the amplification. The reverse primer '1' could amplify all the forms in combination with the gene specific forward primer 50 ^{0}C annealing temperature. It was interesting to note that the primers 2 and 3 could not amplify all the forms. In combination with the promoter specific forward primer, reverse primer 2 could amplify Gln 2, 5 & 6 , while reverse primer 3 could amplify Gln 2 only. This may be due to the differences in the downstream 3'UTR sequences among all these forms of β-1,3-glucanases. Fig. 64. A & B shows the amplification of the different forms of glucanase obtained with its 3'UTR regions.



M- Marker (λ DNA *EcoR*I/ *Hind* III double digest)
1 - Gln 4.1 – 1.6 kb
2 - Gln 4.2
3 - Gln 4. 3
4 - Gln 5.1 – 2kb
5 - Gln 5.2 – 2.1 kb
6 - Gln 5.3
7 - Gln 6.1 – 1.8 kb
8 - Gln 6. 2 – 1.81 kb
9 - Gln 6. 3

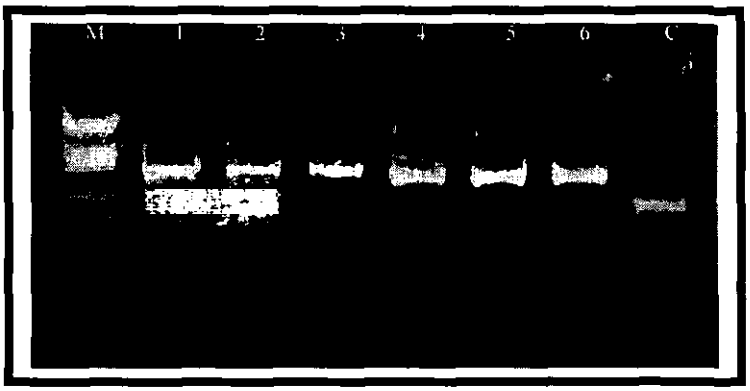
Fig. 64. A . Amplification of 3'UTR regions of the different forms of β-1,3-glucanases



1 - Gln 3.1 – 1.4 kb
2 - Gln 3.2
3 - Gln 3. 3
4 - Gln2. 1 – 1.5 kb
5 - Gln2. 2 – 1.51 kb
6 - Gln2. 3 – 1.52 kb
M - Marker (λ DNA *EcoR*1/ *Hind* III double digest)

Fig. 64.B. Amplification of the 3'UTR region of different forms of β-1,3-glucanases

The amplified bands were cloned into pGEM-T® Easy vector system and transformed to chemically competent DH5α cells. The transformed colonies were screened from the antibiotic selection plate (ampicillin 50 μg/ml) through colony PCR with sequence specific primers. Recombinant plasmids were later isolated from the positive colonies (fig. 65) and sequenced at M/S Macrogen, Korea.



M - Marker : (λ DNA *EcoR*1/ *Hind* III double digest)
Plasmids with the inserts
1- Gln 5.3
2- Gln 6.3
3- Gln 2. 4
4- Gln 3. 3
5- Gln 5. 3
6- Gln 4. 3
7- Control plasmid without the insert

Fig. 65. Recombinant plasmids isolated for sequencing

The sequence analysis showed the presence of full length gene of all the five forms with partial 3'UTR. The comparison of the 3'UTRs of five forms of β-1,3- glucanase is shown in the fig. 66.

Fig. 66. Comparison of the 3'UTR regions of the five forms of β-1,3-glucanases. *The stop codon is highlighted in red colour. The yellow font in the highlighted stop codon is the amber codon of Gln 4.*

```
Gln5.1          GATATG[redacted]GATT--GTGAGAATTTAAGTACTATATATATTTCCAATGTATGCATGTATC
Gln2.2          GATATG[redacted]GATG--GTGAGAATTTAAATACTATATATATTTTCAATGTATGCATGTATC
Gln4.1          GATATT[redacted]GATATTGTGAGAATTTAAGTGCTATATGTATTTCCAATGTATGCATGTATC
Gln3.1          GACATG[redacted]GATG--GTGAGAATTTAAGTACTACATACATTTCCAATGTATGCATGTATC
Gln6.1          GATATG[redacted]GATT--GTGAGAATTTAAGTACTATATATATTTCCAATGTATGCATGTATC
                ** ** * ****   ************  * *** **  ***  ****************

Gln5.1          C-------------------
Gln2.2          CATGTATTAAATAAGAGAACC
Gln4.1          C-------------------
Gln3.1          C-------------------
Gln6.1          C-------------------
                *
```

On alignment of the 3'UTR sequences of the five forms 2, 3, 4, 5 & 6, it is found that the Gln 4 sequence is having the amber stop codon (TAA) rather than the ochre stop codon in others (TAG). A region of 50 3'UTR nucleotides has been obtained with Gln 3, 5 & 6; 52 nucleotides with Gln 4 and 70 nucleotides with Gln2. Out of these five forms, the Gln 4 shows a difference of 5 nucleotides with respect to other sequences thus showing a difference of 10 % in the 50 bp nucleotides in the 3'UTR region. Gln 2 is the only one with which a 70 nucleotides in the 3'UTR region. This may be due to the presence of the region complementary for the primer 4 in Gln 2 which will be absent in the other forms. The alignment proves the difference in the sequence of the downstream 3'UTR region of all the forms of glucanase.

4.4.1. Sequence characterization of Gln 4

It was possible to obtain the full length genomic sequence of Gln 4 sequence (with the 582 bp promoter) when the 3'UTR reverse primer '1' was used. Previously when attempts for the amplification of all the genomic forms with promoter region was made with the promoter specific forward and a gene specific reverse primer (common primer from the coding region including the stop codon) Gln 4 could not be amplified. The sequence of Gln 4 is shown in fig . 67.

AGATATATTCAGTTTCATTCTTAAAGATATTATAAAAAAAATTAAAAAGATAAAAATTATTAA
ATTAAAAAAATCTCAATTTTTTTTTTAAATATCTCTTAATAAAATAATACCCGTGATTATAAA
TCTTAGTTTTCAATTATTAGAGGATAAAAAATAATTATGTTGAAGAGTGAATTTAATCTTATT
TTCTCAGTGTATTGTATTGCTGTGCTTGGACAAAATCTTATCCTTTATCTTTTAAGGAGATTT
CTAGGTGCAGAAATATGACTTTTAAGTCTTTAACAGATAGGGACATTCATCTGTCCAACGAAA
ATTGAAAGATGGCTAAGACATTTTCCAAACTAAATTAATTGGCATTAATTGTCATGATTGGTA
AAGATGCAAGGAGAGTAATTGATAATTTTTAATTCAGTGATTGTTTAAAATTCCAATCTCAAT
TAAAGCCAAATAAATAAATTAATAAACTCCAGCAACAGTATGGAAAAGGGTCCCTATATAAAG
CATTGACATGGTAGTAACATAACACATATGCATGCCCCAAATTAAGCTACTTTCTTCTAATTT
CCCCCCTTCTTA**ATG**GCTATCTTCTCTTCAAATTCAGCAACTAGTAGTTCCTTGCCCTCAAG
AACTACTTCCATACTTCTGGTTCTCTTTATAGCAAGCCTTAGTATAACAGGTCTCTCTCTCTC
TCTCTCTCTCTCTCTCTACTCTCATGTTAAAGTTGATAATGCATTTTTTTTTTCTTGTTCTAA
AAGGTTTCAACTAATATCTGTATTTGGAATTCAGATGCCCAGGTAGGTGTTTGCTATGGAATG
CTAGGCAACAACCTTCCACCTGCTTCACAGGTTATCTCTCTCTATAAACAAGCGAACATTAAG
AGAATGAGAATTTATGATCCAAATCAAGCAGCATTGCAAGCCCTTAGAGGCTCAAACATTCAA
CTCATGCTTGGTGTTCCAAACTCAGATCTCCAAAGCCTTACCAATCCTTCTAATGCAAACTCA
TGGATACAAAGAAATGTTCGAGCCTTCTGGCCAAGTGTCAGATTCAGATATATAGCAGTTGGA
AACGAAATTAGTCCAGTGAATGGAGGCACAGCTTCCTTGGCTAAATTTGTTTTGCCTGCCATG
AGAAATATATATAATGCAATAAGATCAGCTGGTCTTCAGGATCAAATAAAGGTCTCTACCGCA
ATTGACATGACCCTGATAGGAAATTCATACCCTCCTTCTGCAGGTGCTTTCAGGGGTGATGTT
AGATCATACTTGGACCCAATTATAGGATTCCTATCCTCTATTAGGTCACCTTTACTTGCCAAT
ATTTATCCTTACTTTAGTTATGCTGGTAATCCAAGGGATATTTCCCTTCCCTATGCTTTGTTC
ACTTCACCATCAATAGTTGTATGGGATGGTCAGCGAGGTTATAAGAACCTTTTCGATGCAATG
TTGGATGCATTGTACTCTGCTCTTGAGAGGGCTGGTGGTGGTTCTCTGGAGGTAGTTGTGTCG
GAAAGTGGCTGGCCGTCCGCCGGAGCTTTTGCAGCCACATTTGATAATGGGCGCACTTATCTC
TCAAATTTGATCCAGCATGTTAAAGGAGGTACTCCTAAGAGGCCTGGCCGATTTATAGAGACT
TACCTATTTGCCATGTTTGATGAAAATCAAAAGCAACCGGAGTTTGAGAAACACTTTGGACTT
TTCTTTCCAAATAAACAGCAAAAATATAATCTCAATTTTGGTGCAAAAAAGAACTGGGATATT
TCCGCTGATTACAATGCAACAGTACTTTTACTTAAGAGTGATATT**TAA**GATATTGTGAAAAT
TTAAGTGCTATATGTATTTCCAATGTATGCATGTATC

Total region amplified: 1671 nucleotides
Promoter region – 388 bp amplified within the 582 bp promoter
5'UTR – 37 bps
3'UTR - 51 bps
Gene portion (from ATG- stop codon) – 1232 bps
Introns - 110
CT repeats- 16 no's
Start codon (ATG)
Stop codon (TAA)
Primer sequences underlined

Fig. 67. The coding gene sequence of Gln 4 along with the promoter and 3'UTR

The amplified sequence designated as Gln 4 (NCBI Acc. No. JN251022) comprises 1671 nucleotides with 388 bp promoter region comprising a 37 bp 5'UTR region, 51 bps 3'UTR region. This sequence is also a single intronic one with 110 intronic nucleotides including the 16 number

CT repeats. A unique feature found with this sequence is the presence of the amber codon instead of the ochre stop codon found in other forms.

The translated amino acid sequence shows the presence of 24 acidic amino acid and 33 basic amino acid residues with a molecular weight of 40.9 kDa. The pI of the protein is predicted to be 9.48 (fig. 68). It comprises a total of 373 amino acid residues showing a deletion of a single amino acid residue at the 21st position in comparison with others.

Fig. 68. Details of the amino acid sequence of Gln 4

```
        10         20         30         40         50         60         70
         |          |          |          |          |          |          |
MAIFSSNSATSSSLPSRTTSILLVLFIASLSITDAQVGVCYGMLGNNLPPASQVISLYKQANIKRMRIYD
PNQAALQALRGSNIQLMLGVPNSDLQSLTNPSNANSWIQRNVRAFWPSVRFRYIAVGNEISPVNGGTASL
AKFVLPAMRNIYNAIRSAGLQDQIKVSTAIDMTLIGNSYPPSAGAFRGDVRSYLDPIIGFLSSIRSPLLA
NIYPYFSYAGNPRDISLPYALFTSPSIVVWDGQRGYKNLFDAMLDALYSALERAGGGSLEVVVSESGWPS
AGAFAATFDNGRTYLSNLIQHVKGGTPKRPGRFIETYLFAMFDENQKQPEFEKHFGLFFPNKQQKYNLNF
GAKKNWDISADYNATVLLLKSDI

Number of amino acids: 373
Molecular weight: 40936.6 Da
Theoretical pI: 9.48
Total number of negatively charged residues (Asp + Glu): 24
Total number of positively charged residues (Arg + Lys): 3
```

Since the Gln 4 peptide sequence is showing good amino acid variations on comparison with the other forms, a conserved domain search was performed (fig.69).

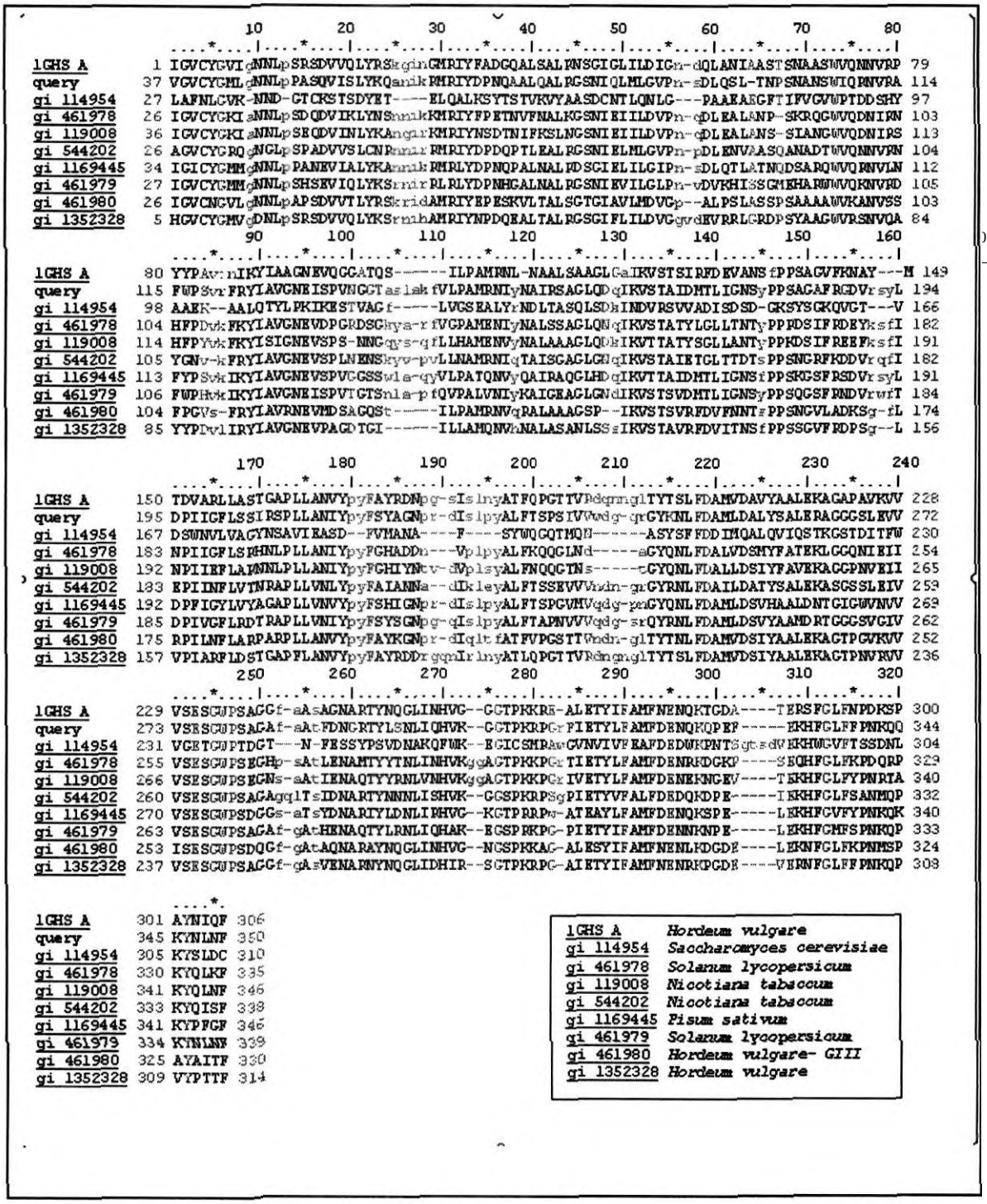


Fig. 69. Details of the conserved domain search of Gln 4. *Red colour indicates the conservation of identical amino acid residues.*

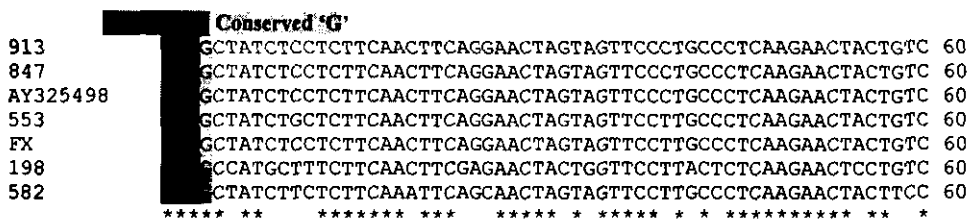
The conserved domain search shows that it is sharing positional conservation of the amino acids with other β-1,3-glucanase sequences reported from *H. vulgare*, *S. cerevisiae*, *S. lycopersicum* and different forms reported from a single species of *N. tabaccum* etc. Apart from that it is also

showing 24 unique variations which is highlighted in magenta colour upon comparison with the other reports.

BLASTp analysis of Gln 4 showed 85-89% similarity with the amino acid sequences of the other reports from *Hevea*. But Gln 4 peptide sequence is the one which is showing slightly more homology towards (2-3% higher) the reported amino acid sequences from species other than *Hevea brasiliensis*. 65% similarity has been observed with β-1,3-glucanase from *Vitis vinifera* (CAB91554). 64% similarity with a basic β-1,3-glucanase from *N. tabaccum* (ACF93731.1).63% similarity with another basic β-1,3-glucanase from *N. tabaccum* (AAA63541).65% similarity with the β-1,3-glucanase from *Pisum sativum* (AAB24398).79 % with a basic vacuolar isoform of β-1,3-glucanase *Vitis vinifera* (XP002277169). 65% with an acidic glucanase from *Medicago sativa* (AAB41551). 66% with another acidic glucanase from *Medicago sativa* (ABY47902). 73% similarity with a β-1,3-glucanase regulator from *N. plumbaginifolia* (AAA34078).

The comparison of the genomic sequence of the six different forms of β-1,3-glucanase with the earlier reported genomic DNA sequence by Thanseem *et al.*, (2003) (AY325498), from Rubber Research Institute of India, showed that the maximum alignment score (99) is with Gln 5 (fig. 70). The least alignment (90) is with Gln 3 (having the 198 bp promoter) and Gln 2 (with 553 bp promoter); Gln 7 (from FX 516) is in alignment with Gln 4 (having the 582 bp promoter). Gln 6 is showing maximum similarity with Gln 3 and Gln 5 apart from the intron nucleotide variations; showing an alignment score of 98.

Fig. 70. Comparison of the nucleotide sequence of all the six forms of β -1, 3- glucanases with the earlier report (Acc No: AY325498)



```
                                                                  Introns
913          ATGCTTCTTCTGATTTTCTTTACAGCAAGCCTTGGTATAACAGGTCTCTCTCTCTCTCTC 120
847          ATGCTTCTTCTGATTTTCTTTACAGCAAGCCTTGGTATAACAGGTCTCTCTCTCTCTCTC 120
AY325498     ATGCTTCTTCTGATTTTCTTTACAGCAAGCCTTGGTATAACAGGTCTCTCTCTCTCTCTC 120
553          ATGCTTCTTCTGTTTTTCTTTACAGCAAGCGTTGGTATAACAGGTCTCTCTCTCTCTCTC 120
FX           ATGCTTCTTCTGATTTTCTTTACAGCAAGCCTTGGTATAACAGGTCTCTCTCTCTCTCTC 120
198          ATGCTTCTTCTGATTCTCTATATAGCAAGCCTTGGTATAACAGGTGTCTCTCTCTCTCTA 120
582          ATACTTCT---GGTTCTCTTTATAGCAAGCCTTAGTATAACAGGTCTCTCTCTCTCTCTC 117
             ** *****   * ** *** ** ******* ** ********** ************

913          TCTCTCTC---------------CATCTATCTACTCTCATGTTAAAGTTGACGATGCTCTT 166
847          TCTCTCTCTCTCTCTCTCTCCATCTATCTACTCTCATGTTAAAGTTGACGATGCTCTT 180
AY325498     TCTCTCTCTCTCTCTCTCTCCATCTATCTACTCTCATGTTAAAGTTGACGATGCTCTT 180
553          TCTCTCTCTC-------------------------ATGTTAAAGTTGACGATGCTCTT 153
FX           TCTCTCTCTCTCTC----------TCTCTCT-TTCTCATGTTAAAGTTGACGATGCTCTT 169
198          TCTATCT-----------------------ACTCTCATGTTAAAGTTGACCATGCTTTT 156
582          TCTCTCTCTC------------------TCTACTCTCATGTTAAAGTTGATAATGCATTT 159
             *** ***                              *************  ****  **

913          TTTTTCCCTCCTTTTTCTAAAAGGTTTCAACTAATACCTGTATTT-GGAATTTAGATGCC 225
847          TTTTTCTCTCCTTGTTCTAAAAGGTTTCAACTAATACCTGTATTT-GGAATTTAGATGCC 239
AY325498     TTTTTCTCTCCTTGTTCTAAAAGGTTTCAACTAATACCTGTATTTAGGAATTCAGATGCC 240
553          TTTTTCTCTCCTTGTTCTAAAAGGTTTCAACTAATACCTGTATTT-GGAATTTAGATGCC 212
FX           TTTTTCTCTCCTTGTTCTAAAAGGTTTCAACTAATACCTGTATTT-GGAATTTAGATGCC 228
198          TTTCT---TCCTCGTTCTGAAGGGTTTCAACTAATACCTGTATTT-GGAATTCAGATGCC 212
582          TTTTT---TTCTTGTTCTAAAAGGTTTCAACTAATATCTGTATTT-GGAATTCAGATGCC 215
             *** *   * **  **** ** ************** ******** ****** *******

913          CAGGTAGGTGTTTGCTATGGAATGCAAGGCAACAACCTTCCATCTGTTTCAGAGGTCATA 285
847          CAGGTAGGCGTTTGCTATGGAATGCAAGGCAACAACCTTCCATCTGTTTCAGAGGTCATA 299
AY325498     CAGGTAGGTGTTTGCTATGGAATGCAAGGCAACAACCTTCCACCTGTTTCAGAGGTCATA 300
553          CAGGTAGGTGTTTGCTATGGAATGCAAGGCAACAACCTTCCACCTGTTTCAGAGGTCATA 272
FX           CAGGTAGGTGTTTGCTATGGAATGCAAGGCAACAACCTTCCACCTGTTTCAGAGGTCATA 288
198          CAGGTAGGTGTTTGCTATGGAAAGCTAGGCAACAACCTTCCACCTGCTTCAGAGGTCATA 272
582          CAGGTAGGTGTTTGCTATGGAATGCTAGGCAACAACCTTCCACCTGCTTCACAGGTTATC 275
             ******** ************* ** ***************** *** **** **** **

913          GCTCTCTATAAACAATCTAACATCAAGAGAATGAGAATTTATGATCCAAATCAAGCAGTA 345
847          GCTCTCTATAAACAATCTAACATCAAGAGAATGAGAATTTATGATCCAAATCGAGCAGTA 359
AY325498     GCTCTCTATAAACAATCTAACATCAAGAGAATGAGAATTTATGATCCAAATCGAGCAGTA 360
553          GCTCTCTATAAAAAATCTAACATCACGAGAATGAGAATTTATGATCCAAATCAAGCAGTA 332
FX           GCTCTCTATAAAAAATCTAACATCACGAGAATGAGAATTTATGATCCAAATCGAGCAGTA 348
198          GCTCTCTATAAACAATCTAACATCAAGAGAATGAGAATTTATGATCCAAATCAAGAAGTC 332
582          TCTCTCTATAAACAAGCGAACATTAAGAGAATGAGAATTTATGATCCAAATCAAGCAGCA 335
             *********** ** * ***** * ************************** ** **

913          TTGGAAGCCCTTAGAGGCTCAAACATTGAACTCATACTAGGTGTTCCAAACTCAGATCTC 405
847          TTGGAAGCCCTTAGAGGCTCAAACATTGAACTCATACTAGGTGTTCCAAACTCAGATCTC 419
AY325498     TTGGAAGCCCTTAGAGGCTCAAACATTGAACTCATACTAGGTGTTCCAAACTCAGATCTC 420
553          TTGGAAGCCCTTAGAGGCTCAAACATTGAACTCATACTAGGTGTTCCAAACTCAGATCTC 392
FX           TTGGAAGCCCTTAGAGGCTCAAACATTGAACTCATACTAGGTGTTCCAAACTCAGATCTC 408
198          TTGCAAGCCCTTAGAGGCTCAAACATTGAACTCATACTAGGTGTTCCAAACTCAGATCTC 392
582          TTGCAAGCCCTTAGAGGCTCAAACATTCAACTCATGCTTGGTGTTCCAAACTCAGATCTC 395
             *** ********************** ******* ** **********************


913          CAAAGCCTTACCAATCCTTCCAATGCAAACTCATGGGTACAAAAAAATGTTCGTGGGTTC 465
847          CAAAGCCTTACCAATCCTTCCAATGCAAACTCATGGGTACAAAAAAATGTTCGTGGCTTC 479
AY325498     CAAAGCCTTACCAATCCTTCCAATGCAAACTCATGGGTACAAAAAAATGTTCGTGGCTTC 480
553          CAAAGCCTTACCAATCCTTCCAATGCAAAATCATGGGTACAAAAAAATGTTCGTGGCTTC 452
FX           CAAAGCCTTACCAATCCTTCCAATGCAAAATCATGGGTACAAAAAAATGTTCGTGGCTTC 468
198          CAAAGCCTTACCAATCCTTCCAATGCAAACTCATGGGTACAAAAAAATGTTCGTGACTTC 452
582          CAAAGCCTTACCAATCCTTCTAATGCAAACTCATGGATACAAAGAAATGTTCGAGCCTTC 455
             ******************** ******** ****** ****** ********* *  ***


913          TGGTCAAGTGTCAGGATCAGATATATAGCAGT-GGCAACGAAATTAGTCCCTGTAAATGG 524
847          TGGTCAAGTGTCAGGTTCAGATATATAGCAGTTGGCAACGAAATTAGTCC-TGTAAATGG 538
AY325498     TGGTCAAGTGTCCTGTTCAGATATATAGCAGTTGGCAACGAAATTAGTCC-TGTCAATGG 539
553          TGGTCAAGTGTCAGGTTCAGGTATATAGCAGTTGGCAACGAAATTAGTCC-TGTCAATAG 511
FX           TGGTCAAGTGTCAGGTTCAGATATATAGCAGTTGGCAATGAAATTAGTCC-TGTAAATAG 527
198          TGGTCAAGTGTCAGGCTCAGGTATATAGCAGTCGGCAACGAAATTAGTCC-TGTAAATGG 511
582          TGGCCAAGTGTCAGATTCAGATATATAGCAGTTGGAAACGAAATTAGTCC-AGTGAATGG 514
             *** ********    **** *********** ** ** ***********  ** *** *
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913          AGGCACAGCTTGGTTGGCCCAATTTGTTTTGCCTGCCATGAGAAATATACATGATGCTAT 584
847          AGGCACAGCTTGGTTGGCCCAATTTGTTTTGCCTGCCATGAGAAATATACATGATGCTAT 598
AY325498     AGGCACAGCTTGGTTGGCTCAATTTGTTTTGCCTGCCATGAGAAATATACATGATGCTAT 599
553          AGGCACAGCTTGGTTGGCTCAATTTGTTTTGCCTGCCATGAGAAATATACATGATGCTAT 571
FX           AGGCACAGCTTGGTTGGCTCAGTTTGTTTTGCCTGCCATGAGAAATATACATGATGCTAT 587
198          AGGCACAGCTTGGTTGGCTCAATTTGTTTTGCCTGCCATGAGAAATATACATGATGCTAT 571
582          AGGCACAGCTTCCTTGGCTAAATTTGTTTTGCCTGCCATGAGAAATATATATAATGCAAT 574
             ***********  *****  * ************************** ** **** **

913          AAGATCAGCTGGTCTTCAAGATCAAATCAAGGTCTCCACTGCAATTGACTTGACCCTGGT 644
847          AAGATCAGCTGGTCTTCAAGATAAAATCAAGGTCTCCACTGCAATTGACTTGACCCTGGT 658
AY325498     AAGATCAGCTGGTCTTCAAGATCAAATCAAGGTCTCCACTGCAATTGACTTGACCCTGGT 659
553          AAGATCAGCTGGTCTTCAAGATCAAATCAAGGTCTCCACTGCAATTGACTTGACCCTGGT 631
FX           AAGATCAGCTGGTCTTCAAGATCAAATCAAGGTCTCCACTGCAATTGACTTGACCCTGGT 647
198          AAGATCAGCTGGTCTTCAAGATCAAATCAAGGTCTCTACTGCGATTGACTTGACCCTGAT 631
582          AAGATCAGCTGGTCTTCAGGATCAAATAAAGGTCTCTACCGCAATTGACATGACCCTGAT 634
             ****************** *** **** ******** ** ** ****** ******** *

913          AGGAAATTCCTACCCTCCTTCTGCAGGTGCTTTCAGGGATGATGTTAGATCATACTTGGA 704
847          AGGAAATTCCTACCCTCCTTCTGCAGGTGCTTTCAGGGATGATGTTAGATCATACTTGGA 718
AY325498     AGGAAATTCCTACCCTCCTTCTGCAGGTGCTTTCAGGGATGATGTTAGATCATACTTGGA 719
553          AGGAAATTCCTACCCTCCTTCTGCAGGTGCTTTCAGGGATGATGTTAGATCATACTTGAA 691
FX           TGGAAATTCCTACCCTCCTTCTGCAGGTGCTTTCAGGGATGATGTTAGATCATACTTGAA 707
198          GGGAAATACCTACCCTCCTTCTGCAGGTGCTTTCAGGGATGATGTTAGATCATATTTGGA 691
582          AGGAAATTCATACCCTCCTTCTGCAGGTGCTTTCAGGGGTGATGTTAGATCATACTTGGA 694
             ****** * ***************************** *************** *** *

913          CCCAATTATTGGATTTCTATCCTCTATCAGGTCACCTTTACTTGCCAATATTTATCCTTA 764
847          CCCAATTATTGGATTTCTATCCTCTAGCAGGTCACCTTTACTTGCCAATATTTATCCTTA 778
AY325498     CCCAATTATTGGATTTCTATCCTCTATCAGGTCACCTTTACTTGCCAATATTTATCCTTA 779
553          CCCAATTATTAGATTTCTATCTTCTATCAGGTCACCTTTACTTGCCAATATTTATCCTTA 751
FX           CCCAATTATTAGATTTCTATCCTCTATCAGGTCACCTTTACTTGCCAATATTTATCCTTA 767
198          CCCAATTATTGGATTCCTATCCTCTATCAGGTCACCTTTACTTGCCAATATTTATCCTTA 751
582          CCCAATTATAGGATTCCTATCCTCTATTAGGTCACCTTTACTTGCCAATATTTATCCTTA 754
             *********  **** ***** ****  *********************************

913          CTTTACTTATGCTGATAATCCAAGGGATATTTCCCTTCCCTATGCTTTGTTCACTTCACC 824
847          CTTTACTTATGCTTATAATCCAAGGGATATTTCCCTTCCCTATGCTTTGTTCACTTCACC 838
AY325498     CTTTACTTATGCTGGTAATCCAAGGGATATTTCCCTTCCCTATGCTTTGTTCACTTCACC 839
553          CTTTACTTATGCTGGTAATCCAAGGGATATTTCCCTTCCCTATGCTTTGTTCACTTCACC 811
FX           CTTTACTTATGCTGGTAATCCAAGGGATATTTCCCTTCCCTATGCTTTGTTCACTTCACC 827
198          CTTTACTTATGCTGGTAATCCAAGGGATATTTCCCTTCCCTATGCTTTGTTCACTTCACC 811
582          CTTTAGTTATGCTGGTAATCCAAGGGATATTTCCCTTCCCTATGCTTTGTTCACTTCACC 814
             ***** *******  ********************************************

913          ATCAGTTGTTGTGTGGGATGGTCAGCGAGGTTATAAGAACCTTTTTGATGCAACGTTGGA 884
847          ATCAGTTGTTGTGTGGGATGGTCAGCGAGGTTATAAGAACCTTTTTGATGCAACGTTGGA 898
AY325498     ATCAGTTGTTGTGTGGGATGGTCAGCGAGGTTATAAGAACCTTTTTGATGCAACGTTGGA 899
553          ATCAGTTGTTGTGTGGGATGGTCAGCGAGGTTATAAGAACCTTTTTGATGCAACGTTGGA 871
FX           ATTCGTTGTTGTGTGGGATGGTCTGCGAGGTTATAAGAACCTTTTTGATGCAACGTTGGA 887
198          ATCAGTTGTTGTGTGGGATGGTCAGCGAGGTTATAAGAACCTTTTTGATGCAACGTTGGA 871
582          ATCAATAGTTGTATGGGATGGTCAGCGAGGTTATAAGAACCTTTTCGATGCAATGTTGGA 874
             **   * ***** ********** ********************** ******* ******

913          TGCATTGTACTCTGCTCTTGAGAGGGCTAGTGGTGGTTCTCTGGAGGTGGTTGTTTCGGA 944
847          TGCATTGTACTCTGCTCTTGAGAGGGCTAGTGGTGGTTCTCTGGAGGTGGTTGTTTCGGA 958
AY325498     TGCATTGTACTCTGCTCTTGAGAGGGCTAGTGGTGGTTCTCTGGAGGTGGTTGTTTCGGA 959
553          TGCATTGTACTCTGCTCTTGAGAGGGCTAGTGGTGGTTCTCTGGAGGTGGTTGTTTCGGA 931
FX           TGCATTGTACTCTGCTCTTGAGAGGGCTAGTGGTGGTTCTCTGGAGGTGGTTGTTTTGGA 947
198          TGCATTGTACTCTGCTCTTGAGAGGGCTAGTGGTGGTTCTCTGGAGGTGGTTGTTTCGGA 931
582          TGCATTGTACTCTGCTCTTGAGAGGGCTGGTGGTGGTTCTCTGGAGGTAGTTGTGTCGGA 934
             **************************** ******************** ***** * ***

913          AAGTGGCTGGCCGTCTGCCGGAGCATTTGCTGCCACATTTGACAATGGGCGTACTTATCT 1004
847          AAGTGGCTGGCCGTCTGCCGGAGCATTTGCTGCCACATTTGACAATGGGCGTACTTATCT 1018
AY325498     AAGTGGCTGGCCGTCTGCCGGAGCATTTGCTGCCACATTTGACAATGGGCGTACTTATCT 1019
553          AAGTGGCTGGCCGTCTGCCGGAGCATTTGCTGCCACATTTGACAATGGGCGTACTTATCT 991
FX           AAGTGGCTGGCCGTCTGCCGGAGCATTTGCTGCCACATTTGACAATGGGCGTACTTATCT 1007
198          AAGTGGCTGGCCGTCTGCCGGAGCATTTGCTGCCACATTTGACAATGGGCGTACTTATCT 991
582          AAGTGGCTGGCCGTCCGCCGGAGCTTTTGCAGCCACATTTGATAATGGGCGCACTTATCT 994
             *************** ******** ***** *********** ******** ********
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913          CTCAAATTTGATCCAGCATGTTAAAGGAGGTACTCCTAAGAGGCCTAACAGAGCTATAGA 1064
847          CTCAAATTTGATCCAACATGTTAAAGGAGGTACTCCTAAGAGGCCTAACAGAGCTATAGA 1078
AY325498     CTCAAATTTGATCCAGCATGTTAAAGGAGGTACTCCTAAGAGGCCTAACAGAGCTATAGA 1079
553          CTCAAATTTGATCCAGCATGTTAAAAGAGGTACTCCTAAGAGGCCTAAAAGAGCTATAGA 1051
FX           CTCAAATTTGATCCAGCATGTTAAAAGAGGTACTCCTAAGAGGCCTAACAGAGCTATAGA 1067
198          CTCAAATTTGATCCAGCATGTCAAAGGAGGTACTCCTAAGAGGCCTGACAGAGCTATAGA 1051
582          CTCAAATTTGATCCAGCATGTTAAAGGAGGTACTCCTAAGAGGCCTGGCCGATTTATAGA 1054
             *************** ***** *** ********************    **  ******

913          GACTTACTTATTTGCCATGTTTGATGAAAATAAGAAGCAACCAGAGGTTGAGAAACACTC 1124
847          GACTTACTTATTTGCCATGTTTGATGAAAATAAGAAGCAACCAGAGGTTGAGAAACACTT 1138
AY325498     GACTTACTTATTTGCCATGTTTGATGAAAATAAGAAGCAACCAGAGGTTGAGAAACACTT 1139
553          GACTTACTTATTTGCCATGTTTGATGAAAATAAGAAGCAACCAGAGGTTGAGAAACACTT 1111
FX           GACTTACTTATTTGCCATGTTTGATGAAAATAAGAAGCAACCAGAGGTTGAGAAACACTT 1127
198          GACTTACTTATTTGCCATGTTTGATGAAAATCAGAAGCAACCAGAGGTTGAGAAACACTT 1111
582          GACTTACCTATTTGCCATGTTTGATGAAAATCAAAAGCAACCGGAGTTTGAGAAACACTT 1114
             ******* ********************** * ******** *** ************

913          TGGACTTTTCTTTCCTGATAAACGGCCAAAATATAATCTCAATTTTGGTGCAGAAAAGAA 1184
847          TGGACTTTTCTTTCCTGATAAACGGCCAAAATATAATCTCAATTTTGGTGCAGAAAAGAA 1198
AY325498     TGGACTTTTCTTTCCTGATAAACGGCCAAAATATAATCTCAATTTTGGTGCAGAAAAGAA 1199
553          TGGACTTTTCTTTCCTAATAAATGGCAAAAATATAATCTCAATTTTAGTGCAGAAAAGAA 1171
FX           TGGACTTTTCTTTCCTGGTAAACGGCAAAAATATAATCTCAATTTTGGTGCAGAAAAGAA 1187
198          TGGACTTTTCTTTCCTGATAAACGGCCAAAATATAATCTCAATTTTGGTGCAAAAAAGAA 1171
582          TGGACTTTTCTTTCCAAATAAACAGCAAAAATATAATCTCAATTTTGGTGCAAAAAAGAA 1174
             ***************   ****  ** ******************** ***** *******

913          CTGGGATATTTCTACTGAACACGATGCAACAATACTTTTCCTTAAGAGTGATATG[redacted] 1242
847          CTGGGATATTTCTACTGAACACAATGCAACAATACTTTTCCTTAAGAGTGATATG[redacted] 1256
AY325498     CTGGGATATTTCTACTGAACACAATGCAACAATACTTTTCCTTAAGAGTGATATG[redacted] 1257
553          CTGGGATATTTCTACTGAACACAATGCAACAATACTTTTCCTTAAGAGTGATATG[redacted] 1229
FX           CTGGAATATTTCTACCGAACACAATGCAACAATACTTTTCCTTAAGAGTGATATG[redacted] 1245
198          CTGGGATATTTCTACTGAACACAATGCAACAGTACTTTTCCTTAAGAGTGATATG[redacted] 1229
582          CTGGGATATTTCCGCTGATTACAATGCAACAGTACTTTTACTTAAGAGTGATATT[redacted] 1232
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Sequences (1:7) Aligned. Score:  98
Sequences (2:3) Aligned. Score:  94
Sequences (2:4) Aligned. Score:  95
Sequences (2:5) Aligned. Score:  94
Sequences (2:6) Aligned. Score:  90
Sequences (2:7) Aligned. Score:  96
Sequences (3:4) Aligned. Score:  97
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Sequences (3:6) Aligned. Score:  90
Sequences (3:7) Aligned. Score:  98
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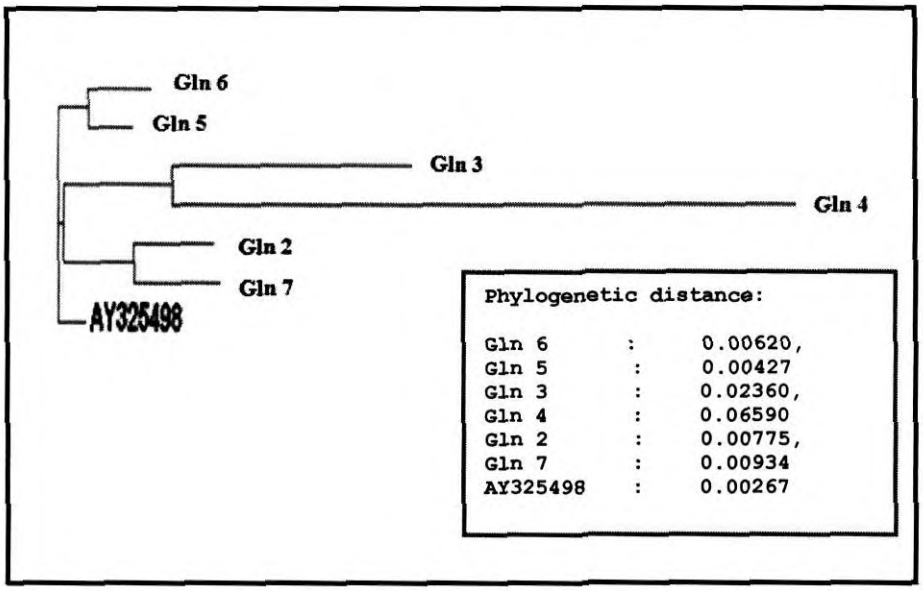
Sequence format is Pearson
Sequence 1: 913 - Gln 6 - 1242 bp
Sequence 2: 198 - Gln 3 - 1229 bp
Sequence 3: 553 - Gln 2 - 1229 bp
Sequence 4: 847 - Gln 5 - 1256 bp
Sequence 5: FX - Gln 7 - 1245 bp
Sequence 6: 582 - Gln 4 - 1232 bp
Sequence 7: AY325498 - Gln 1-1257 bp
(earlier report)

The Gln 5 is highly similar to the reported sequence AY325498 (99 score), Gln 2 is highly homologous to Gln 7 and the earlier report (98 score), Gln 3 share maximum similarity with the reported sequence (96) but less than the Gln 5 which showed a score of 99. It is least homologous with Gln 4

(score - 90). Gln 7 is highly similar to Gln 2 and the reported sequence. Gln 4 gives a maximum alignment score of 92 with that of the report. With all the others it is showing a score of 90 (with Gln 7, 2, 3) and 91 (Gln 5 & 6).

Thus sequences reported in this study (Gln2-7) are not 100% homologous with each other. It is interesting to find that the new sequences are not 100 % similar to the earlier reported sequences also. The Guanine ('G') nucleotide immediately following the ATG is conserved in all the sequences obtained to make the Kozak consensus, thus making it an adequate consensus in all. The result of the alignment tree is in par with the nucleotide sequence alignment described as in previous paragraphs (fig. 71).

Fig. 71. Phylogenetic tree showing the pattern of clustering of the different genomic forms of glucanase and the earlier report



The phylogenetic tree also shows that the Gln 5 nucleotide sequence with the 847 bp promoter is highly similar to the earlier report. Gln 4 is showing the maximum distance of 0.06590 on comparison with others. It also shows the higher similarity of Gln 7 with Gln 2 and Gln 5 with Gln 6 as shown by the ClustalW alignment score.

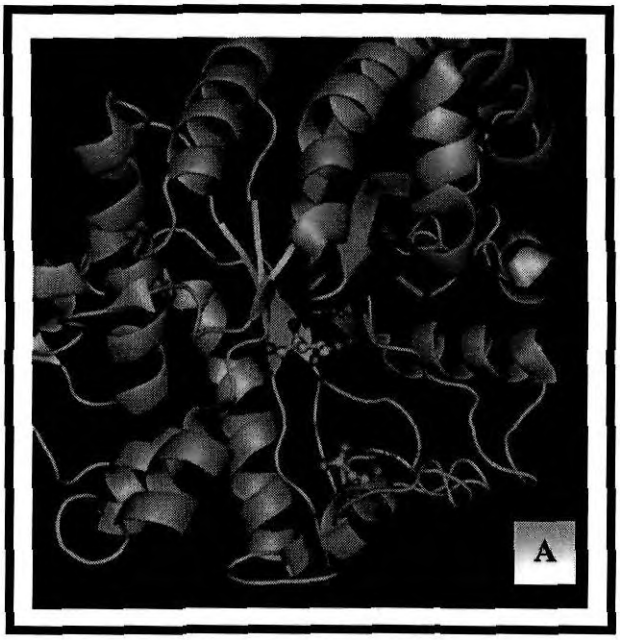
4.5. Bioinformatic analysis of β-1,3-glucanase

4. 5.1. Comparison of the 3D molecular structures of β-1,3-glucanases

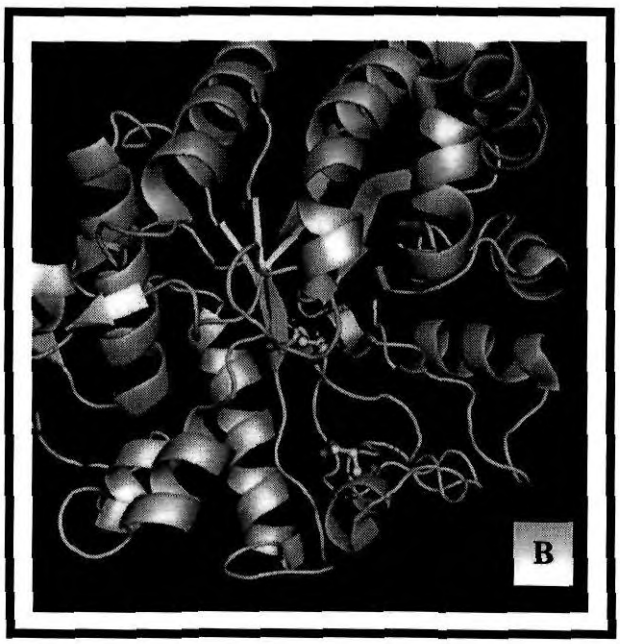
The 3D structure of the protein also determines its function. Therefore an attempt has been made to construct the 3-Dimensional model of the protein using the 'modeller' software. The protein model was later visualized using 'Pymol' software. The predicted peptide sequence was analysed with earlier reported sequences based on the X-ray crystallographic studies from *Hevea brasiliensis* (PDB ID : 3EM5 and 3F55). These reported sequences are 100 % similar in their amino acid sequence. So one of the reported sequence is taken as standard here. *ie*, 3EM5 which belongs to another β-1,3-glucanase from *H. brasiliensis*. An X-ray crystallographic 3D structure of barley β-1,3-glucanase (accession No: 1GHS) was also used to compare (fig. 72) the structure of β-1,3-glucanase from *Hevea.*

The 3D models show similar pattern of the α-helices and β-sheets arranged in a regular pattern in both *Hevea* as well as barley (fig. 72. A & B), although amino acid sequence of both the barley (1GHS) and *Hevea* β-1,3-glucanase (3EM5) differs upon the alignment, with an alignment score of 50 only (fig. 73). The figure also shows the similarities of the amino acid sequences at certain regions. But on superimposing the modelled 3D structures (fig. 72.C), it is very interesting to observe that both the glucanase of this distantly related groups shows a similar structural pattern thereby forming a completely superimposed structure. It shows a 100% structural alignment. In barley the catalytic cleft resides within 231E-288E (E= glutamic acid), while in *Hevea* 3EM5, it resides within 240E-297E. The location of catalytic residues forming the catalytic cleft in the final tertiary structure of the protein is exactly aligning though there is positional difference in the primary peptide sequence of both. The superimposed image of the barley and *Hevea* glucanase shows the structural alignment of both. The catalytic amino acid residues are located within deep grooves which extend across the enzymes and which probably bind the substrates. However, the number of amino acid residues (57 no's) within the catalytic cleft is found to be conserved in both.

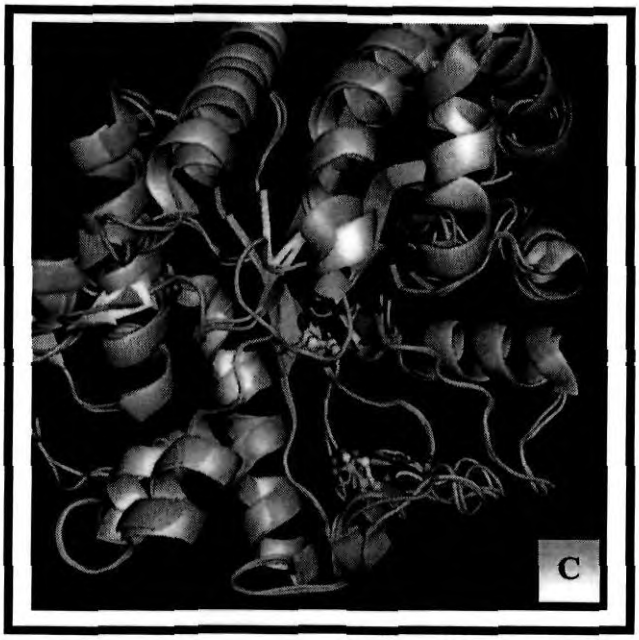
Fig. 72. A-G: The earlier reported 3D crystallographic structure of β-1,3-glucanase from barley, *Hevea* and their superimposed structures with Gln 2 from the present study



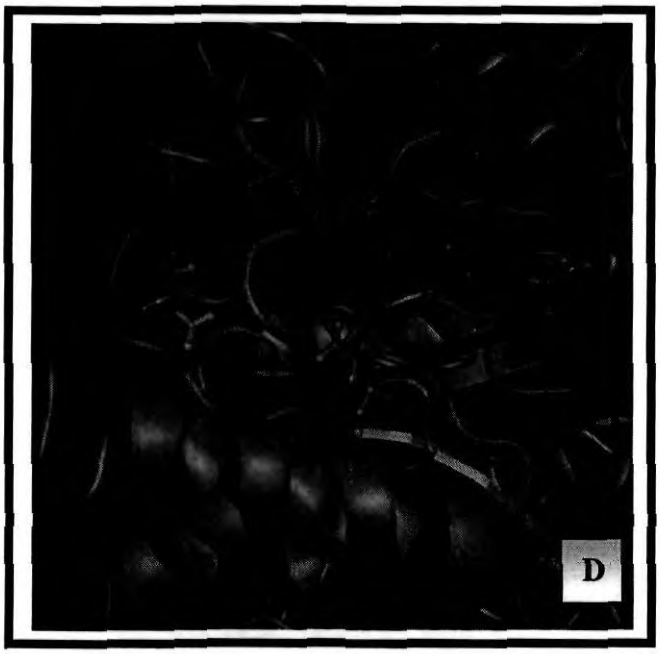
A. 3D crystallographic structure of Barley β-1,3-glucanase showing the catalytic cleft: (PDB ID: 1GHS). Catalytic cleft: 231E – 288E (E= Glutamic acid)



B. 3D crystallographic structure of *Hevea* β-1,3-glucanase : (PDB ID: 3EM5) showing the catalytic cleft : 240E- 297E



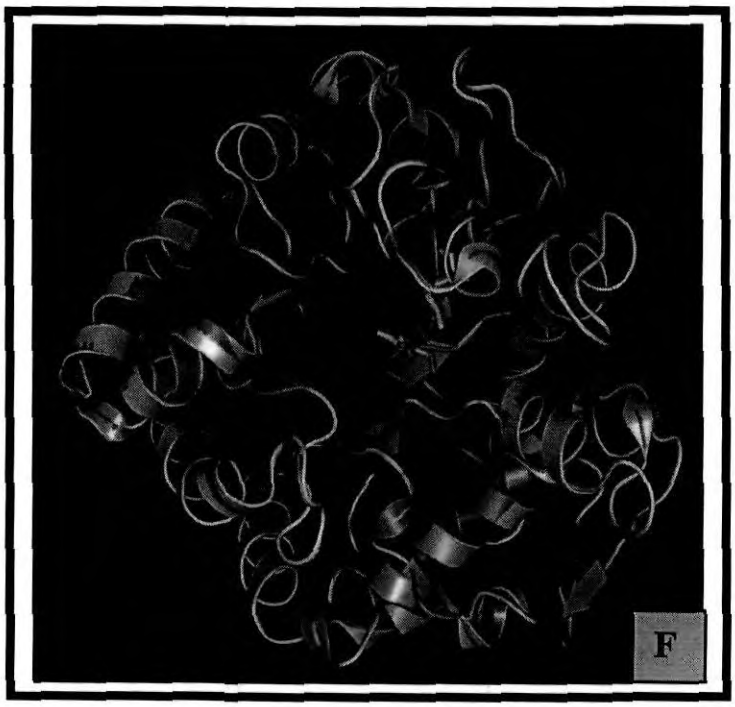
C. Superimposed 3D structures of β-1,3-glucanases of barley and *Hevea* β-1,3-glucanase showing alignment of the catalytic clefts



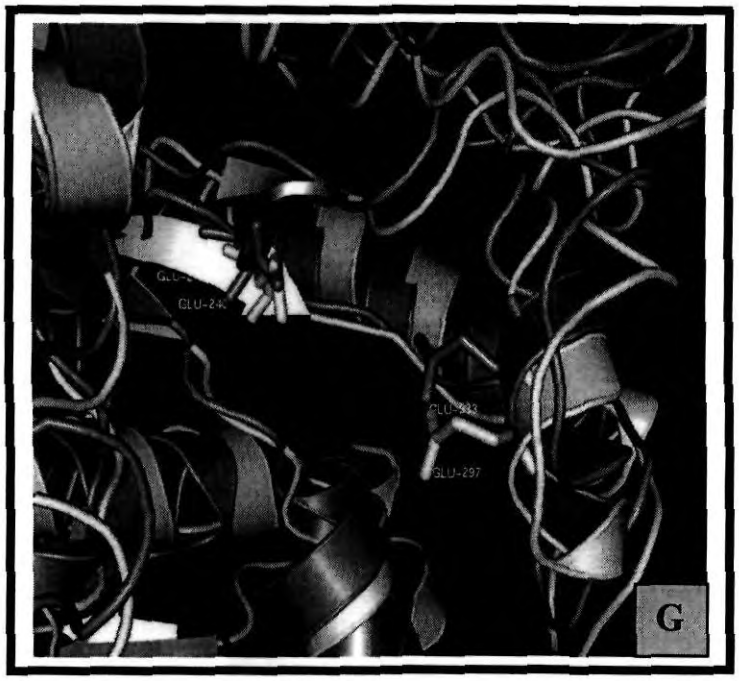
D. 3D Structure modelled for β-1,3-glucanase from *Hevea (AY325498).*
Catalytic cleft: 333E- 276E (it is including the 36 amino acid residues of the signal peptide also). If it is not taken into account the cleft lies between 240E (276- 36) and 297E (333- 36)



E. Superimposed structure of AY325498 and 3EM5 superimposed (*Hevea*) showing the alignment of the catalytic clefts of both the β-1,3-glucanases



F. Superimposed 3D images of Gln 2 (yellow) (obtained in the present study) and 3EM5 (orange)



G. Alignment of the catalytic clefts of Gln 2 (yellow) and 3EM5 (orange)

Like wise the superimposed modelled structures of the report from *Hevea* AY325498 (modelled using the modeller software) (fig. 72. D) and 3EM5 also showed similar results. Here also the catalytic clefts are aligning in par with respect to each other (fig. 72. D & E).

It was observed that among the newly isolated glucanases, Gln 2 is showing 100% amino acid sequence similarity with the reported amino acid sequence of 3EM5. The amino acid sequences residing within the catalytic clefts are also found to be similar in this case. The modeled structure for Gln 2 and its superimposed structure with 3EM5 is also shown (fig. 72 F & G).

Fig. 73. shows the amino acid sequence alignment of barley (PDB ID - 1GHS) & and *Hevea* (3EM5). Although differences exist in their alignment, the alignment of the catalytic cleft is identical in both. It is also seen that despite the amino acid sequence differences, the 3D structures of both barley and *Hevea* β-1,3-glucanases are similar. This similarity of the 3D structures of β-1,3-glucanases shows evolutionary structural conservation of β-1,3-glucanases within these widely related groups.

Fig.73. Clustal W alignment of Barley (1GHS) and *Hevea* (3EM5) β-1,3-glucanases

```
Barley      -IGVCYGVIGNNLPSRSDVVQLYRSKGINGMRIYFADGQALSALRNSGIGLILDIGNDQL 59
Hevea       XVGVCYGMQGNNLPPVSEVIALYKKSNITRMRIYDPNQAVLEALRGSNIELILGVPNSDL 60
            :*****: *****. *:*: **:...*. **** .:  .*.***.*.* ***.: *.:*

Barley      ANIAASTSNAASWVQNNVRPYYPAVNIKYIAAGNEVQGG------ATQSILPAMRNLNAA 113
Hevea       QSLTN-PSNAKSWVQKNVRGFWSSVRFRYIAVGNEISPVNRGTAWLAQFVLPAMRNIHDA 119
            .::  .*** ****:*** ::.:*.::***.***:.         :* :******:: *

Barley      LSAAGLG-AIKVSTSIRFDEVANSFPPSAGVFKN---AYMTDVARLLASTGAPLLANVYP 169
Hevea       IRSAGLQDQIKVSTAIDLTLVGNSYPPSAGAFRDDVRSYLNPIIRFLSSIRSPLLANIYP 179
            : :***   *****:* :  *.**:*****.*::   :*:. : *:*:*  :*****:**

Barley      YFAYRDNPGSISLNYATFQ-PGTTVRDQNNGLTYTSLFDAMVDAVYAALEKAGAPAVKVV 228
Hevea       YFTYAGNPRDISLPYALFTSPSVVVWDGQRG--YKNLFDATLDALYSALERASGGSLEVV 237
            **:* .** .*** ** *  *...* * :.*  *..**** :**:*:***:*.. :::**

Barley      VS SGWPSAGGFAASAGNARTYNQGLINHVGGGTPKK-REALETYIFAMFNENQKTGDAT 287
Hevea       VS SGWPSAGAFAATFDNGRTYLSNLIQHVKRGTPKRPKRAIETYLFAMFDENKKQ-PEV 296
            **********.***: .*.*** ..**:**  ****: :.*:***:****:**:*    .

Barley      RSFGLFNPDKSPAYNIQF- 306
Hevea       KHFGLFFPNKWQKYNLNFS 316
            *: **** *:*   **::*
```

Glutamic acid residues forming the catalytic sites

The ClustalW multiple sequence alignment of the different forms of β-1,3- glucanases with the other reports was done (fig. 74). In the alignment also it is very clear to observe the positional conservation of the catalytic cleft in both the new sequences isolated and also with the other earlier reports.

Fig. 74. Comparison of the amino acid sequence of different forms of β-1,3- glucanases with the earlier reports

**The red coloured regions indicate the antigenic epitopes. The blue colour amino acid residues residing within the red coloured regions show the variations in the conserved regions with each other. The highlighted green colour indicates the positional conservation of glutamic acid residues forming the catalytic cleft in the aligned peptide sequences*

```
Gln 6       MAISSSTSGTSSSLPSRTTVMLLLIFFTASLGITDAQVGVCYGMQGNNLPSVSEVIALYK 60
Gln 5       MAISSSTSGTSSSLPSRTTVMLLLIFFTASLGITDAQVGVCYGMQGNNLPSVSEVIALYK 60
Thanseem    MAISSSTSGTSSSLPSRTTVMLLLIFFTASLGITDAQVGVCYGMQGNNLPPVSEVIALYK 60
Sponer      MAISSSTSGTSSSLPSRTTVMLLLFFFAASVGITDAQVGVCYGMQGNNLPPVSEVIALYK 60
Yeang       ------------------------------------QVGVCYGMQGNNLPPVSEVIALYK 24  NNLPPVSEVIALYK24
Gln 2       MAICSSTSGTSSSLPSRTTVMLLLFFFTASVGITDAQVGVCYGMQGNNLPPVSEVIALYK 60
3EM5A       ------------------------------------QVGVCYGMQGNNLPPVSEVIALYK 24  NNLPPVSEVIALYK24
GLN 7       MAISSSTSGTSSSLPSRTTVMLLLIFFTASLGITDAQVGVCYGMQGNNLPPVSEVIALYK 60
Chye        MAISSSTSGTSSSFPSRTTVMLLLFFFAASVGITDAQVGVCYGMQGNNLPPVSEVIALYK 60
Gln 3       MAMLSSTSRTTGSLLSRTPVMLLLILYIASLGITDAQVGVCYGKLGNNLPPASEVIALYK 60
Gln 4       MAIFSSNSATSSSLPSRTTS-ILLVLFIASLSITDAQVGVCYGMLGNNLPPASQVISLYK 59
                                             *******  *****..*:**:***
```

```
Gln 6       QSNIKRMRIYDPNQAVLEALRGSNIELILGVPNSDLQSLTNPSNANSWVQKNVRGFWSSV 120
Gln 5       QSNIKRMRIYDPNRAVLEALRGSNIELILGVPNSDLQSLTNPSNANSWVQKNVRGFWSSV 120
Thanseem    QSNIKRMRIYDPNRAVLEALRGSNIELILGVPNSDLQSLTNPSNANSWVQKNVRGFWSSV 120
Sponer      KSNITRMRIYDPNRAVLEALRGSNIELILGVPNSDLQSLTNPSNAKSWVQKNVRGFWSSV 120
Yeang       KSNITRMRIYDPNRAVLEALRGSNIELILGVPNSDLQSLTNPSNAKSWVQKNVRGFWSSV 84
Gln 2       KSNITRMRIYDPNQAVLEALRGSNIELILGVPNSDLQSLTNPSNAKSWVQKNVRGFWSSV 120
3EM5A       KSNITRMRIYDPNQAVLEALRGSNIELILGVPNSDLQSLTNPSNAKSWVQKNVRGFWSSV 84
GLN 7       KSNITRMRIYDPNRAVLEALRGSNIELILGVPNSDLQSLTNPSNAKSWVQKNVRGFWSSV 120
Chye        KSNITRMRIYDPNRAVLEALRGSNIELILGVPNSDLQSLTNPSNAKSWVQKNVRGFWSSV 120
Gln 3       QSNIKRMRIYDPNQEVLQALRGSNIELILGVPNSDLQSLTNPSNANSWVQKNVRDFWSSV 120
Gln 4       QANIKRMRIYDPNQAALQALRGSNIQLMLGVPNSDLQSLTNPSNANSWIQRNVRAFWPSV 119
            ::**.********: .*:*******:*:*****************:**:*:*** **.**

Gln 6       RIRYIAVATKLVPVNGGTAWLAQFVLPAMRNIHDAIRSAGLQDQIKVSTAIDLTLVGNSY 180
Gln 5       RFRYIAVGNEISPVNGGTAWLAQFVLPAMRNIHDAIRSAGLQDKIKVSTAIDLTLVGNSY 180
Thanseem    LFRYIAVGNEISPVNGGTAWLAQFVLPAMRNIHDAIRSAGLQDQIKVSTAIDLTLVGNSY 180
Sponer      LFRYIAVGNEISPVNRGTAWLAQFVLPAMRNIHDAIRSAGLQDQIKVSTAIDLTLVGNSY 180
Yeang       LFRYIAVGNEISPVNRGTAWLAQFVLPAMRNIHDAIRSAGLQDQIKVSTAIDLTLVGNSY 144
Gln 2       RFRYIAVGNEISPVNRGTAWLAQFVLPAMRNIHDAIRSAGLQDQIKVSTAIDLTLVGNSY 180
3EM5A       RFRYIAVGNEISPVNRGTAWLAQFVLPAMRNIHDAIRSAGLQDQIKVSTAIDLTLVGNSY 144
GLN 7       RFRYIAVGNEISPVNRGTAWLAQFVLPAMRNIHDAIRSAGLQDQIKVSTAIDLTLVGNSY 180
Chye        LFRYIAVGNEISPVNRGTAWLAQFVLPAMRNIHDAIRSAGLQDQIKVSTAIDLTLVGNSY 180
Gln 3       RLRYIAVGNEISPVNGGTAWLAQFVLPAMRNIHDAIRSAGLQDQIKVSTAIDLTLMGNTY 180
Gln 4       RFRYIAVGNEISPVNGGTASLAKFVLPAMRNIYNAIRSAGLQDQIKVSTAIDMTLIGNSY 179
            : *****..:: *** *** **:*********::*********:********:**:**:*

Gln 6       PPSAGAFRDDVRSYLDPIIGFLSSIRSPLLANIYPYFTYADNPRDISLPYALFTSPSVVV 240
Gln 5       PPSAGAFRDDVRSYLDPIIGFLSSSRSPLLANIYPYFTYAYNPRDISLPYALFTSPSVVV 240
Thanseem    PPSAGAFRDDVRSYLDPIIGFLSSIRSPLLANIYPYFTYAGNPRDISLPYALFTSPSVVV 240
Sponer      PPSAGAFRDDVRSYLNPIIRFLSSIRSPLLANIYPYFTYAGNPRDISLPYALFTSPSVVV 240
Yeang       PPSAGAFRDDVRSYLNPIIRFLSSIRSPLLANIYPYFTYAGNPRDISLPYALFTSPSVVV 204
Gln 2       PPSAGAFRDDVRSYLNPIIRFLSSIRSPLLANIYPYFTYAGNPRDISLPYALFTSPSVVV 240
3EM5A       PPSAGAFRDDVRSYLNPIIRFLSSIRSPLLANIYPYFTYAGNPRDISLPYALFTSPSVVV 204
GLN 7       PPSAGAFRDDVRSYLNPIIRFLSSIRSPLLANIYPYFTYAGNPRDISLPYALFTSPFVVV 240
Chye        PPSAGAFRDDVRSYLDPIIGFLSSIRSPLLANIYPYFTYAYNPRDISLPYALFTSPSVVV 240
Gln 3       PPSAGAFRDDVRSYLDPIIGFLSSIRSPLLANIYPYFTYAGNPRDISLPYALFTSPSVVV 240
Gln 4       PPSAGAFRGDVRSYLDPIIGFLSSIRSPLLANIYPYFSYAGNPRDISLPYALFTSPSIVV 239
            ********.******:*** **** ************:** *************** :**

Gln 6       WDGQRGYKNLFDATLDALYSALERASGGSLEVVVSPSGWPSAGAFAATFDNGRTYLSNLI 300
Gln 5       WDGQRGYKNLFDATLDALYSALERASGGSLEVVVSPSGWPSAGAFAATFDNGRTYLSNLI 300
Thanseem    WDGQRGYKNLFDATLDALYSALERASGGSLEVVVSPSGWPSAGAFAATFDNGRTYLSNLI 300
Sponer      WDGQRGYKNLFDATLDVLYSALERASGGSLEVVVSPSGWPSAGAFAATFDNGRTYLSNLI 300
Yeang       WDGQRGYKNLFDATLDVLYSALERASGGSLEVVVSPSGWPSAGAFAATFDNGRTYLSNLI 264
Gln 2       WDGQRGYKNLFDATLDALYSALERASGGSLEVVVSPSGWPSAGAFAATFDNGRTYLSNLI 300
3EM5A       WDGQRGYKNLFDATLDALYSALERASGGSLEVVVSPSGWPSAGAFAATFDNGRTYLSNLI 264
GLN 7       WDGLRGYKNLFDATLDALYSALERASGGSLEVVVLPSGWPSAGAFAATFDNGRTYLSNLI 300
Chye        WDGQRGYKNLFDATLDALYSALERASGGSLEVVVSPSGWPSAGAFAATFDNGRTYLSNLI 300
Gln 3       WDGQRGYKNLFDATLDALYSALERASGGSLEVVVSPSGWPSAGAFAATFDNGRTYLSNLI 300
Gln 4       WDGQRGYKNLFDAMLDALYSALERAGGGSLEVVVSPSGWPSAGAFAATFDNGRTYLSNLI 299
            *** ********* **.********.******** ************************

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Gln 5       QHVKGGTPKRPNRAIETYLFAMFDENKKQPEVEKHFGLFFPDKRPKYNLNFGAEKNWDIS 360
Thanseem    QHVKGGTPKRPNRAIETYLFAMFDENKKQPEVEKHFGLFFPDKRPKYNLNFGAEKNWDIS 360
Sponer      QHVKRGTPKRPNRAIETYLFAMFDENKKQPEVEKQFGLFFPDKWQKYNLNFG-------- 352
Yeang       QHVKRGTPKRPNRAIETYLFAMFDENKKQPEVEKQFGLFFPDKWQKYNLNFG-------- 316
Gln 2       QHVKRGTPKRPKRAIETYLFAMFDENKKQPEVEKHFGLFFPNKWQKYNLNFSAEKNWDIS 360
3EM5A       QHVKRGTPKRPKRAIETYLFAMFDENKKQPEVEKHFGLFFPNKWQKYNLNFS-------- 316
GLN 7       QHVKRGTPKRPNRAIETYLFAMFDENKKQPEVEKHFGLFFPGKRQKYNLNFGAEKNWNIS 360
Chye        QHVKGGTPKRPNRAIETYLFAMFDENKKQPEVEKHFGLFFPDKRPKYNLNFGAEKNWDIS 360
Gln 3       QHVKGGTPKRPDRAIETYLFAMFDENQKQPEVEKHFGLFFPDKRPKYNLNFSAKKNWDIS 360
Gln 4       QHVKGGTPKRPGRFIETYLFAMFDENQKQPEFEKHFGLFFPNKQQKYNLNFGAKKNWDIS 359
            **** ****** * ************:****.**: *****.*  ******.
```

```
Gln 6       TEHDATILFLKSDM 374
Gln 5       TEHNATILFLKSDM 374
Thanseem    TEHNATILFLKSDM 374
Sponer      --------------
Yeang       --------------
Gln 2       TEHNATILFLKSDM 374
3EM5A       --------------
GLN 7       TEHNATILFLKSDM 374
Chye        TEHNATILFLKSDM 374
Gln 3       TEHNATVLFLKSDM 374
Gln 4       ADYNATVLLLKSDI 373
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Sequences (4:7)  Aligned. Score:  94
Sequences (4:8)  Aligned. Score:  96
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Sequences (4:10) Aligned. Score:  97
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Sequences (7:9)  Aligned. Score:  94
Sequences (7:10) Aligned. Score:  94
Sequences (7:11) Aligned. Score:  94
Sequences (8:9)  Aligned. Score:  97
Sequences (8:10) Aligned. Score:  97
Sequences (8:11) Aligned. Score:  96
Sequences (9:10) Aligned. Score: 100
Sequences (9:11) Aligned. Score:  97
Sequences (10:11) Aligned. Score: 97
```

```
Sequence 1: 913          374 aa
Sequence 2: 198          374 aa
Sequence 3: 582          373 aa
Sequence 4: 553          374 aa
Sequence 5: Thanseem     374 aa
Sequence 6: FX           374 aa
Sequence 7: 847          374 aa
Sequence 8: Chye         374 aa
Sequence 9: Sponer      352 aa
Sequence 10: Yeang       316 aa
Sequence 11: 3EM5A       316 aa
```

Chye and Cheung (1996) - U2214
Sponer *et al.* (2005) - AJ1334
Yeang and Chow (2000) - AF311
Thanseem and Thulaseedharan (2003) - AY325

Reported sequences of β-1,3- glucanase from *Hevea* i NCBI with their accession no's.

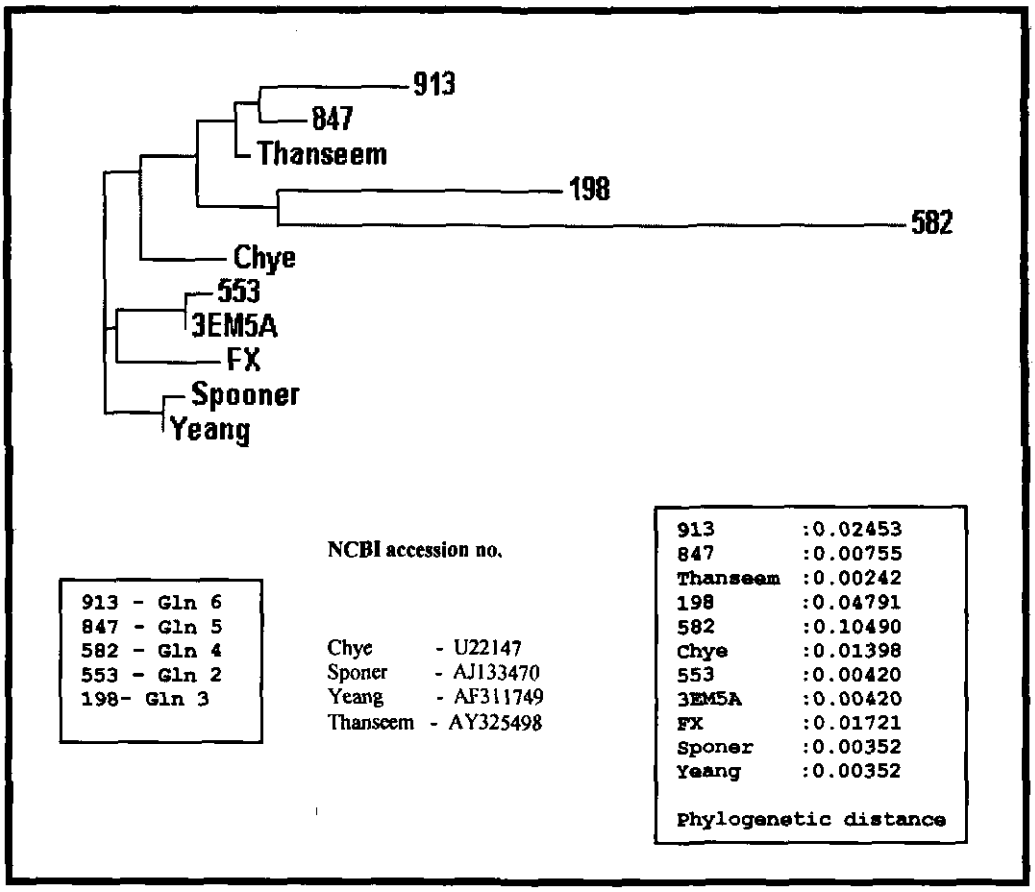
The amino acid sequence alignment of all the six different forms of β-1,3- glucanase with the other reports from *Hevea* (the details of the reported

sequences is shown in the box above) show that though a major portion of the sequence is conserved, it is also showing differences in amino acid residues at certain regions, which may finally contributes to the variation in the protein functions. However as described earlier, the tertiary structures of these variants of β-1,3-glucanase will be the same. From the comparison it is understood that the reported sequence by Sponer *et al.* (2005) and Yeang & Chow (2000) is highly similar with each other (100 %), Gln 2 is exactly similar to 3EM5 sequence-chain A sequence of *Hevea* β-1,3-glucanase. The Gln 6 is showing the maximum alignment score with the report by Thanseem (AY325498) (2003) and Gln 5 like that of its nucleotide similarity. Gln 5 is showing high similarity with the report – AY325498 (98 score) followed by a score of 96 with Gln 6 and the report U22147 (Chye and Cheung, 1996). Gln 4 is showing the maximum alignment score of 87 with the reports U22147 and AJ133410 (Sponer *et al.*, 2005) from *Hevea*. With the other isolated forms, the alignment score exists within a range of 84-86. The Gln 3 sequence which harboured the 198 bp promoter shows a maximum alignment score of 93 with the report (AY325498). Gln 2 shows 100% alignment with the 3EM5A sequence of *Hevea* β-1,3-glucanase. It then shows an alignment score of 97 with the reports U22147 and AJ133410 from *Hevea*; with all the other isolated forms the score is less than 97. The Gln 7 sequence isolated from the *Hevea* - FX 516 is highly similar with a score of 97 with that reported by Sponer and 3EM5A and a score of 96 with Gln2. The 57 amino acid residues occupying the catalytic cleft is highly conserved except for very minor (2-3 aa') sequence variations with each other. The catalytic clefts of Gln 2 and 3EM5 is 100 % similar like that of the alignment shown by the reported sequence of β-1,3-glucanase by Sponer *et al.*, (2005), with the sequence reported by Yeang and Chow (2000). The amino acid sequences in the catalytic clefts are also similar in Gln 5 and Gln 6.

The phylogenetic tree shows the pattern of clustering of the earlier reported sequences and the peptide sequences of the isolated genomic forms of β-1,3- glucanases in the present study (fig. 75). From this it is clear that sequence reported by Sponer and Yeang is highly similar with the same phylogenetic distance (0. 00352). Likewise Gln 2 and 3EM5A also shares the

same phylogenetic distance. The maximum distance of 0.10490 is kept by Gln 4 which is also proved by the nucleotide and amino acid sequence with respect to other forms reported in the present study and earlier reports from *Hevea.*

Fig. 75. Phylogenetic analysis of the amino acid sequence of the six forms of glucanase identified with the other reports.



The individual variation of the amino acid residues shown by the predicted peptide sequence of the isolated glucanases is shown in table. 9

Table.9. shows that Gln 4 is showing the maximum variation of 45 amino acids with the earlier report (Thanseem and Thulaseedharan (2003) - NCBI Acc. No. AY325498) followed by Gln 3 with 20 amino acids. Gln 5 shows the minimum number of variations (3 aa'). Gln 7 is showing 11 amino acid variations while Gln 6 is showing 8 amino acid variations with the report. Gln 2 shows 12 amino acid variations with the earlier report.

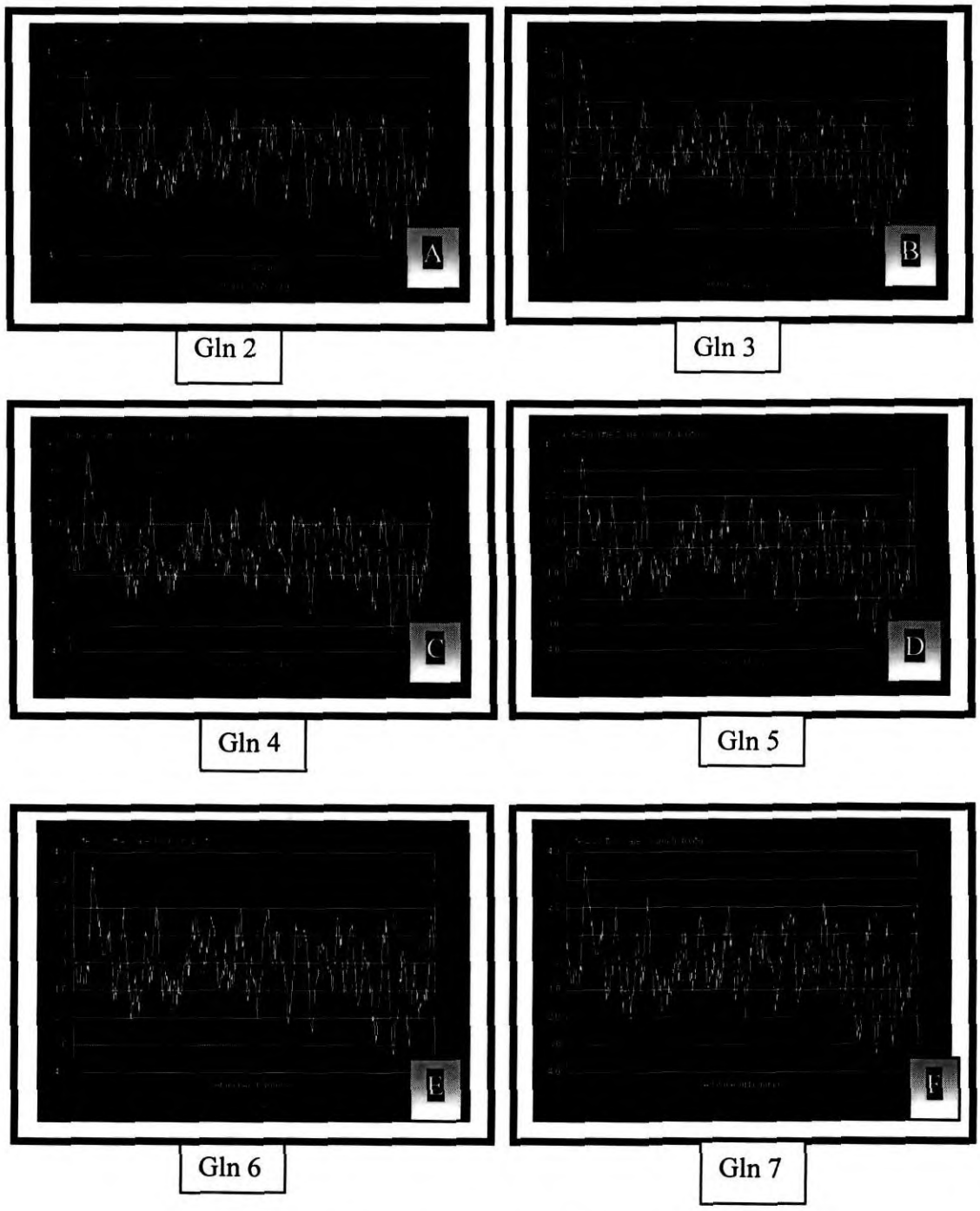
Table.9. Details of the number of amino acids showing variations in different forms of glucanase with the earlier reported sequence

Sl No:	Forms	Amino acid variations with the earlier report
1	RRII Gln 2	12 amino acids
2	RRII Gln 3	20 amino acids
3	RRII Gln 4	45 amino acids
4	RRII Gln 5	3 amino acids
5	RRII Gln 6	8 amino acids
6	RRII Gln 7	11 amino acids

4.5.2. Hydropathy plots

Hydropathy plots depicts the hydrophobicity/ hydrophilicity of the concerned protein. Kyte-Doolittle is a widely applied scale for delineating hydrophobic character of a protein. Regions with values above 0 are hydrophobic in character. Hopp-Woods scale predicts potentially antigenic regions of polypeptides. Hopp-Woods scale values greater than 0 are hydrophilic and thus likely to be exposed on the surface of a folded protein. The plot has amino acid sequence of a protein on its x-axis, and degree of hydrophobicity and hydrophilicity on its y-axis. The hydropathy plot of all the six glucanases is shown in fig. 76 (A-F).

Fig.76. The Hydropathy plots for all the six forms of glucanase A-Gln 2, B- Gln3, C- Gln 4, D- Gln 5, E-Gln 6, F- Gln 7.



**Yellow colour indicates the Kyte - Doolittle Hydropathy plot for hydrophobicity while the magenta colour indicates the Hopp-woods scale for hydrophilicity.*

The hydropathy plot for all the six forms show very high potential hydrophophobic amino acid residues residing at the N-terminal end of the

protein which occupies the transmembrane region. In this, it is residing within the signal peptide region. There are also less potential sites of hydrophobicity in the peptide. The Hopp-Wood scale shows the potential antigenic sites. There are several peaks of potential hydrophilicity in all the forms. It is observed that while the maximum region of hydrophobicity resides at the N-terminal region, the maximum region of hydrophilicity occur towards the C-terminal end.

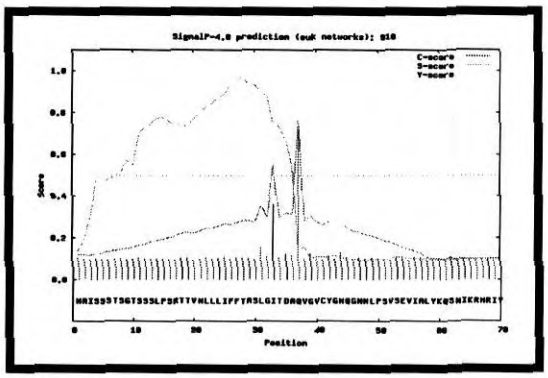
The Kyte-Doolittle hydrophobicity plot shows that Gln 4 is showing the maximum hydrophobicity (the peak is very close to 4.0) at the N-terminal end while others were not showing this high peak. The least level of hydrophobicity observed was with the Gln 2 (those with the 553 bp promoter sequence). Though hydrophilic peaks are present in all the six peptides, they are strongly hydrophobic.

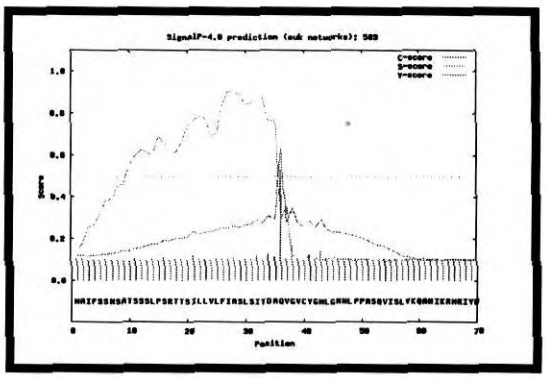
4.5.3. Signal Peptide analysis

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 (Jannick *et al.*, 2004). The method Signal P 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 signal P prediction for the presence of signal peptide in all the six forms except in Gln 4, of glucanase showed positive with a signal peptidase cleavage site between the 36^{th} and 37^{th} amino acid (between alanine and glutamine) as shown with Gln 6 (fig: 77). In the graph, it is seen that at the

particular site the potential crosses the threshold line (dotted line) showing the maximum potential threshold for signal peptide cleavage.





```
Fig : Signal P (version: 4) results for Gln 6
Measure   Position  Value    Cutoff   signal
peptide?
  max. C    37       0.7613
  max. Y    37       0.7662
  max. S    28       0.966
  mean S     1-36    0.695
       D     1-36    0.7626   0.450    YES
Name=913  SP='YES' Cleavage site between pos.
36 and 37
```

A

Fig : Signal P (version: 4) results for Gln 4

```
Measure   Position  Value    Cutoff   signal
peptide?

  max. C    36       0.523
  max. Y    36       0.631
  max. S    27       0.905
  mean S     1-35    0.628
       D     1-35    0.630    0.450    YES
Name=583 SP='YES' Cleavage site between
pos. 35 and 36:
```

B

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

Fig 77. Signal P prediction for Gln 6(A) and Gln 4(B)

In all forms the C-score is found to be around 0.76. However in Gln 4 the cleavage site is found in between the 35th and 36th amino acid. This upward shift is caused due to the deletion of the amino acid at the 21st position in the peptide sequence. The C-score is 0.523, while all the others showed approximately a 0.76 value. These basic β-1,3-glucanases are often vacuolar and can be excreted through the secretory pathway.

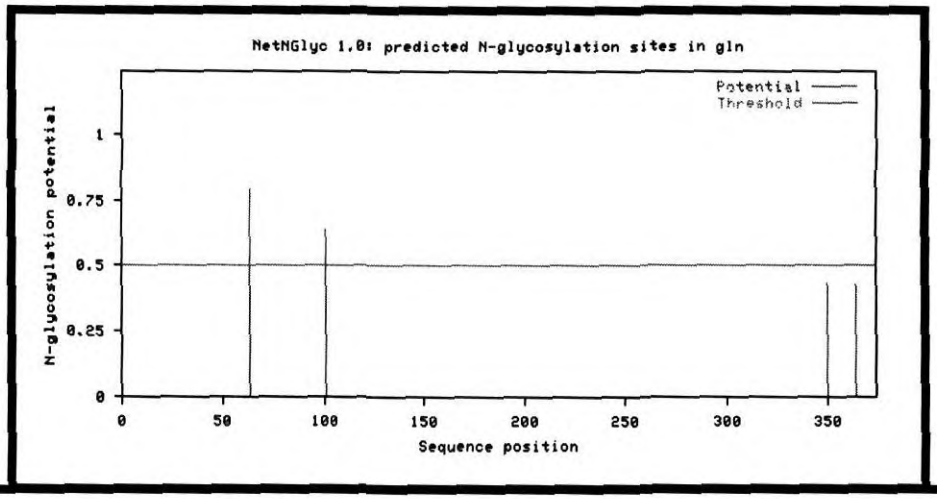
4.5.4. NetNGlyc prediction

It is being identified that some proteins do not fold correctly unless they are glycosylated first. Experiments have also shown that in some cases, glycosylation is not a strict requirement for proper folding, but the un-glycosylated protein degrades quickly. β-1,3-glucanases are also glycosylated proteins. Here an attempt has been made to compare the glycosylation potential of the six different forms of glucanase with the NetNGlyc online software (fig. 78. A- F).

The NetNGlcosylation prediction of Gln 2 showed the presence of four Asn-Xaa-Ser/Thr sequons in the sequence at 63rd (NITR), 101th (NPSN), 350th (NFSA) and 364th (NATI) positions (fig. 78. A). But the potential glycosylation site identified was with the 63rd and 101st asparagine. However the 101st asparagine is immediately followed by a proline (NPSN) which might mask asparagine residue and make it inaccessible to glycosylation. As the point crosses the mentioned threshold 0.5, this region is also predicted to be glycosylated by the NetNglyc server.

Fig. 78. A. NetNGlyc prediction for the potential glycosylation sites in Gln 2

Asn-Xaa-Ser/Thr sequons in the sequence output below are highlighted in blue.Asparagines predicted to be N-glycosylated are highlighted in red.



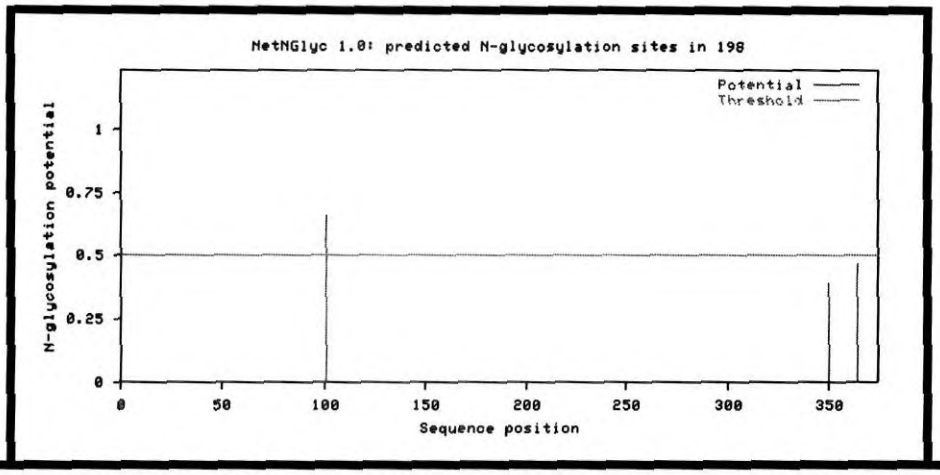
A

```
Name:  Gln 2       Length:  374
MAICSSTSGTSSSLPSRTTVMLLLFFFTASVGITDAQVGVCYGMQGNNLPPVSEVIALYKKSNITRMRIYDPNQAVLEAL      80
RGSNIELILGVPNSDLQSLTNPSNAKSWVQKNVRGFWSSVRFRYIAVGNEISPVNRGTAWLAQFVLPAMRNIHDAIRSAG     160
LQDQIKVSTAIDLTLVGNSYPPSAGAFRDDVRSYLNPIIRFLSSIRSPLLANIYPYFTYAGNPRDISLPYALFTSPSVVV     240
WDGQRGYKNLFDATLDALYSALERASGGSLEVVVSESGWPSAGAFAATFDNGRTYLSNLIQHVKRGTPKRPKRAIETYLF     320
AMFDENKKQPEVEKHFGLFFPNKWQKYNLNFSAEKNWDISTEHNATILFLKSDM
.....................................................................N.....      80
.....................N.....................................................      160
.....................................................................N.....      240
.....................................................................N.....      320
.....................................................                          400

(Threshold=0.5)
-----------------------------------------------------------------------
SeqName      Position  Potential    Jury    N-Glyc
                                    agreement result
-----------------------------------------------------------------------
Gln            63 NITR   0.76962     (9/9)   +++
Gln           101 NPSN   0.6412      (9/9)   ++   WARNING: PRO-X1.
Gln           350 NFSA   0.4337      (7/9)   -
Gln           364 NATI   0.4313      (7/9)   -
```

The NetNGlcosylation prediction of Gln 3 showed the presence of three Asn-Xaa-Ser/Thr sequons in the sequence at 101st (NPS), 350th (NFSA) and 364th (NATV) positions (fig. 78.B). But the potential glycosylation site identified was with 101st asparagine. Here the sequence is NPS that is, Asn-Pro-Ser. As stated earlier here also the proline can mask asparagine and make it inaccessible to glycosylation. However the point crossing the threshold 0.5, is predicted to be glycosylated by the NetNGlyc server. From what we know so far, only asparagines within Asn-Xaa-Ser/Thr (and in some cases, Asn-Xaa-Cys) are N-glycosylated *in vivo*.

Fig.78.B. NetNGlyc prediction for the potential glycosylation sites in Gln 3



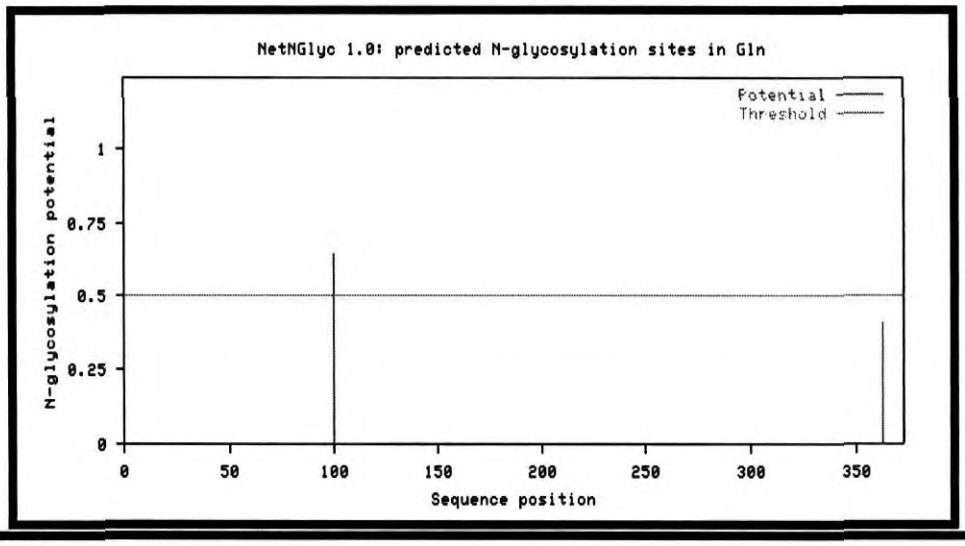
B

```
MAMLSSTSRTTGSLLSRTPVMLLLILYIASLGITDAQVGVCYGKLGNNLPPASEVIALYKQSNIKRMRIYDPNQEVLQAL     80
RGSNIELILGVPNSDLQSLTNPSNANSWVQKNVRDFWSSVRLRYIAVGNEISPVNGGTAWLAQFVLPAMRNIHDAIRSAG    160
LQDQIKVSTAIDLTLMGNTYPPSAGAFRDDVRSYLDPIIGFLSSIRSPLLANIYPYFTYAGNPRDISLPYALFTSPSVVV    240
WDGQRGYKNLFDATLDALYSALERASGGSLEVVVSESGWPSAGAFAATFDNGRTYLSNLIQHVKGGTPKRPDRAIETYLF    320
AMFDENQKQPEVEKHFGLFFPDKRPKYNLNFSAKKNWDISTEHNATVLFLKSDM
........................................................................................     80
.....................N..................................................................     160
........................................................................................     240
........................................................................................     320
..............................................                                          400

(Threshold=0.5)
--------------------------------------------------------------------------------
SeqName      Position  Potential    Jury    N-Glyc
                                            agreement result
--------------------------------------------------------------------------------
Gln          101 NPSN   0.6587      (9/9)   ++   WARNING: PRO-X1.
Gln          350 NFSA   0.3936      (9/9)   --
Gln          364 NATV   0.4701      (6/9)   -
--------------------------------------------------------------------------------
```

In Gln 4, the potential glycosylation sites are at the 100th asparagine (NPSN), and 363rd asparagine (NATV) (fig. 78. C). However the asparagine residue identified at the 100th position is immediately followed by a proline which can prevent the glycosylation to some extend *in vivo*. Eventhough the asparagine present at the 363rd position tends to be glycosylated, it is showing only a weak glycosylation potential in Gln 4. The figure shows the single potential glycosylation site at the 100th asparagine which crossed the threshold 0.5.

Fig. 78. C. NetNGlyc prediction for the potential glycosylation sites in Gln 4



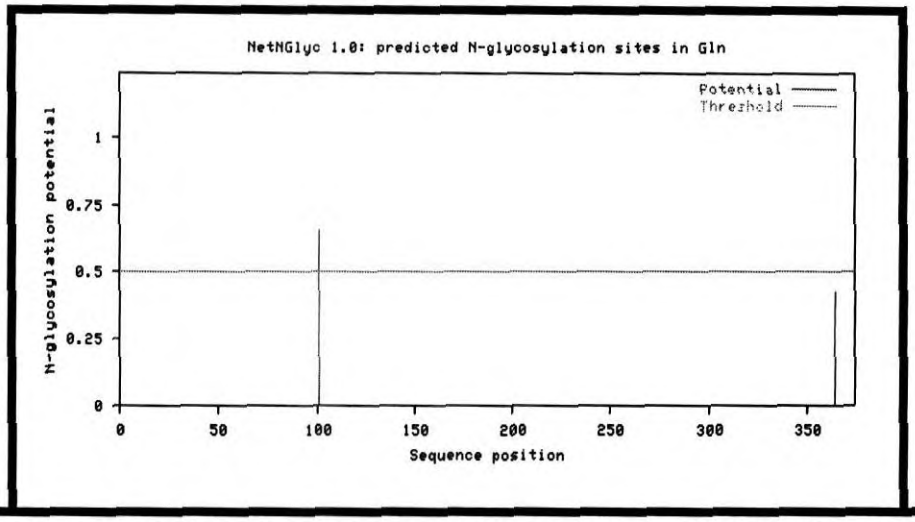
C

```
Name:  Gln          Length:  372
MAIFSSNSATSSSLPSRTTSILLVLFIASLSITDAQVGVCYGMLGNNLPPASQVISLYKQANIKRMRIYDPNQAALQALR      80
GSNIQLMLGVPNSDLQSLTNPSNANSWIQRNVRAFWPSVRFRYIAVGNEISPVNGGTASLAKFVLPAMRNIYNAIRSAGL     160
QDQIKVSTAIDMTLIGNSYPPSAGAFRGDVRSYLDPIIGFLSSIRSPLLANIYPYFSYAGNPRDISLPYALFTSPSIVVW     240
DGQRGYKNLFDAMLDALYSALERAGGGSLEVVVSESGWPSAGAFAATFDNGRTYLSNLIQHVKGGTPKRPGRFIETYLFA     320
MFDENQKQPEFEKHFGLFFPNKQQKYNLNFGAKKNWDISADYNATVLLLKSD
.....                                                                              80
.....N.....                                                                       160
.....                                                                             240
.....                                                                             320
.....
400

(Threshold=0.5)
-------------------------------------------------------------------------
SeqName      Position  Potential    Jury    N-Glyc
                                    agreement result
-------------------------------------------------------------------------
Gln          100 NPSN   0.6453     (9/9)   ++   WARNING: PRO-X1.
Gln          363 NATV   0.4072     (8/9)   -
-------------------------------------------------------------------------
```

In Gln 5 two potential sites can be observed at the 101st and the 364th asparagines residue. Here also the 101st asparagine is immediately followed by proline which may reduce the glycosylation potentiality of Gln 5 (fig. 78. D).

Fig. 78. D. NetNGlyc prediction for the potential glycosylation sites in Gln 5



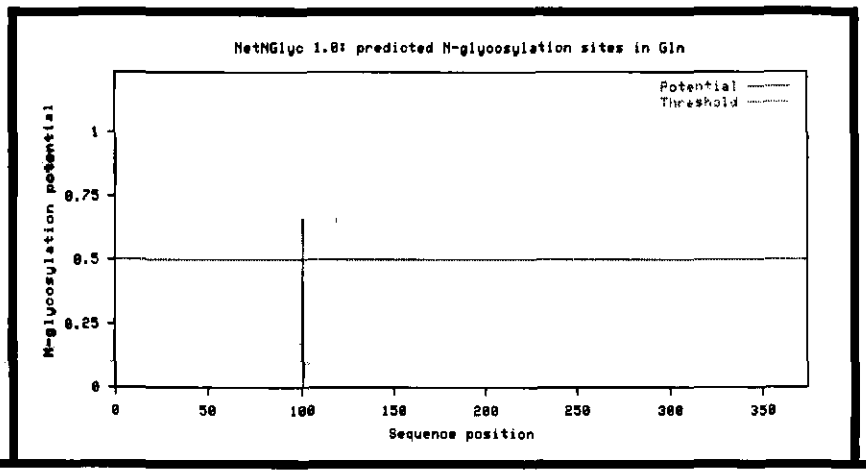
D

```
MAISSSTSGTSSSLPSRTTVMLLLIFFTASLGITDAQVGVCYGMQGNNLPSVSEVIALYKQSNIKRMRIYDPNRAVLEAL     80
RGSNIELILGVPNSDLQSLTNPSNANSWVQKNVRGFWSSVRFRYIAVGNEISPVNGGTAWLAQFVLPAMRNIHDAIRSAG    160
LQDKIKVSTAIDLTLVGNSYPPSAGAFRDDVRSYLDPIIGFLSSSRSPLLANIYPYFTYAYNPRDISLPYALFTSPSVVV    240
WDGQRGYKNLFDATLDALYSALERASGGSLEVVVSESGWPSAGAFAATFDNGRTYLSNLIQHVKGGTPKRPNRAIETYLF    320
AMFDENKKQPEVEKHFGLFFPDKRPKYNLNFGAEKNWDISTEHNATILFLKSDM
........................................................................................  80
.....................N..................................................................  160
........................................................................................  240
........................................................................................  320
.....................................................                                  400

(Threshold=0.5)
-------------------------------------------------------------------------
SeqName      Position  Potential   Jury    N-Glyc
                                          agreement result
-------------------------------------------------------------------------
Sequence     101 NPSN  0.6586      (9/9)   ++   WARNING: PRO-X1.
Sequence     364 NATI  0.4314      (7/9)   -
-------------------------------------------------------------------------
```

The NetNglycosylation prediction of the Gln 6 amino acid sequence shows the presence of a potential N-glycosylation site at the 101st position with the asparagine residue (fig. 78. E). But the 101st asparagine is immediately followed by a proline (NPSN) which can sometimes mask asparagine residue and make it inaccessible to glycosylation.

Fig: 78. E. NetNGlyc prediction for the potential glycosylation sites in Gln 6



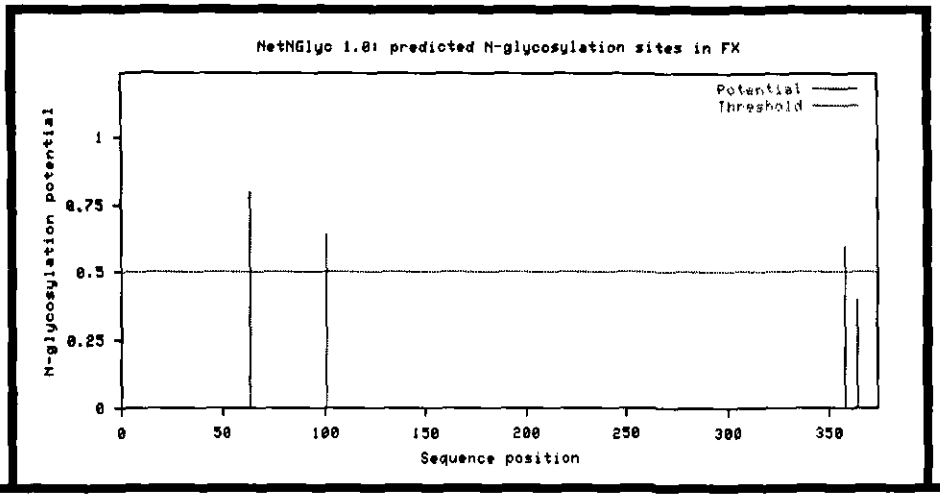
E

```
Output for 'Gln'
Name:  Gln          Length:  374
MAISSSTSGTSSSLPSRTTVMLLLIFFTASLGITDAQVGVCYGMQGNNLPSVSEVIALYKQSNIKRMRIYDPNQAVLEAL      80
RGSNIELILGVPNSDLQSLTNPSNANSWVQKNVRGFWSSVRIRYIAVATKLVPVNGGTAWLAQFVLPAMRNIHDAIRSAG     160
LQDQIKVSTAIDLTLVGNSYPPSAGAFRDDVRSYLDPIIGFLSSIRSPLLANIYPYFTYADNPRDISLPYALFTSPSVVV     240
WDGQRGYKNLFDATLDALYSALERASGGSLEVVVSESGWPSAGAFAATFDNGRTYLSNLIQHVKGGTPKRPNRAIETYLF     320
AMFDENKKQPEVEKHSGLFFPDKRPKYNLNFGAEKNWDISTEHDATILFLKSDM
.............................................................................           80
.....................N.....................................................           160
.............................................................................           240
.............................................................................           320
.....................................................                                   400

(Threshold=0.5)
-------------------------------------------------------------------------
SeqName      Position  Potential    Jury    N-Glyc
                                    agreement result
-------------------------------------------------------------------------
Gln          101 NPSN   0.6588      (9/9)    ++    WARNING: PRO-X1.
-------------------------------------------------------------------------
```

Among all the six forms of glucanase obtained from *Hevea*, Gln 7 is the only form having three glycosylation sites (fig. 78. F). The potential glycosylation sites are at the 63rd asparagine (NITR), 101st asparagine (NPSN), 358th asparagines (NIST) and 364th asparagines (NATI). At all these sites the threshold crossed the 0.5 value. However the asparagine residue identified at the 101st position is immediately followed by a proline which can prevent the glycosylation to some extend *in vivo*.

Fig. 78. F. NetNGlyc prediction for Gln 7



F

```
Name:  Sequence     Length:  374
MAISSSTSGTSSSLPSRTTVMLLLIFFTASLGITDAQVGVCYGMQGNNLPPVSEVIALYKKSNITRMRIYDPNRAVLEAL      80
RGSNIELILGVPNSDLQSLTNPSNAKSWVQKNVRGFWSSVRFRYIAVGNEISPVNRGTAWLAQFVLPAMRNIHDAIRSAG     160
LQDQIKVSTAIDLTLVGNSYPPSAGAFRDDVRSYLNPIIRFLSSIRSPLLANIYPYFTYAGNPRDISLPYALFTSPFVVV     240
WDGLRGYKNLFDATLDALYSALERASGGSLEVVVLESGWPSAGAFAATFDNGRTYLSNLIQHVKRGTPKRPNRAIETYLF     320
AMFDENKKQPEVEKHFGLFFPGKRQKYNLNFGAEKNWNISTEHNATILFLKSDM
.............................................................N.................      80
.....................N.....................................................N.....      160
.....................N.....................................................N.....      240
.....................N.....................................................N.....      320
.....................N.....................................................N.....      400

(Threshold=0.5)
-------------------------------------------------------------------------------
SeqName      Position  Potential Jury    N-Glyc
                                        agreement result
-------------------------------------------------------------------------------
Sequence       63 NITR   0.76961   (9/9)   +++
Sequence      101 NPSN   0.6410    (9/9)   ++   WARNING: PRO-X1.
Sequence      358 NIST   0.5964    (8/9)   +
Sequence      364 NATI   0.3999    (7/9)   -
-------------------------------------------------------------------------------
```

The prediction of C-terminal glycosylation (Julenius, 2007) through NetCGlyc server showed no probability of anchorage of these proteins in the memberane. Therefore it is assumed that these proteins may be secreted into the surrounding medium without anchorage.

4.5.5. Isochore patterns

The newly obtained β-1,3-glucanases in the present study showed major variations in their promoter sequences as well as in their intron lengths. Minor variations were observed in the exon portion. Here an attempt had been made to understand the structural variations caused by these minor variations in the exon and that of the introns to the DNA architecture for each of these different forms.

The isochore plots GC content in windows over a DNA sequence. Though isochore patterns are usually implied upon long stretches of DNA, sometimes small segments of DNA also give interesting results. Isochores have been associated to a plethora of genetic and genomic features like gene/intron lengths, gene and retrotransposon densities, staining of chromosome bands, methylation patterns, or tissue specificity of gene products (Bernardi, 2004). The isochore patterns of all the six new glucanases from *Hevea* showed more or less similar pattern of organization. Hence for a general idea, only the patterns generated with only 2-3 forms are given here. Fig: 79 shows the isochore pattern generated with Gln 4, Gln 5, Gln 6 and the report (Acc. No. AY325498). Fig.79. A. shows the isochore plot showing the variations in Gln 6 in the gene portion (i) and the enlarged intron region (ii) which can be due to the variations in the GC content in the prescribed regions.

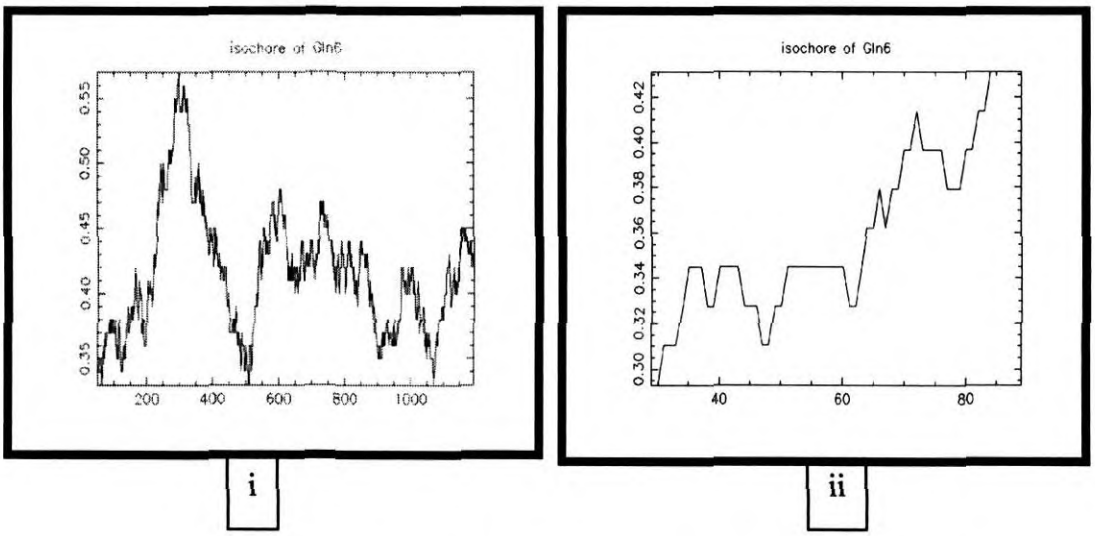


Fig. 79. A. The isochore pattern generated with the gene portion (i) and with the intron portion alone (ii) of Gln 6 (which is having the 913 bp promoter)

The fig. 79. A. (i) shows that the graph is at its peak at the region between 200 and 500 bps with the gene. The region can offer good folding for the nucleosome packaging and chromatin structuring thereby can play a role in the gene transcription events. Whereas fig.79.A. (ii) shows very weak isochore potential with the intron which is going to a high peak as the intron–exon splice junction arrives.

The isochore pattern of Gln 5 gene sequence (fig. 79. B. (i)) is exactly similar with the reported sequence of β-1,3-glucanase-AY325498 (fig.79. B.(ii)). This shows the similarity of the genomic sequences.

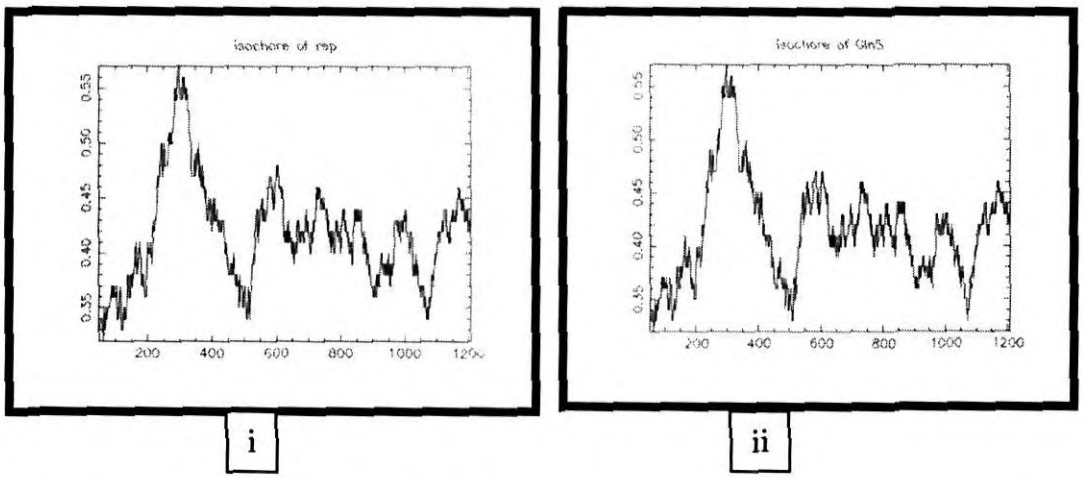


Fig 79. B. The isochore pattern of the genomic DNA sequence of Gln 5 (i) and the report - AY325498 (ii). The patterns are highly similar with respect to each other.

The rest of the different forms of glucanses also show similar isochore patterns with not much variation in the DNA architecture. All shows a potential nucleosome potential region before the intron region. Within the intron it shows a very weak potential for the nucleosome binding which suggests that the introns of the *Hevea* glucanases might not participate in the nucleosome formation. This can be apart of the regulatory mechanism for the gene expression. The Gln 4 which showed the maximum variations also showed similar architecture like the other forms (fig: 79.C).

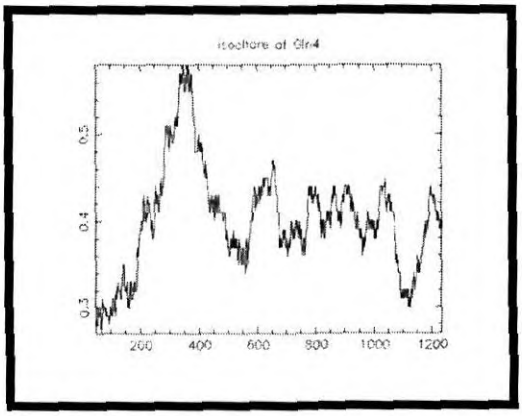


Fig. 79. C. The isochore pattern of Gln 4

4.5.6. Determination of antigenic sites

The antigenic sites of all the β-1,3-glucanase forms were determined using the EMBOSS- antigenic software (http://150.185.138.86/cgi-bin/emboss/antigenic) showing the antigenic domains with respect to the position and the number of amino acids. The table.10 shows the details of the antigenic epitope obtained using the EMBOSS – antigenic software.

Table.10. Comparison of the antigenic epitope sites in all the forms of *Hevea* β-1,3-glucanases (new 6 forms and one earlier report)

Sl. No.	Gln-7	Gln-6	Gln-5	Gln-4	Gln-3	Gln-2	Gln-1 (Earlier Rep)
1	11-17	11-17	11-17	11-17	**12-44**	11-17	11-17
2	19-43	19-43	19-43	19-43	--	19-43	19-43
3	49-61	49-61	49-61	49-61	**50-61**	49-61	49-61
4	73-80	**72-80**	73-80	**71-79**	73-80	**72-80**	73-80
5	85-94	85-94	85-94	**85-93**	85-94	85-94	85-94
6	116-134	**116-135**	116-134	**114-133**	116-134	116-134	116-134
7	140-149	140-149	140-149	**138-148**	140-149	140-149	140-149
8	161-179	161-179	161-179	**160-169**	**161-174**	161-179	161-179
9	191-219	191-219	**191-221**	**190-219**	191-219	191-219	191-219
10	**226-243**	226-242	226-242	**225-241**	226-242	226-242	226-242
11	252-263	252-263	252-263	**251-261**	252-263	252-263	252-263
12	269-277	269-277	269-277	**268-276**	269-277	269-277	269-277
13	282-287	282-287	282-287	**281-286**	282-287	282-287	282-287
14	296-305	296-305	296-305	**295-304**	296-305	296-305	296-305
15	316-322	316-322	316-322	**315-321**	316-322	316-322	316-322
16	330-341	330-341	330-341	**334-340**	**329-341**	330-341	330-341
17	365-371	365-371	365-371	**363-370**	365-371	365-371	365-371

The EMBOSS-antigenic software detects antigenic epitopes of different forms of the glucanase protein. The software detected 17 epitopes in Gln 2, 4, 5, 6, and 7. But only 16 had been detected in Gln 3. Though the maximum variations of the amino acids in the the ORF has been shown by Gln4, the number of antigenic epitopes was found to be same in it like the other forms. From the table it is understood that eventhough the number and position of the epitope is moreover similar, it shows 1-2 number of amino acids forming the epitope. The least number of variation of one is being shown by Gln7, Gln 5 and Gln 2. A difference of two has been noted in Gln6 and 3 in Gln 3.

4.6. Functional validation of the promoters of β-1,3-glucanase isoforms

Promoters are the DNA sequence present 5' upstream to the gene which control gene expression. In eukaryotic systems, the spatial, temporal and quantitative expression of genes are regulated by the promoters. Promoters with the required regulatory elements are essential to make the gene functional. The relative tolerance of plants towards a particular pathogen is mainly determined by the interactions between the disease resistant 'R' genes and the associated pathogen avirulence 'avr' genes. Most of the 'R' genes are induced ones - induced upon a biotic/abiotic stressed condition showing a controlled regulation by their specific promoter regions. Since different isoforms of β-1,3-glucanase gene controlled by independent promoters were identified in this study, an attempt has been made to understand whether all the promoter forms identified are functional so that all the gene forms identified will also be functional. Therefore, promoter: reporter gene fusion binary vectors were constructed and the reporter gene expression was studied in the heterologous system, tobacco, through *Agrobacterium* mediated genetic transformation. Attempts were also done to understand the reporter gene expression in *Hevea* callus through transient assay. β-glucuronidase gene was selected as the reporter gene.

Five promoter forms of β-1,3-glucanase isolated from a single *Hevea* clone, RRII 105 were selected in this study. Within the isolated promoter sequences of β-1,3-glucanase, several promoter-related elements including TATA-box, CAAT-box, WRKY motif and GT1 consensus sequences *etc.* were found. Total 11 binary vectors were developed which are as follows: five basic constructs with 913, 847, 582, 553 and 198 bps; two deletions of the 913 bp promoter with 550 and 200 bps; two deletions of the 847 bp promoter with 550 and 200 bps and one 200 bp deletion with 582 and 553 bp promoter. The vector used was pCAMBIA 1381 Z TDNA vector, a plant expression vector to check for the promoter efficiency.

The plasmid vector pCAMBIA 1381Z is designed for promoter analysis in plants, containing a fully functional promoterless *gus*A reporter gene with catalase intron, which is immediately downstream of a truncated lacZα containing a polylinker. Efficiency and tissue specificity characteristics of promoter, which is introduced 5` to the *gus*A gene can be studied in terms of its ability to drive gus gene expression. The vector has bacterial selection with kanamycin (*npt II*) and plant selection with hygromycin (*hpt II*).

The pCAMBIA vectors are a series of vectors derived from the pZP family of vectors (Hajdukiewicz *et al.*, 1994) and they offer several advantages over other traditional vectors, which include: 1) high copy number in *E.coli* for high DNA yields, 2) pVS1 replicon for high stability in *Agrobacterium,* 3) small size, 7-12 kb depending on plasmid, 4) restriction sites designed for modular plasmid modifications and small but adequate poly-linkers for introducing the DNA of interest, 5) bacterial selection with chloramphenicol or kanamycin, 6) plant selection with hygromycin B or kanamycin, 7) simple means to construct translational fusions to *gusA* reporter genes.

4.6.1.Construction of binary plasmid vectors

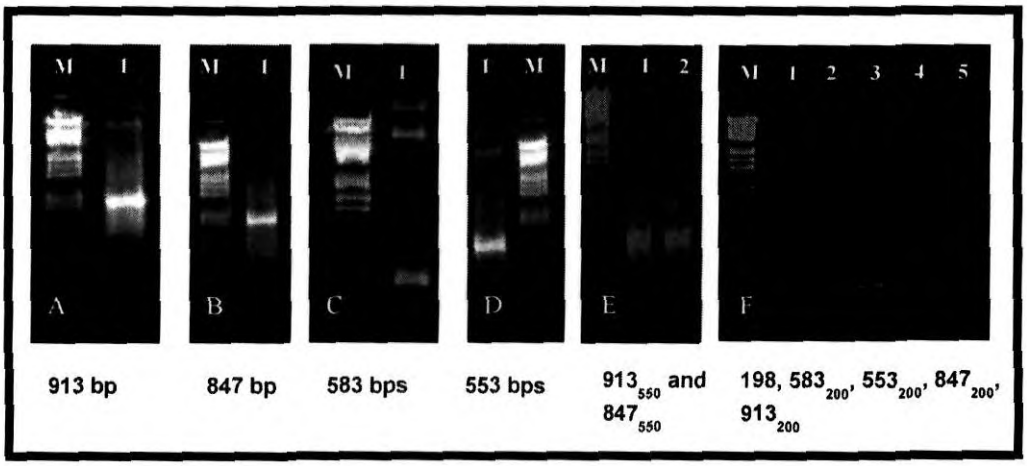
4.6.1.1. PCR amplification of the inserts from the cloned TA vector to develop the binary vector

vector to

The inserts for developing the binary vector for the transformation had been PCR amplified from the already cloned TA cloning vector using specific primers tagged with appropriate restriction sites. The inserts were the promoter sequences of the different forms of β-1,3- glucanase. A series of different 5' deletion fragments were also amplified for the corresponding promoter sequences using the appropriate primers as mentioned before (fig. 80 A- F).

Enzymes used to tag the primers were *EcoR*1 for the forward primer and *Bgl* II for the reverse primer. A good single band amplicon of the expected size was obtained for all inserts attempted. The gel purified amplicon was used for double digestion with the corresponding restriction enzymes.

Fig: 80. A- F: PCR amplification of the inserts to be cloned into the binary vector constructs



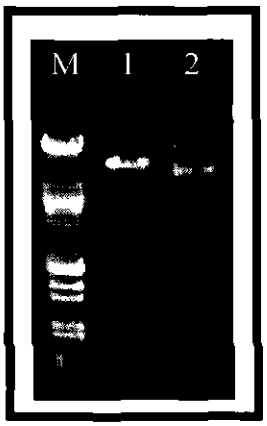
The lane M denotes the DNA marker (λ DNA *Eco*R1/*Hind* III double digest) in all the gels. A- amplification of 913 bp promoter, B-847 bp promoter, C- 583 bp promoter, D- 553 bp promoter, E- 550 bp deletions of 913 and 847 bp promoters, F- lane 1: 198 bp promter, F- lanes 2-5 : 200 bp deletions of 583 (2), 553 (3), 847 (4) and 913 bp (5) promoters.

4.6.1.2. Isolation of the binary plasmid vectors pCAMBIA 1381Z

The binary vector pCAMBIA 1381Z available in the laboratory had been transformed to the chemically competent *E.coli* DH5α cells and the plasmids were isolated after multiplication in the cells.

4.6.1.3. Digestion of the plasmid vectors and the insert

Double digestion of the plasmid and the gel purified inserts were carried out with the restriction enzymes, *EcoR* 1 and *Bgl* II. The use of two different restriction enzymes ensures the insert to be in correct orientation in the double digested vector. After first digestion both the insert and the vector was purified by phenol: chloroform extraction followed by precipitation of DNA. This ensures perfect digestion with the consecutive second restriction enzyme. After the second digestion also the DNA was purified. This avoids any hindrance to ligation due to residual restriction enzyme activity. Proper digestion of the vectors was confirmed after each digestion in an agarose gel after electrophoresis (fig. 81).



M – Marker (λ DNA *Eco*R1/*Hind* III double digest)

1 - Double digested vector- pCAMBIA 1381Z

2 - Non- digested pCAMBIA 1381Z

Fig. 81. Double digested pCAMBIA 1381Z vector prior to ligation

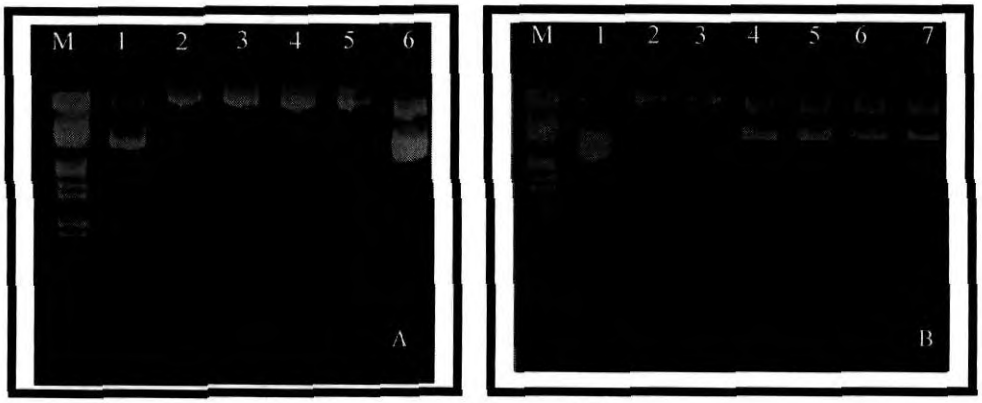
The double digested vector was later de-phosphorylated and used for the ligation of the inserts. It is especially useful when both protruding terminii of the linearised plasmid have been cut with the same enzyme (sticky ends) but is also used in blunt-ended ligations. Since DNA ligase will catalyze the formation of a phosphodiester bond between adjacent nucleotides only if one nucleotide contains a 5'-phosphate group and the other a 3'-hydroxyl group during the ligation. Hence recircularization of plasmid DNA can be minimized

by removing the 5' phosphates from both ends of the linear DNA with calf intestinal phosphatase and as a result, neither strand of the duplex can form a phosphodiester bond. However, a foreign DNA segment with 5'-terminal phosphates can be ligated efficiently to the dephosphorylated plasmid DNA to give an open circular molecule containing two nicks. Because circular DNA (even nicked circular DNA) transforms much more efficiently than linear plasmid DNA, most of the transformants will contain recombinant plasmids. The dephosphorylation was followed by a phenol extraction and an ethanol precipitation to inactivate the phosphatase since residual active phosphatase can cause failure in subsequent ligation/transformation experiments.

4.6.1.4. Ligation and construction of new binary vectors

The linearized double digested vector (pCAMBIA 1381Z) and the PCR amplified inserts (promoter) were ligated together using T4DNA ligase enzyme (M/S New England Biolabs, UK). An insert: vector ratio of 3:1 was used for ligation which was found to be good. The ligated mixture was used to transform *E. coli*. Several colonies were obtained in kanamycin containing LB plates. Here, the blue/ white screening by the insertional inactivation of the lacZα gene could not be performed. Therefore, the preliminary screening of the colonies with recombinant plasmids was confirmed through colony PCR. A bit of the bacterial colony as such was used for the template instead of DNA for the normal PCR. The colonies selected for the PCR reaction showed a band of expected size corresponding to the eleven inserts and plasmids were isolated from the positive colonies (fig. 82. A & B). PCR of the recombinant binary vectors was carried out for further confirmation using the insert specific primers. PCR for the confirmation of their respective promoter sequence (insert) using promoter specific forward and reverse primers was carried out (fig. 83. A). PCR of the recombinant plasmids to confirm the presence of the marker gene *hpt* II and the reporter gene GUS was also carried out and amplifications with the approximate sizes were obtained correspondingly for *hpt II* (≈ 610 bp) and GUS genes (≈ 700 bp) (fig: 83. B & C)

Fig: 82. A & B. Different binary vectors isolated from DH5α cells for transformation to *Agrobacterium* strain EHA 105



A:

M- Marker (λ DNA *Eco*R1/*Hind* III double digest)
1 - 198 bp promoter harbouring binary vector
2 - 913 bp
3 - 847 bp
4 - 582 bp
5 - 553 bp
6 - Non-inserted vector

B:

M- Marker (λ DNA *Eco*R1/*Hind* III double digest)

1 - Non- inserted binary vector
2 - 913_{550}
3 - 847_{550}
4 - 913_{200}
5 - 847_{200}
6 - 553_{200}
7 - 582_{200}



1 - 198 bp
2 - 553_{200}
3 - 553
4 - 582_{200}
5 - 582
6 - 847_{200}
7 - 847_{550}
8 - 847
9 – 913_{200}
10- 913_{550}
11- 913
M- Marker (λ DNA *Eco*R1/*Hind* III double digest)

Fig. 83. A. Plasmid PCR for the presence of inserts in the eleven constructs using insert specific forward and reverse primers

Fig. 83. B & C. *hpt* II (B) and GUS (C) amplification of all the 11 constructs using specific primers



M - Marker (λ DNA *Eco*R1/*Hind* III double digest)
1- 11 - *hpt* II gene amplification in the 11 constructs



M - Marker (λ DNA *Eco*R1/*Hind* III double digest)
1- 11 - GUS gene amplification in the 11 constructs

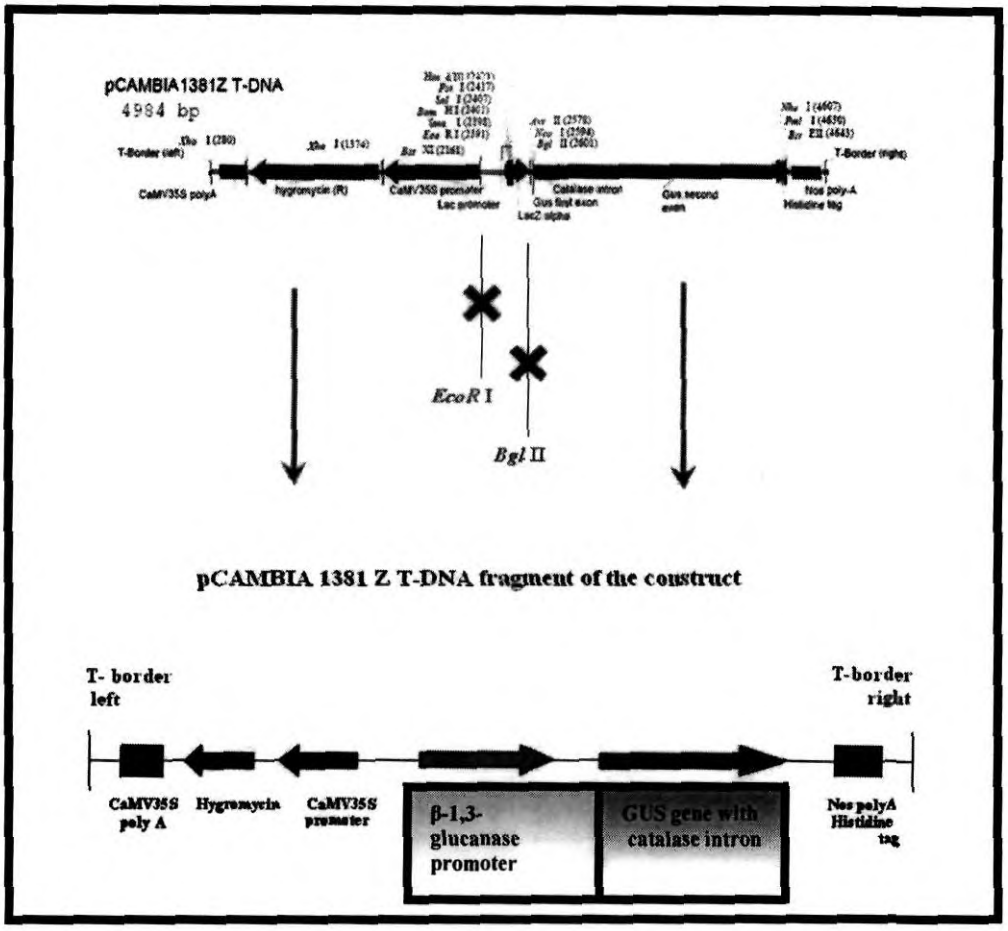
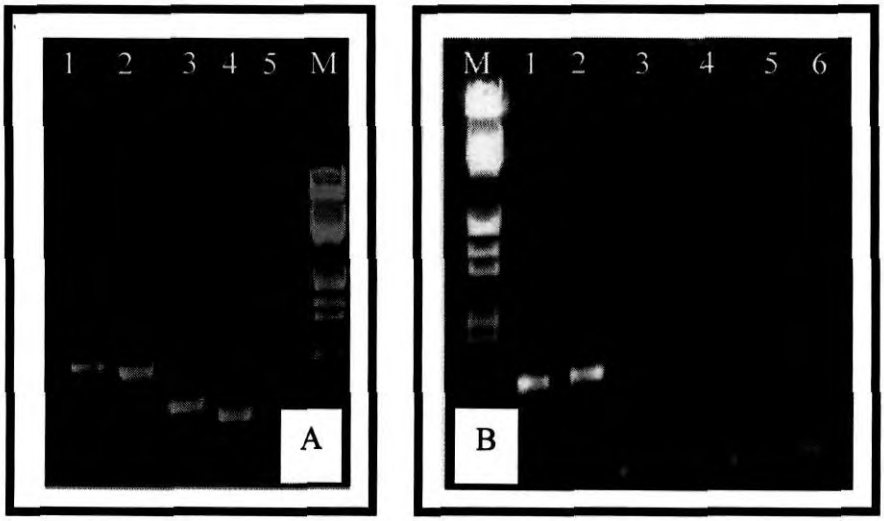


Fig. 84. Schematic representation of the construction of binary vector cassettes of β-1,3-glucanase gene

4.6.2. Transformation of Agrobacterium with the recombinant binary vectors

The recombinant plasmids containing promoter: reporter gene fusions were introduced into competent *Agrobacterium* strain EHA 105 by freeze thaw method. Relatively good transformation efficiency was observed. A transformation reaction with 100 µl competent *Agrobacterium* cells and 1.0 µg plasmid (binary vector), 50- 75 colonies were obtained on selection plate when 100 µl cell suspension of the transformed mixture was plated. Control plasmid without the insert (promoter) was also transferred into *Agrobacterium.* Transformed colonies obtained on the LB selection plate containing rifampicin 20 µg/ ml and kanamycin 50 µg/ ml were screened initially by colony PCR using respective forward and reverse primers of the insert (fig. 85. A & B).

Fig. 85. *Agrobacterium* colony PCR to ensure the presence of recombinant binary vector



A :
1 - 913 bp
2 - 847bp
3 - 582 bp
4 - 553 bp
5 - 198 bp
M - DNA Marker
(λ DNA *Eco*R1/*Hind* III double digest)

B :
M- DNA Marker (λ DNA *Eco*R1/*Hind* III double digest)
1 - 913 $_{550}$
2 - 847 $_{550}$
3 - 913 $_{200}$
4 - 847 $_{200}$
5 - 582 $_{200}$
6 - 553 $_{200}$

4.6.3. Agrobacterium mediated genetic transformation of *Hevea brasiliensis* and *Nicotiana tabaccum*

For the functional validation of the promoters, the promoter : reporter gene binary vectors were transformed to tobacco leaf discs and *Hevea* callus through *Agrobacterium* mediated transformation. The GUS expression was studied in tobacco through transient assay as well as stable expression in regenerated transgenic tobacco. In *Hevea* callus, GUS expression was studied through transient assay only since transgenic plant regeneration in *Hevea* takes 2-3 years.

4.6.3.1. *Agrobacterium* mediated transformation of *Hevea* callus and transient assay

The transient assay using *Hevea* callus showed a very good GUS activity with all the constructs made, from the least minimum base pairs (198 bp) to the highest (913 bp) (fig. 86). The control with the empty vector, untransformed callus and the *Agrobacterium* broth culture did not show any signs of GUS activity which clearly indicates the specificity of the promoter in driving the GUS reporter gene in the transformed tissue. The GUS activity was found to be higher with the callus transformed with the 913 bp promoter construct (blue arrow). This can be attributed to the length of the promoter which can hold diverse / multiple transcription factors/ regulatory elements.

During early stages of co-cultivation, single-stranded T-DNA is transferred from the bacteria to plant cells (Zupan and Zambryski, 1995). Once moved into the plant cell by bacterial and plant encoded proteins (Tzfira *et al.*, 2004), this T-DNA becomes double-stranded and migrates to the nucleus and integrates into the host chromosomes leading to stably transformed cells that can subsequently be regenerated into transgenic plants. Although the long-term fate of the T-DNAs that do not integrate into the chromosomes is unclear, for a time, these pieces of DNA are transcriptionally competent; this is the basis of the *Agrobacterium*-mediated transient expression systems (Voinnet, 2003).

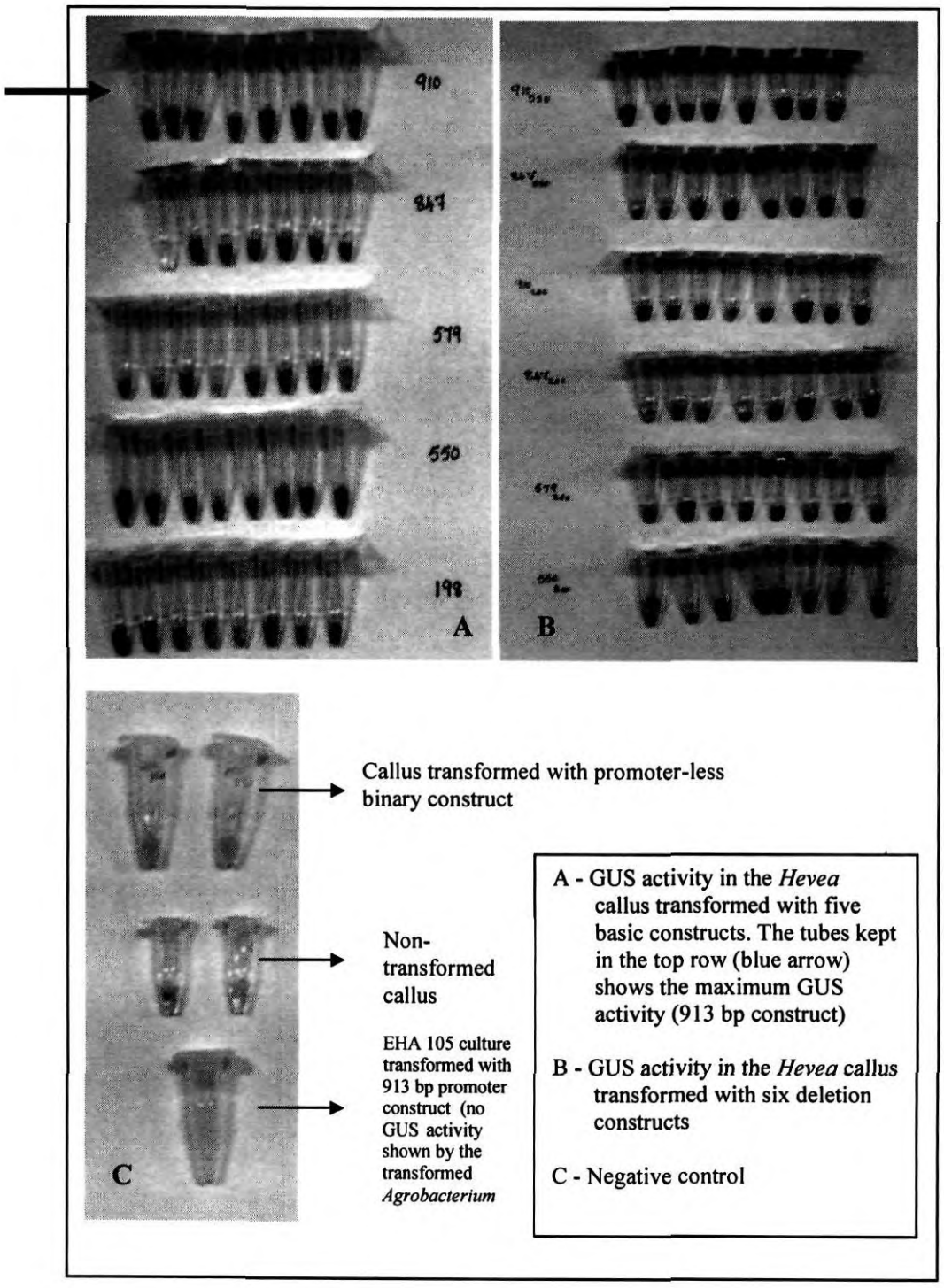


Fig. 86. Transient GUS activity of *Hevea* callus with all the 11 binary constructs

**Blue arrow points the row showing the GUS activity of the callus with the 913 bp promoter*

4.6.3.2. *Agrobacterium* mediated transformation of Tobacco

Agrobacterium mediated transformation of leaf discs (Horch *et al.*, 1985) was found as an efficient method for the transformation of tobacco plants. The binary construct was introduced into tobacco by co-cultivation of leaf discs with *Agrobacterium* for 2 days. Here a convenient, *Agrobacterium*-mediated transient expression assay was evaluated for rapid analysis of β-1,3-glucanase gene promoters *in vitro*. By simple infiltration of *Agrobacterium* cells carrying appropriate plasmid constructs into tobacco leaves and *Hevea* callus, reproducible expression assays could be conducted in as little as 2-3 days without using expensive equipment (e.g. biolistic gun or electroporation apparatus) or complicated procedures (e.g. preparation of protoplasts).

Transient assay of the transformed *Nicotiana* leaf discs does not showed any significant levels of GUS activity. The negative controls (non-transformed leaf discs, leaf discs transformed with promoterless construct) also did not show any signs of GUS activity. The leaf discs were later transformed to selection plate containing hygromycin (30 μg/ml) for the selection of transformants and 350 μg/ml cefotoxime to prevent the *Agrobacterium* overgrowth. The hygromycin used was found effective for the selection of transformants. After 10-15 days, transformed shoots were emerged from the putatively transformed tissues in the selection medium (fig. 87. A- F).

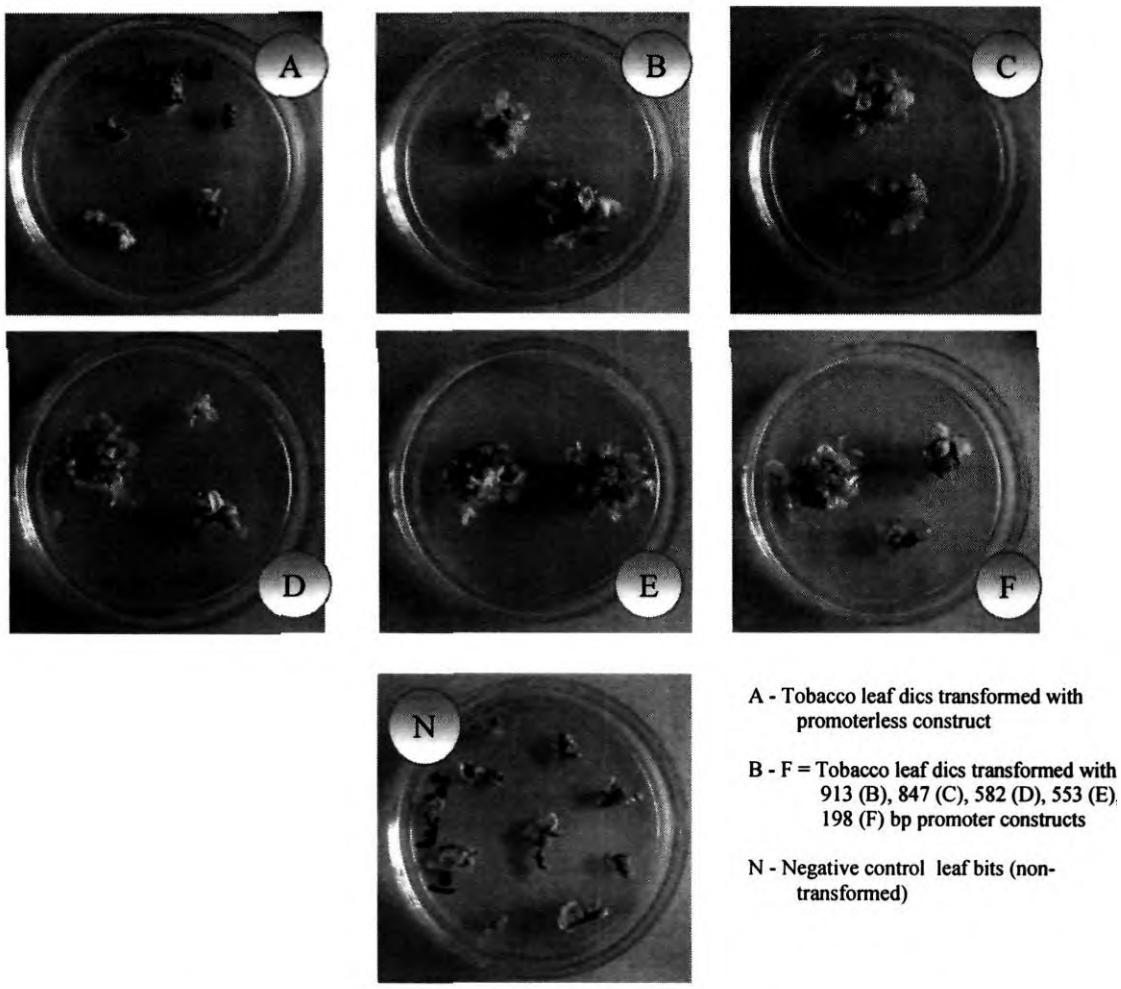


Fig. 87. Regenerating tobacco plantlets in the selection plate

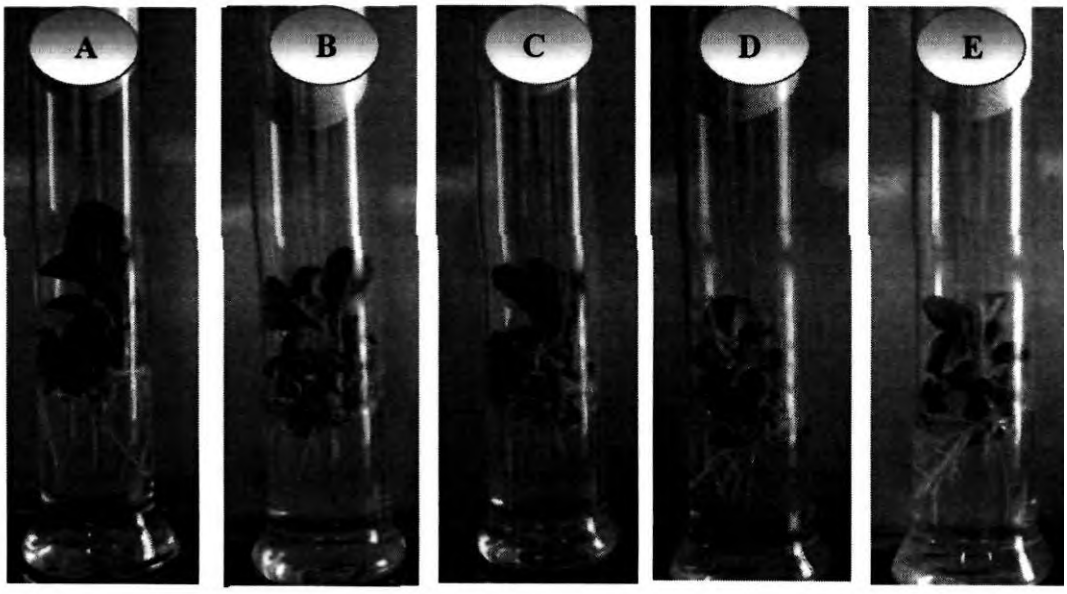


Fig. 88. Transgenic tobacco plants obtained with the basic constructs. A –E: Transgenic tobacco harbouring 913 (A), 847 (B), 582(C), 553(D) and 198 bp promoter constructs.

When the GUS activity of these shootlets were checked, it did not show any GUS activity, the result of which is matching with the nil expression of the GUS reporter gene in the transient assay done initially. Whereas the non-transformed tissue got gradually bleached and finally turned dark (fig. 88 G).

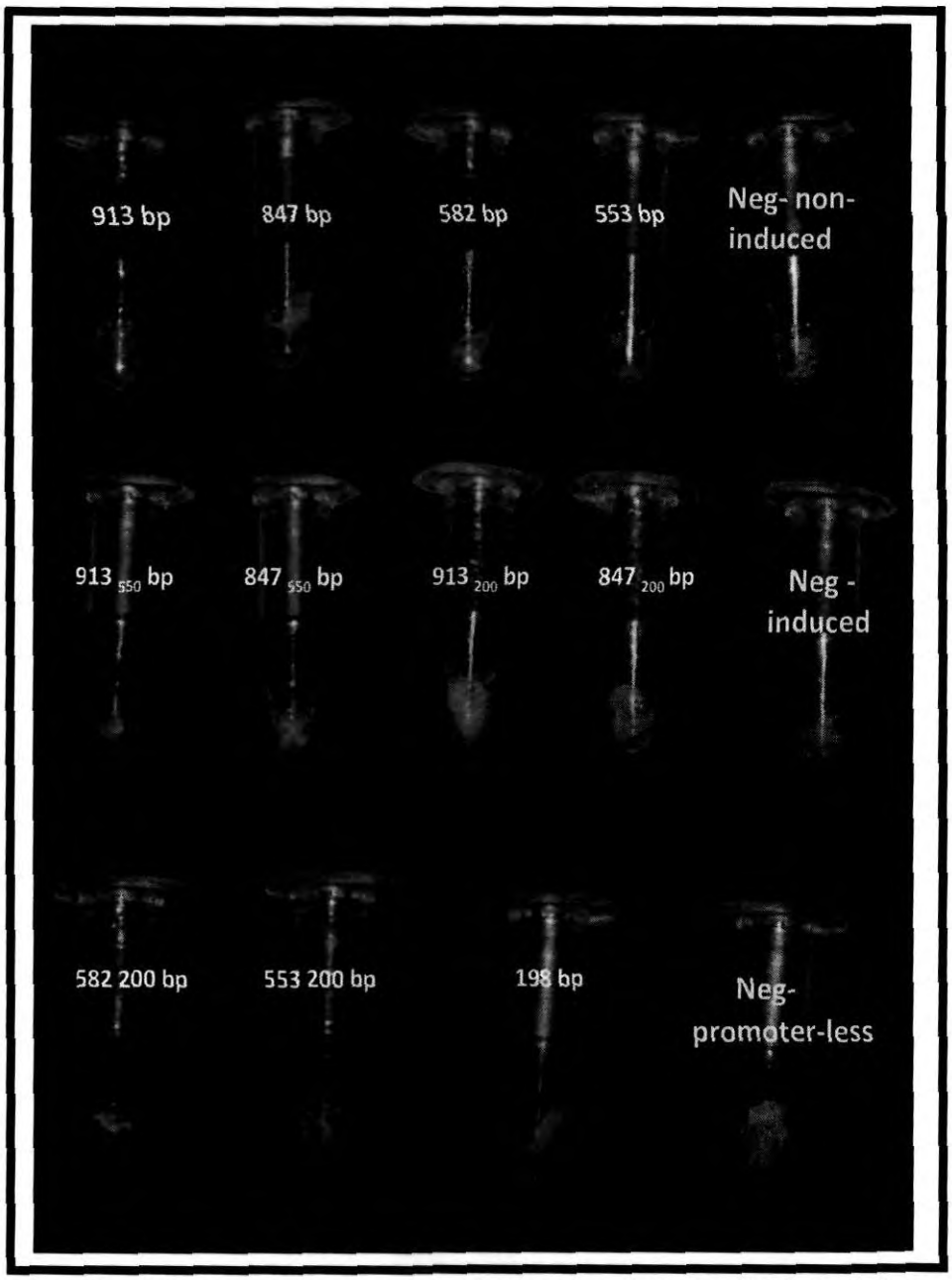


Fig. 89. Transgenic tobacco leaf bits showing no GUS activity upon induction

Induction level studies were made in the emerging shoots (fig. 88. A-E) of the transformed tobacco with 0.1% salicylic acid. Though the studies

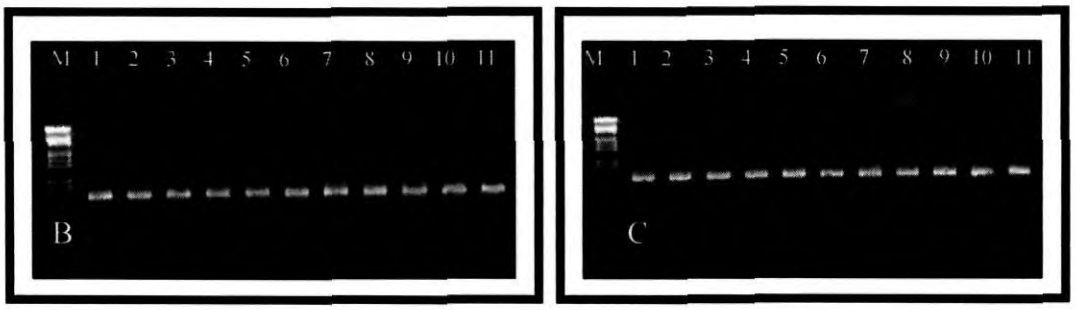
initially showed a slight GUS activity, upon treatment with the alcohol no visible coloration as a part of the GUS staining was observed (fig. 89). The transformed plants showed amplification of the inserts and also for the reporter and marker genes (fig. 90. A, B & C).

Fig.90.A. Transgene promoter regions amplified from the transformed tobacco



1 - 198 bp
2 - 553 $_{200}$
3 - 553
4 - 582 $_{200}$
5 - 582
6 - 847 $_{200}$
7 - 847 $_{550}$
8 - 847
9 - 913 $_{200}$
10 - 913 $_{550}$
11 - 913
M - Marker (λ DNA *Eco*R1/*Hind* III double digest)

Fig. 90. B & C. Amplification of GUS (B) and *hpt* II (C) from the transformed tobacco plants with the 11 constructs.



B:
M - Marker (λ DNA *Eco*R1/*Hind* III double digest)
1-11 : Amplification of GUS gene from transformed tobacco with the 11 constructs

C:
M- Marker (λ DNA *Eco*R1/*Hind* III double digest)
1-11 : Amplification of *hpt* II gene from transformed tobacco with the 11 constructs

More studies are required in future in order to identify further upstream promoter sequences of these promoter sequences and also to learn whether the transgenic promoter sequence is an induced one or not.

4.7. Heterologous expression of β-1,3-glucanase isoforms, purification of recombinant proteins and antifungal studies

4.7.1. Construction of functional gDNA clone and purification of recombinant proteins from *Pichia pastoris*

Heterologous expression involves characterization of desired genes, developing expression vectors and transfer of the desired genes to hosts other than the original source, for synthesis of the encoded proteins. Protein isolation, especially from plant sources, can be costly, cumbersome and lengthy, and expression in heterologous system like yeast or bacteria like *E.coli* provides a convenient alternative. Yeast cells have the advantage of expressing genomic DNA with introns.

The different forms of the isolated β-1,3-glucanase gene shows minor variation in their exon sequence and also intron variations- 'CT' additions/ deletions in the introns. The amplification of the individual cDNA representing each of these different forms of β-1,3-glucanase from a pool is extremely difficult as the single band of the PCR product consists of a mixture of DNA sequences which is of a common occurrence in the amplification of multigene familes. Hence to learn the full functional aspects of the gene to its final translational product, the gene is cloned in a eukaryotic host where there is an efficient intron splicing machinery. All the forms of β-1,3-glucanases need not exhibit anti fungal properties. Hence the identification of the correct form of glucanase with higher antifungal activity is necessary for the selection of the right candidate gene for the crop improvement programmes. In the present study an attempt was carried out to express the different forms of the isolated β-1,3-glucanase genes in the eukaryote, *Pichia pastoris,* the new generation yeast. The purified protein can be used for the antifungal studies against the abnormal leaf fall disease causing fungus *Phytophthora meadii.*

4.7.1.1. PCR amplification of the inserts to be cloned with restriction site tagged primers

Two primer pairs were designed for each of the different forms of glucanase obtained with restriction sites to amplify the region coding for the protein in *Pichia pastoris*. The forward primer is having the *EcoR*1 site at 5' end and reverse primer is having the *Kpn* 1 site at the 3' end. All the primer combinations specifically amplified the band of expected size (approximately 1.3 kb) under optimal PCR conditions at an annealing temperature of 55 ^{0}C (fig. 91) for the different forms of six glucanases. These fragments obtained were gel purified for the cloning purpose.

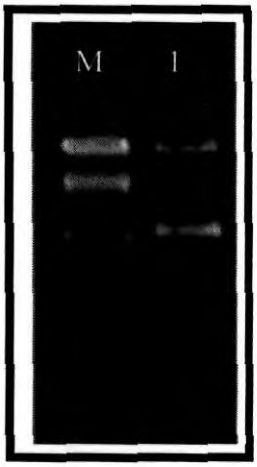


Fig. 91. PCR amplification of the sequences coding for β-1,3-glucanase using primers tagged with restriction sites

4.7.1.2. Cloning in the yeast expression vector- pPICZ A

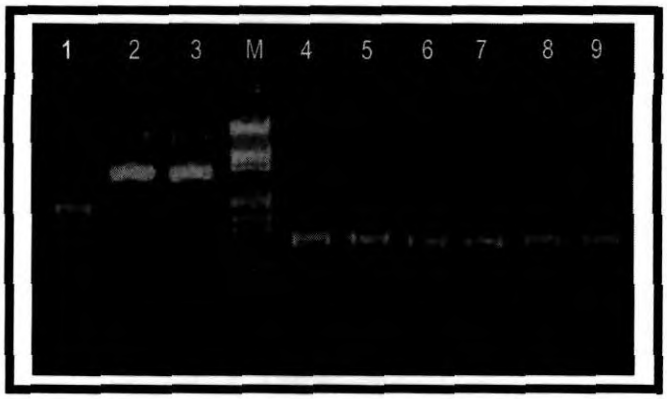
The yeast expression vector pPICZ A (non- secretory vector) was used for the expression studies for the rubber β-1,3-glucanase gene. The expression vector was isolated in large quantities from the transformed DH5α cells carrying the same vector by the alkaline lysis procedure. Concentration and purity of the plasmid preparations was found to be good as observed from the 1 % agarose gels (fig. 92). The pPICZ A vector isolated from DH5α cells were of 3.3 kb size.

The vector and the inserts were digested with the appropriate restriction enzymes to create the cohesive ends and digestion of the vector was ensured on agarose gels (fig. 93). The presence of two different sites on either ends ensures for the unidirectional cloning.



M- Marker (λ DNA *Eco*R1/*Hind* III double digest)
1- pPICZ A vector

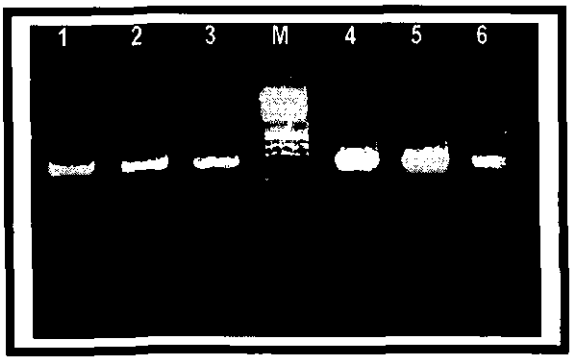
Fig. 92. pPicZ A vector (3.3 kb) isolated from DH5 α cells.



1 - Undigested vector
2& 3 - Double digested vecto –pPICZ A
M - Marker (λ DNA *Eco*R1/*Hind* III double digest)
4 - Gln 2 insert
5 - Gln 3
6 - Gln 4
7 - Gln 5
8 - Gln 6
9 - Gln 7

Fig. 93. Double digested vectors and the inserts of approximately 1.3 kb

The vector was de-phosphorylated before ligation to prevent self ligation. The vector was later ligated to the insert by keeping a vector: insert ratio of 1:3. After ligation and transformation to recombination deficient (recA) and endonuclease A deficient DH5α cells, the cells were spread on Zeocin^{+} (25μg/ml) low salt LB plates. Many colonies of transformed DH5α were obtained with all the ligated mix. For analysis of recombinant colonies, a colony PCR was carried out with the insert specific primers. The PCR showed positive amplification indicating the presence of inserts of nearly 1.3 kb (fig. 94) in the colonies selected.

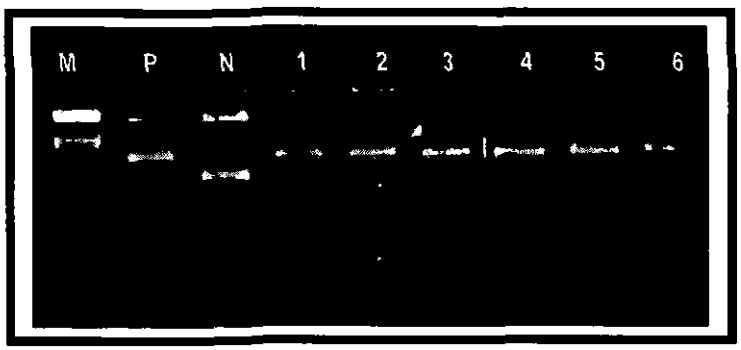


1 - Gln 2
2 - Gln 3
3 - Gln 4
M - Marker (λ DNA *Eco*R1/*Hind* III double digest)
4 - Gln 5
5 - Gln 6
6 - Gln 7

Fig. 94. Colony PCR of the transformed DH5α cells showing positive amplification for the presence of the inserts

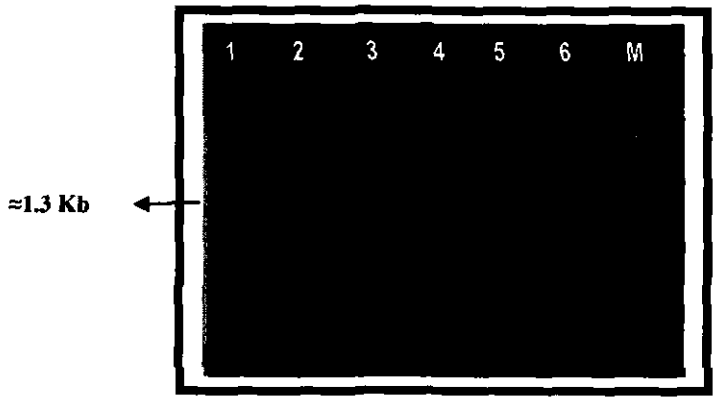
Recombinant plasmids were isoalated from the positive colonies (fig. 95). Compared to the insertless plasmid control, the recombinant plasmid showed higher size indicating the presence of the inserts. When PCR of the recombinant plasmids were conducted, the bands of expected size (≈1.3 Kb) was amplified from the potential recombinant plasmids when insert specific primers were used in the PCR (fig. 96). The sequencing results of the recombinant vectors showed the presence of inserts in its right orientation.

The recombinant plasmids isolated were later linearised using the selective restriction enzyme *Sac* I, the site of which is not present in the inserts. Upon linearization of the plasmids containing the inserts it was found to be approximately 4.7 kb which includes the vector size 3.3 kb (3329 nucleotides) plus the 1.3 kb insert (fig. 97).



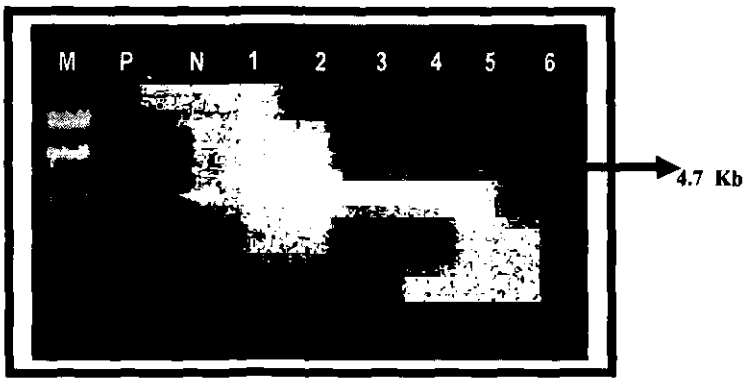
M - Marker (λ DNA *Eco*R1/*Hind* III double digest)
P - Positive control with the insert
N- Negative control insertless plasmid
1 - Gln 2
2 - Gln 3
3 - Gln 4
4 - Gln 5
5 - Gln 6
6 - Gln 7

Fig. 95. Recombinant pPICZ A plasmids isolated from the DH5α cells



1 - Gln 2
2 - Gln 3
3 - Gln 4
4 - Gln 5
5 - Gln6
6 - Gln 7
M - Marker (λ DNA *Eco*R1/*Hind* III double digest)

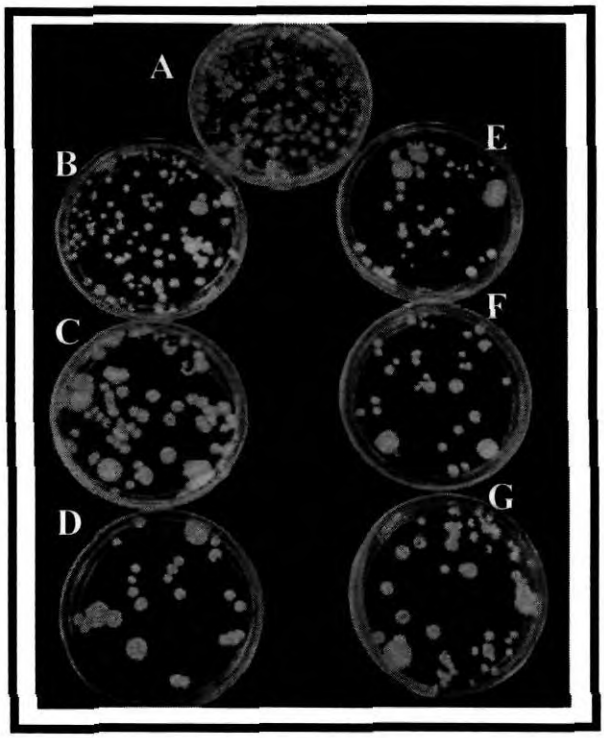
Fig. 96. PCR of the recombinant plasmids isolated from DH5α to ensure the presence of inserts



M - Marker (λ DNA *Eco*R1/*Hind* III double digest)
P - Plasmid with insert (non- linearized)
N - Plasmid without insert (non- linearized)
1-6 - Linearized recombinant vectors
1 - Gln 2
2 - Gln 3
3 - Gln 4
4 - Gln 5
5 - Gln 6
6 - Gln 7

Fig. 97. Agarose gel showing the linearized vectors to transform *Pichia*

The linearized plasmid was later used to transform *Pichia* strain X-33. This is a wild type strain which is positive towards the methanol utilization (Mut^+). Linearized pPICZ A vector without the insert was used as the negative control here. The selection was made in the YPDS plates containing 100 μg/ml zeocin antibiotic. A fair number of colonies were obtained with each transformation events with the six separate inserts of the isolated β-1,3-glucanase genes (fig. 98). The negative control plate showed more number of colonies than the transformed ones.



A - Negative control
B - *Pichia* colonies for Gln2
C - *Pichia* colonies for Gln 3
D - Pichia colonies for Gln 4
E - Pichia colonies for Gln 5
F - Pichia colonies for Gln 6
G - Pichia colonies for Gln 7

Fig. 98. Recombinant *Pichia* colonies in YPDS plates containing the zeocin (100µg/ ml) antibiotic

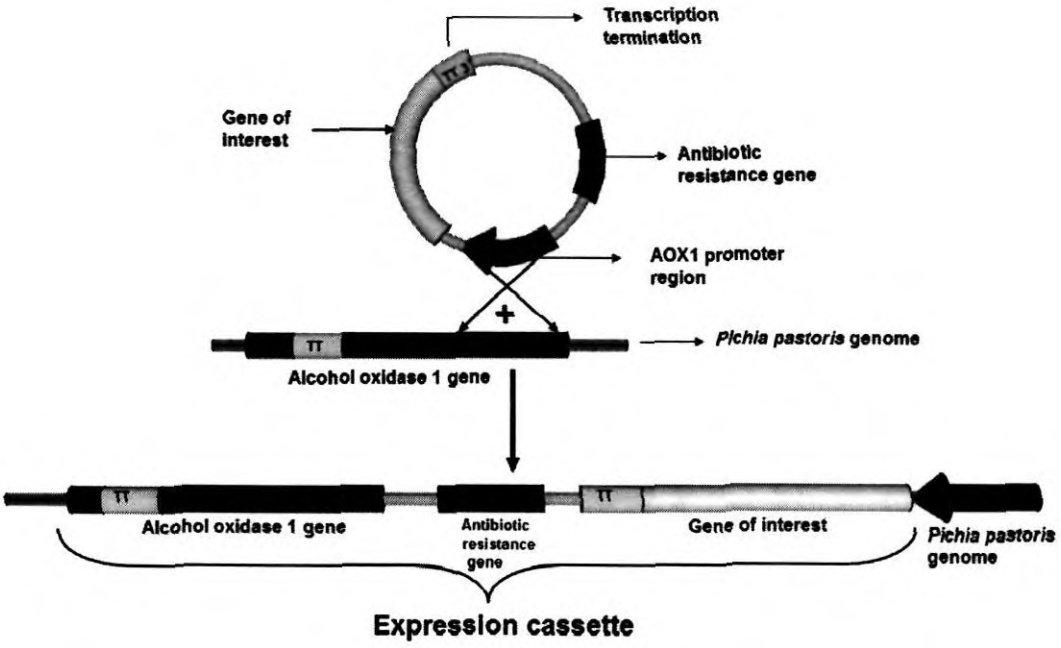


Fig. 99. Schematic representation showing the pattern of integration of the gene in *Pichia* genome

Once the linearized vector is transformed into the yeast host, the free DNA ends promote homologous recombination events, resulting in the integration of the linearised expression vector by gene insertion into the host AOX1 (alcohol oxidase 1 gene) region. Fig. 99 shows the pattern of integration of the vector within the *Pichia* genomic DNA. Only the recombinant hosts will grow in the antibiotic plate containing zeocin antibiotic. The transformed cells were later checked for methanol utilization in the MM (minimal methanol) plates. The positive colonies showed good growth in the MM plates. (fig. 100).

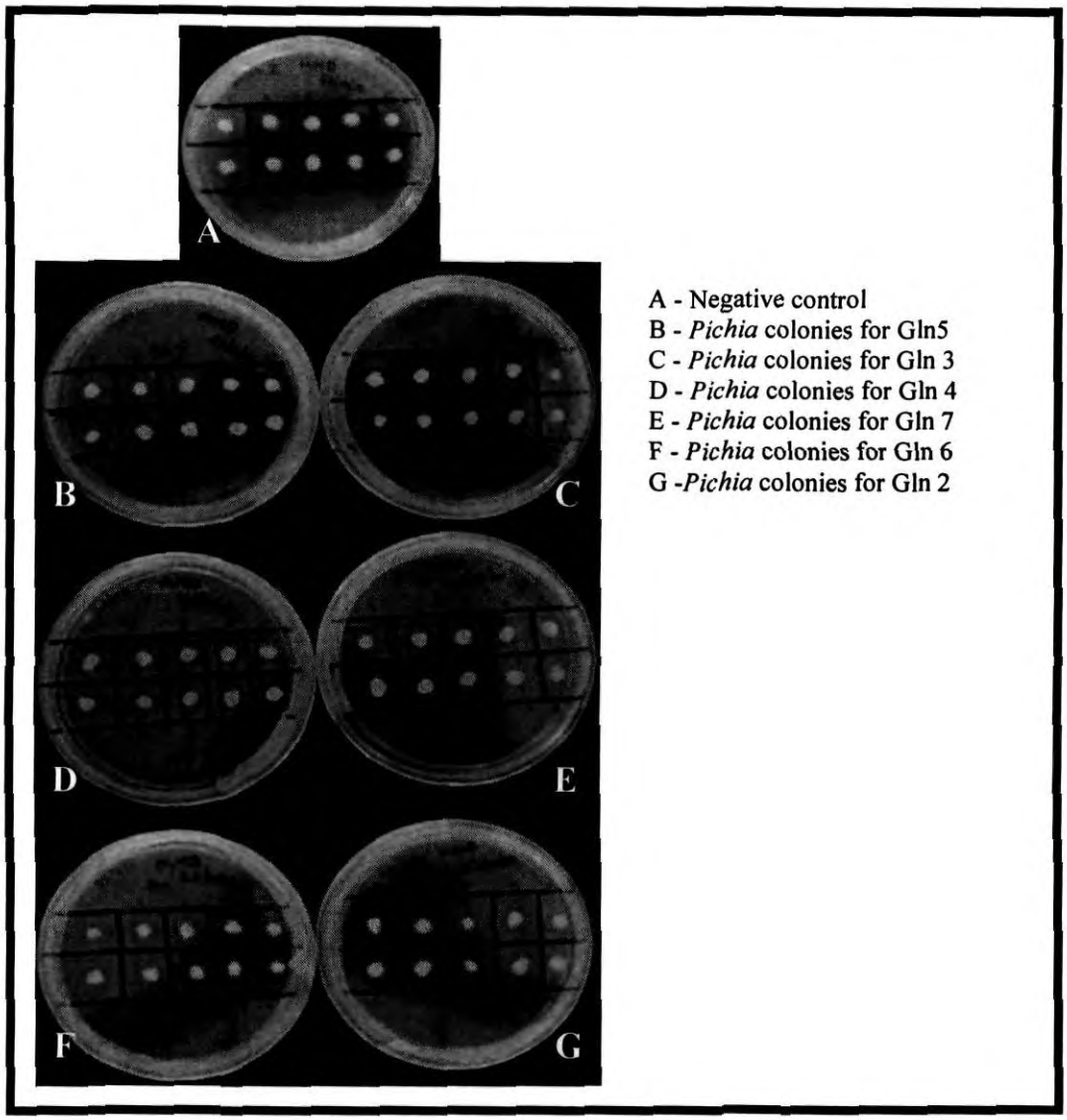
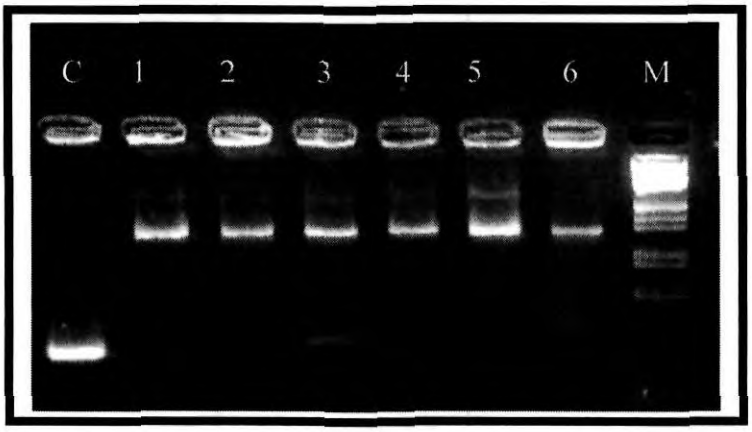


Fig. 100. Colonies harbouring the inserts in MM plates to check for methanol utilization.

Since the X- 33 strain is methanol utilization +ve (Mut $^{+}$) all the colonies selected representing each of the different forms of glucanase showed similar pattern of growth in the MM plates. The growth in size could also be comparable with that of the negative control which posess the pPICZ vector without the insert. Since the strain is methanol utilization +ve, the colonies in the –ve control plate is also showing good growth.

The presence and the orientation of the insert in the transformed *Pichia* cells was ensured by PCR with 5' AOX forward and the 3'AOX reverse primer. The colony PCR showed the presence of the insert and also the native AOX gene (fig. 101). The gel showed the presence of two bands, one lower band that of the presence of the insert and one upper band (2.2 kb) which is indicating the amplification of the native AOX gene in *Pichia*. The negative control shows the amplification of only a 330 bp AOX present in the vector.



C- Negative control
1- Gln 2
2- Gln 3
3- Gln 4
4- Gln 5
5- Gln 6
6- Gln 7
M- Marker (λ DNA *Eco*R1/*Hind* III double digest)

Fig. 101. Colony PCR of *Pichia pastoris* representing each of the six different forms of β-1,3- glucanases

The genomic DNA integration was again confirmed through southern blotting with one of the *Pichia* clone carrying Gln 2 which showed positive amplification in the colony PCR. For southern blotting, DNA was isolated from the *Pichia* colonies carrying the insert (positive control) as well as without insert (negative control) using the protocol reported by Tomitha *et al.*, 2002 which does not require the use of any hydrolytic enzymes for cell wall

breakage. The DNA isolated from *Pichia* was of good quality and quantity. The quantity checked through spectrophotometer showed a 260/280 ratio of >1.7. Fig. 102 shows the DNA isolated from the positive and negative clones of *Pichia.*



M- Marker : (λ DNA *Eco*R1/*Hind* III double digest)

1 - DNA from the positive clone carrying the Gln 2 gene integrated

2 - DNA from the negative clone transformed with the pPICZA vector only

Fig. 102. DNA isolation from *Pichia* clones



1 - *Bcl* I digested negative control DNA (NCD)

2 - *Bam*H 1 digested NCD

3 - *Ass* I digested NCD

I - PCR amplified probe itself

C- Positve control- vector with the insert

4 - *Bcl* 1 digested positive control DNA (PCD)

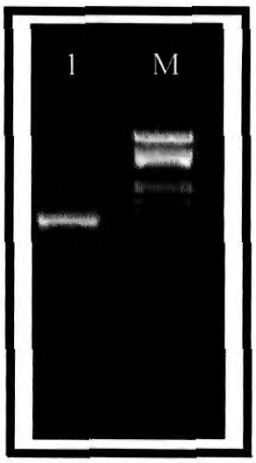
5 - *Bam*H 1 digested PCD

6 - *Ass* 1 digested PCD

Fig. 103. Restriction enzyme digestion of *Pichia* genomic DNA with *Bcl* 1, *Bam* H1 and *Ass* 1 in 1% agarose gel

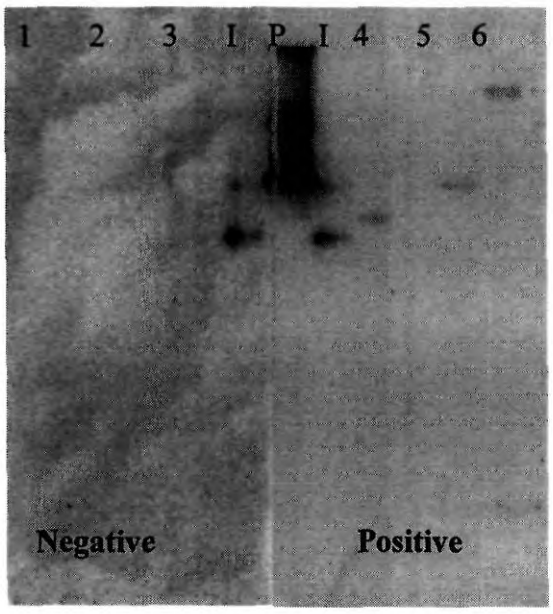
The restriction enzymes chosen did not have any sites in the Gln 2 gene integrated in the *Pichia* genome. These enzymes offered a good digestion

pattern as seen in the 1% agarose gel (fig. 103). This gel was used to prepare the southern blot by the capillary transfer. The probe used for the southern blot was the Gln 2 gene itself which was amplified from the cloned Strataclone vector (fig. 104). The purified Gln 2 gene probe was used for southern hybridization.



1 - 1.3 kb probe (Gln 2)
M - Marker (λ DNA *Eco*R1/*Hind* III double digest)

Fig. 104. Gln 2 gene amplification to be used as probe in southern blot hybridization



1 - *Bcl* 1 digested negative control DNA (NCD)
2 - *Bam*H 1 digested NCD
3 - *Ass* I digested NCD
I - PCR amplified probe itself
P - Positive control- plasmid with insert
4 - *Bcl* 1 digested test sample
5 - *Bam*H 1 digested test sample
6 - *Ass* 1 digested test sample

Fig. 105. Southern blot-autoradiogram of *Pichia* genomic DNA

The southern blot hybridization confirmed the integration of Gln 2 into the genome of *Pichia pastoris* as indicated by the autoradiogram. Signals corresponding to hybridization with α P^{32} dCTP labelled Gln 2 gene probe was observed in all the test samples analyzed showing positive results for the gene

integration in *Pichia*. Different hybridization pattern observed for different restriction digested samples indicated the random integration of the insert into the genome of the *Pichia* host through *transformation* (fig. 105). The positive controls used were the recombinant plasmid (lane P) and the PCR amplified inserts (lanes 'I'). The positive controls showed fair signaling. No hybridization could be detected in the negative control.

The protein expression levels were rapidly tested by SDS-PAGE analysis of the cell extracts followed by staining with coomassie blue. The equal quantity of the denatured protein sample obtained from a 1ml culture of a 72 hour culture belonging to each of the *Pichia* transformants was loaded in a 10% SDS PAGE gel and visualized by coomassie blue staining for the presence of the recombinant proteins. The recombinant proteins obtained were approximately of 43.5 kDa shown as a fade band in the gel. One of the protein was found to be ≈ 42.5 kDa which belonged to the Gln 4 (fig. 106).

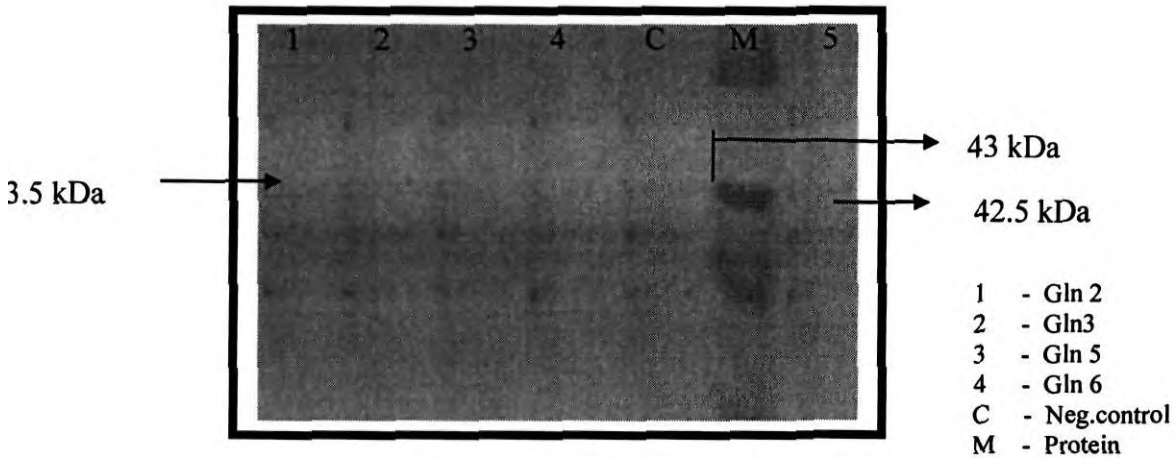


Fig. 106. SDS – PAGE analysis *via* Coomassie blue staining

The protein was later purified using the His-spin trap column. The protein expressed in pPICZ A vector was effectively purified in micro gram quantities with the His-spin trap purification kit (M/S GE Healthcare, USA). The fusion tag contains six histidine residues that will bind with Ni^{2+} cations, which are immobilized on the column resin using a charge buffer containing 50 mM $NiSO_4$. After washing away the unbound proteins, the target protein

was recovered by elution with 500 mM imidazole. No contaminating proteins were seen in purified fractions in 12% acrylamide gel (fig. 107). The eluted fractions were stored at –70 ^{0}C for further analyses.

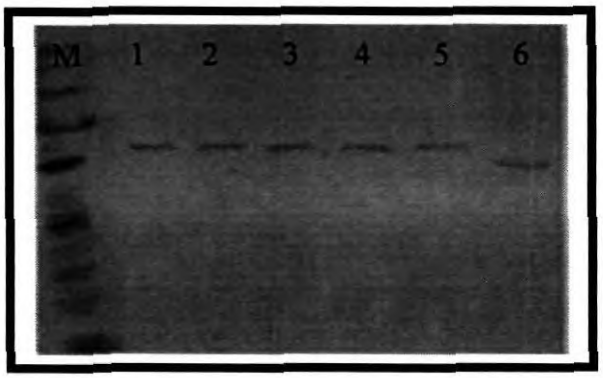


Fig. 107. Confirmation of the induced target proteins

The functional activity of the induced recombinant protein of β-1,3-glucanases was analysed through activity staining as described by Pan *et al.,* (1989) (fig. 108). From the gel it is clear that the lane with Gln 2 is showing more activity than other forms.

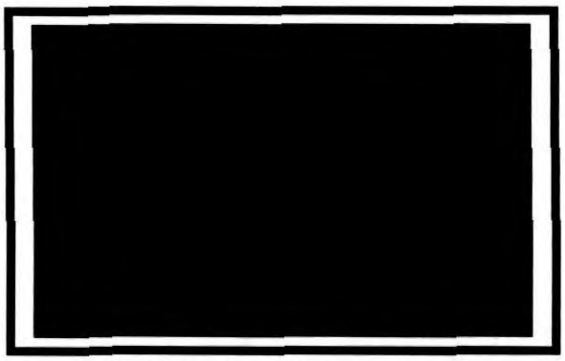
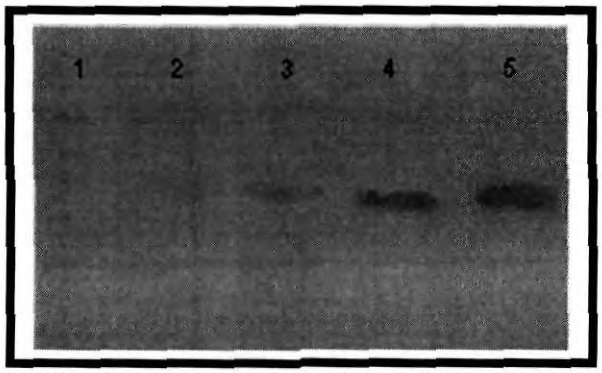


Fig. 108. Activity staining of the isolated protein of the six different glucanases

The activity staining also shows that the proteins purified were β-1,3-glucanase itself. The glucanases could be detected in the gel because laminarin used as substrate can be used by β-1,3-glucanases and the glucose and reducing oligomers released by the enzyme can be stained by the triphenyl tetrazolium reagent. The identity of the induced recombinant purified protein in pPICZ A vector was confirmed through western blotting with the vector encoded fusion partner of the target protein. The development of the western blot was done with the western blot kit (M/S KPL, USA). His-tag monoclonal

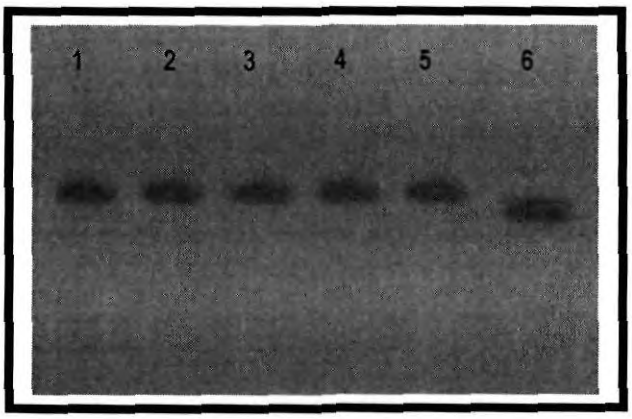
antibody supplied with the kit is a conjugated antbody (the His-tag anibody is coupled with anti-Mouse IgG- AP conjugate) which is having an epitope consisting of 5 consecutive histidine residues, was used as the primary reagent. This will bind with the His-tag sequences encoded by the pPICZ -A vector. Finally the antigen-antibody-anti antibody complexes on nitrocellulose filter were located with the chromogenic substrates NBT and BCIP. The approximately 43.5 kDa and a single 42.5 kDa fusion protein were located separately on the western blot; confirming that the induced proteins are His tagged (fig. 109). The size of the his-tag and c-myc epitope is added with the protein size (approximately 2.5 kb) here.



1 - 0h
2 - 24 h
3 - 48 h
4 - 72 h
5 - 96 h

Fig.109. Western blot analysis of Gln 2 at various time intervals to check for the protein induction

The western blot analysis of the purified protein (Gln 2) at the various intervals has shown that the protein got induced within 24 hrs and the expression got increased in the ascending time intervals (fig. 110). The expression was observed to a maximum by 96 hrs.



1 - Gln 2
2 - Gln 3
3 - Gln 5
4 - Gln6
5 - Gln 7
6 - Gln 4

Fig. 110. Western blot analysis of the purified protein at 96 hrs of all the β-1,3-glucanase forms

The anti-fungal property of purified six *Hevea* β-1,3-glucanases was assayed on PDA plates, which is inoculated with a growing culture of *P.meadii* using the filter paper disc method. The purified recombinant protein preparations were added to the filter discs on agar plate after two days of initial vegetative growth of the fungi. In the following two days distinct inhibition zones were developed around the filters soaked with 12 μg of the purified enzyme solution (fig.111. A & B ; A- after three days of fungal inoculation, B- after fourth day of fungal infection).

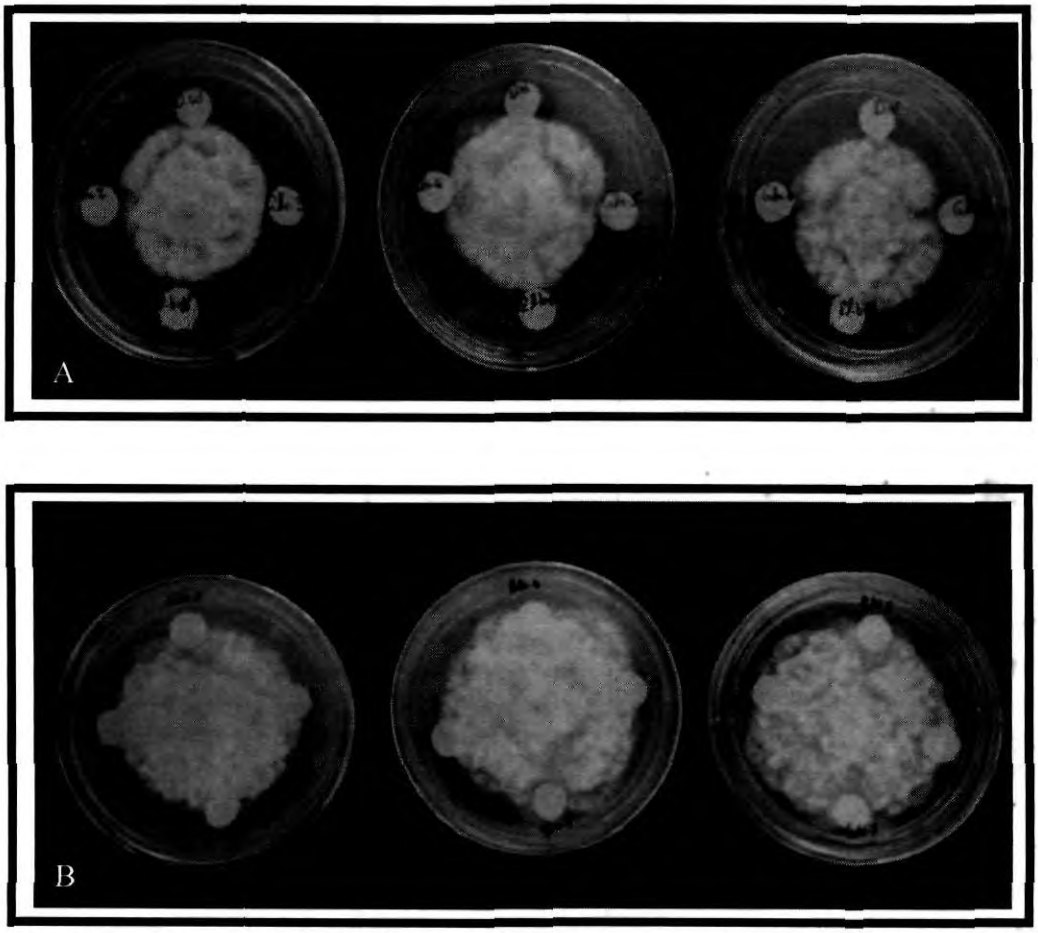


Fig. 111. Antifungal activity of the six glucanases in PDA plates against *Phytophthora meadii* in day 3 (A) and day 4 (B)

Gln 2	**+++**
Gln 3	**+**
Gln 4	**++**
Gln 5	**++-/+**
Gln 6	**++-/+**
Gln 7	**++-/+**

These inhibitory zones remained visible for at least one week and then the fungus started to overgrow the initial inhibition zones. Gln 2 showed the

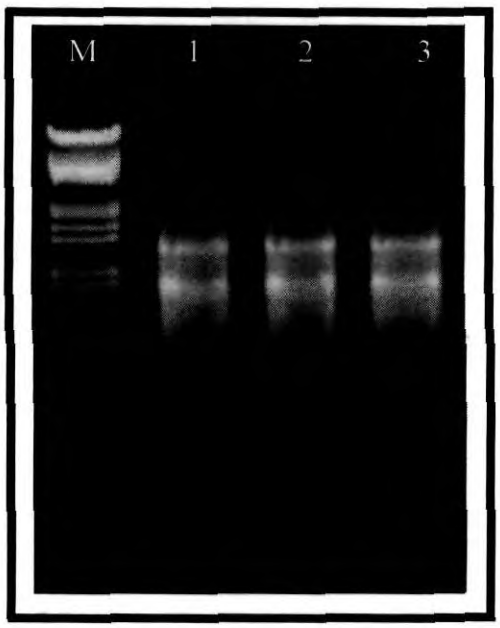
more inhibitory activity compared with the other five forms. Gln 3 and 4 showed less or no antifungal activity at all. The activity can be represented as Gln2 > Gln 5> Gln6> Gln7> Gln4=Gln3.

4.8. Tissue specific expression of Gln 4

Among the six different forms of glucanases isolated, Gln 4 showed maximum variation with respect to nucleotide sequences of the other forms. The present study was aimed to understand the expression of Gln 4 in different *Hevea* tissues. The pathogen induced and the tissue specific expression of this particular isoform was also investigated.

4.8.1. Isolation of RNA from leaf, callus and latex

Total RNA isolated from different tissues like latex, bark and leaf of mature rubber tree was used for the expression studies of Gln 4. The concentration of the isolated RNA was determined and the value of $OD_{260/280}$ was 2.0 indicating that the isolated RNA was pure. Equal amount of total RNA isolated from various tissues were loaded onto a 1% (w/v) agarose gel and fractionated electrophoretically (fig. 112) and the absence of DNA contamination was confirmed.



M - Marker (λ DNA *Eco*R1/*Hind* III double digest)
1 - RNA from leaf
2 - RNA from latex
3 - RNA from callus

Fig. 112. RNA isolated from *Hevea* latex, leaf and callus (*DNase* treated)

4.8.2. Reverse transcriptase PCR (RT-PCR) of Gln 4 from latex, leaf and callus

The RT-PCR was performed using the gene specific primers for Gln 4. Same concentration of the cDNA prepared for each of the three different tissues was used (300 ng) for the RT-PCR. The amplification of the internal control actin gene shows a constitutive pattern in all the three different tissues (Fig 113. A). The results showed that this isoform is constitutively expressing in the latex, callus and in the leaf, but showing a relatively lower level of expression in the leaf as indicated by the band intensities (Fig. 113. B).

Fig. 113. RT-PCR amplification of actin gene and Gln 4 in *Hevea* latex, callus and leaf

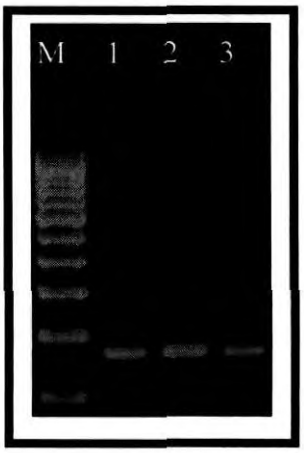


Fig.113.A. Actin gene amplification from the cDNA of *Hevea* latex, callus and uninfected leaf of RRII 105

M- DNA marker (100 bp ladder)
1- Latex
2- Callus
3- Leaf

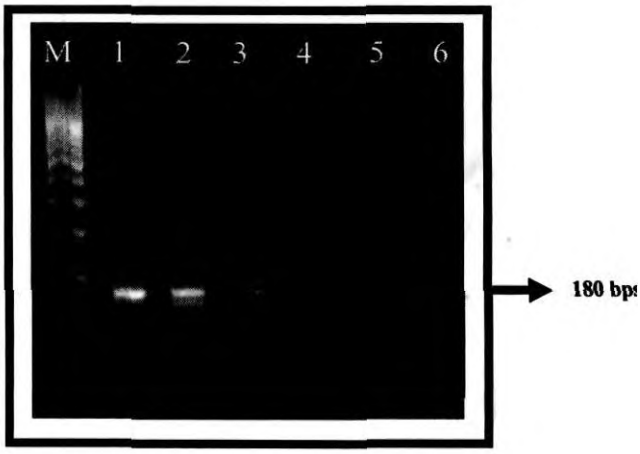


Fig. 113. B. cDNA amplification of Gln 4 from *Hevea* latex, callus and uninfected leaf of RRII 105

M- DNA marker (100 bp ladder)
1 - Latex
2 - Callus
3 - Leaf
4-6 - Amplification using RNA template to check for DNA contamination



+++ +++ +

Among the different tissues such as *Hevea* callus, latex and the uninfected leaves of the clone RRII 105, studies showed that the latex and callus exhibited a higher level of Gln 4 expression than in the leaf tissue. The net intensities of Gln 4 bands amplified from the latex and callus were good and almost similar while in the leaf it gives only a fade band indicating low level of expression when observed in the ethidium bromide stained agarose gels. These results are in agreement with the earlier studies, which reported that β-1,3-glucanases is highly expressed in the latex, than in leaves.

4.9. Phytophthora induced expression of Gln 4 in *Hevea brasiliensis*

4.9.1. Fungal infection to the plant material

When the fresh mycelia of the pathogen were inoculated to 100 ml white oats medium and grown for 2 days under darkness sporulation was occurred. The mycelial mat was washed with sterile water and on exposure to light it was observed that the fungal mat in water produced sporangia profusely after 48 h. At this time the complete mat was found to be studded with sporangia (fig. 114).



Fig. 114. Fungal mat studded with sporangia

A brief heat shock at this stage effectively released the motile zoospores. The medium free zoospore suspension was used for the *in planta*

leaf inoculation of the *Hevea* clones RRII 105 and RRIM 600 under controlled conditions. To maintain the required humidity, the plants were covered with transparent polythene bags and observed for the development of symptoms. The disease symptoms began to appear 2 days after inoculation and were more prevalent in the susceptible clone RRIM 600 than the tolerant one RRII 105. The necrotic spots developed by the hyper sensitive responses induced by the pathogen in the leaf were more in the areas where the inoculum was applied.

RNA was also isolated from uninfected control and the infected leaf samples of both the clones at different time intervals (Fig. 115. A & B). The absence of the DNA contamination was also confirmed.

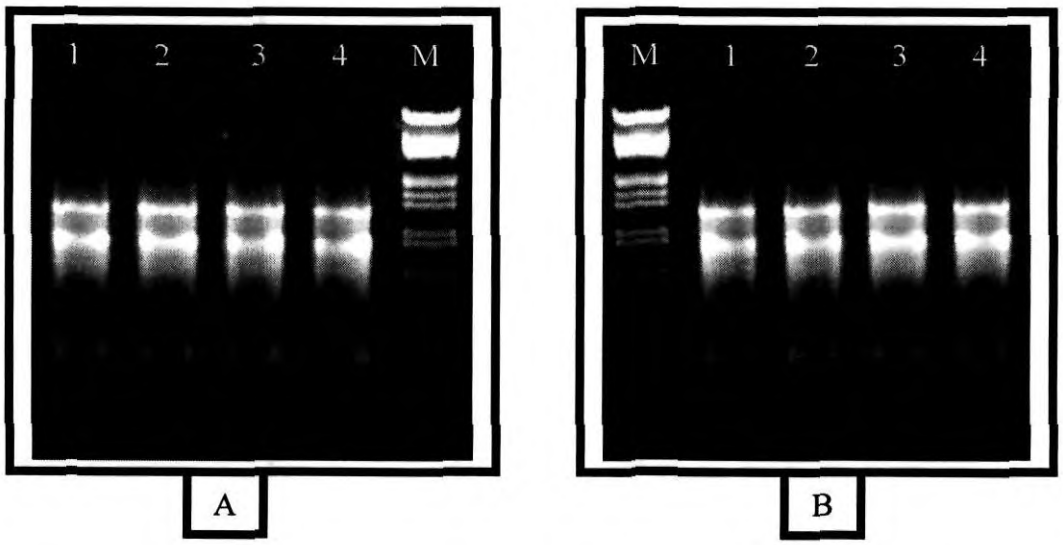


Fig. 115. RNA isolation from RRII 105 and RRIM 600 (*DNase* treated)

A - RNA isolated from RRII 105
Lanes 1- 4: RNA isolated at 0, 24, 48 and 72 hrs
M - Marker(λ DNA *Eco*R1/*Hind* III double digest)

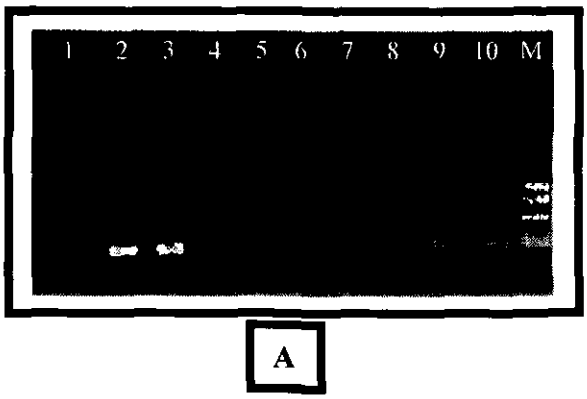
B - RNA isolated from RRIM 600
Lanes 1- 4: RNA isolated at 0, 24, 48 and 72 hrs
M - Marker (λ DNA *Eco*R1/*Hind* III double digest)

4.9.2. RT-PCR

cDNA prepared from the RNA samples of different time intervals representing each clone was used for the RT- PCR (Reverse Transcriptase – PCR). The samples taken from the uninfected leaf was used as the reference at the 0 h. 300 ng of the template cDNA was used for the PCR using the specific primers for Gln 4. Approximately a 180 bp amplicon for Gln 4 and actin were

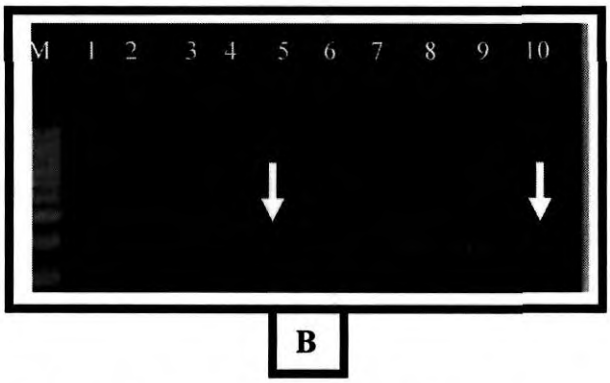
obtained from all the samples. The net intensities of β-glu bands amplified from control samples of both clones were more or less same as observed in the agarose gels. With the Gln 4 specific primers the band intensities were found to be same in the infected samples, of both the RRIM 600 and RRII 105 *Hevea* clones compared to that of the 0h and an increase in band intensity was not observed as reported by Thanseem *et al.*, (2005) with primers specific for a different isoform which was similar to Gln5 in the present study (fig. 116. A & B). However in the susceptible clone RRIM 600, at 96 hrs, a reduction in Gln 4 activity was observed. In RRIM 600, the onset of the disease symptoms and the injury caused to the leaf due to the fungal infection is more compared to that of the less susceptible clones of *Hevea* towards the ALF. The leaf is getting decayed and abscissed within 96 hrs in RRIM 600. This may affect the production and processing of the basic biomolecules in the leaf. May be due to this reason RRIM 600 showed a diminished expression of β-1,3-glucanase and also with the endogenous control actin at 96 hrs.

Fig. 116. Amplification of cDNA sequence of Gln 4 and β-actin gene of the *Hevea* clones RRII 105 and RRIM 600



A: Amplification of cDNA from RRII 105

1-5 : cDNA amplification of the actin gene at 0, 24, 48, 72 and 96 hrs
6- 10 : cDNA amplification of Gln 4 gene at 0, 24, 48, 72 and 96 hrs
M - DNA marker (100 bp ladder)



B: Amplification of cDNA from RRIM 600

1-5 : cDNA amplification of the Gln 4 gene at 0, 24, 48, 72 and 96 hrs
6- 10 : cDNA amplification of actin gene at 0, 24, 48, 72 and 96 hrs
M - DNA marker (100 bp ladder)

Thus the preliminary results show that Gln 4 is not a pathogen inducible form. More detailed study is required to confirm the role and activity of Gln 4 in *Hevea.*

Chapter V

Discussion

Several classes of pathogenesis related (PR) proteins, are induced in response to infection of plants with microbial pathogens. β-1,3-glucanases are included in the family of PR-2 proteins, which are abundant and highly regulated enzymes, widely distributed throughout the plant kingdom. They are able to catalyse the endo-type hydrolytic cleavage of 1,3-β-D- glucosidic linkage in β-1,3-glucans. The cell wall of *Phytophthora*, the causative organism of abnormal leaf fall disease in *H.brasiliensis*, is made principally of β-1,3-linked glucans, which constitutes 80 – 90% of the cell wall dry weight. Therefore, host β-1,3-glucanase enzyme might play a pivotal role in combating the ALF disease in *Hevea*. An understanding about the role of such an established defence-related gene like β-1,3-glucanase would contribute significantly towards the development of sophisticated molecular tools to combat ALF disease in *Hevea*. The antifungal activity of these hydrolase enzymes make them rational candidates for over expression by genetic transformation to produce disease resistant crops (Thanseem *et al.*, 2005). It has already been recognized that the species of higher plants produce a broad range of β-1,3-glucanases differing in primary structure, cellular localization and catalytic activity. Many of them were proved to be effective anti-fungal agents both *in vitro* and when over-expressed in transgenic plants.

Earlier studies at RRII on the expression patterns of the gene coding for pathogenesis-related β-1,3-glucanase (β-glu) enzyme in a tolerant (RRII 105) and a highly susceptible (RRIM 600) clone of rubber tree after infection with ALF-causing *Phytophthora meadii* observed similar expression patterns in both tolerant and susceptible clones initially upon induction with *Phythophthora*. However, the expression was found to be lost or diminished in the susceptible clone RRIM 600 after 48 hrs post – infection period. What governs this process is not well understood. It was assumed that the genomic

sequence variations can be one among the several causes for this differential gene expression in tolerant and susceptible clones. Difference in promoter sequences of the gene may be another reason. Earlier reports on the nucleotide sequences of *H. brasiliensis* β-1,3-glucanase gene from different sources also show nucleotide variations (Accession Nos. AY325498, U22147, AJ133470, AF311749).

Therefore, an attempt has been made for the isolation and characterization of β-1,3-glucanase gene from *Hevea* clones differing in their tolerance and susceptibility so as to find out whether any sequence variations of the gene is imparting tolerance/ susceptibility of the plants against *Phytophthora* attack. Between the ten sequences of the β-1,3-glucanase gene obtained from the corresponding ten different clones, a variation in nucleotide sequence length was observed between the 'ATG' start codon and the 'TGA' stop codon. The variation in their lengths was due to the differences in 'CT' repeats within the intron and minor variations within the exon region.

There are reports saying that the genes coding for β-1,3-glucanase differ in their intron length and also in their repetitive sequences within the introns. In the present study also, the intron sequence were found to have 'CT' repeats and the number of 'CT' repeats vary from 9-21 duplets in length, in the clones studied. The maximum number of CT repeats were observed in the clone TJIR 1 (21 nos). A 131 bp and 137 bp intron was observed in the *Hevea* clones, PR 107 and TJIR 1, which contained 18 and 21 'CT' repeats respectively. The least number was found in the clone RRII 414 with 9 CT repeats. It is observed that although the indel sequences show numerical variations, the location of the particular 'CT' repeats remained the same showing that the splice junctions are conserved among the different clones. While not considering the intron sequences within the full length sequences obtained from the different clones, the exon region alone contained 1125 nucleotides which exactly codes for 374 amino acids and a stop codon. This result is in par with the previously reported sequence of the β-1,3-glucanase

genes from *Hevea* comprising 374 amino acids and the stop codon (Thanseem *et al.*, 2005).

Through the sequence similarity search at NCBI site, 146 Blast hits were obtained with the characterized sequences, including the already reported sequences of β-1,3-glucanase gene in *Hevea.* This indicates that β-1,3-glucanase is encoded by different genomic DNA sequences among the different *Hevea* clones. Very similar findings have been reported for the gene family encoding ribulose- bisphosphate carboxylase small subunit (rbcS), which shows an intraspecific sequence divergence of <10% and an inter-specific divergence of >23% (Sperisen *et al.*, 1991). In eukaryotes, sequence homogeneity can be maintained by DNA exchange resulting from recombination or gene conversion. Several mechanisms have been proposed for maintaining uniformity of amino acid sequences encoded by small gene families. If sequence homogeneity results from selection, then there should be high ratios of silent to nonsilent nucleotide substitutions in pairwise comparisons among different sequences encoding the mature proteins.

All β-1,3-glucanse genes examined so far, perhaps with two exceptions, contain a single intron and similar intron splice junctions also ('GT 'and 'AG'). A β-1,3-glucanase gene (gn1) isolated from *Nicotiana plumbaginifolia* contains two introns (Castresana *et al.*, 1990). The gene encoding the barley β-1,3-glucanase isozyme GI has no intron. The position of the intron is usually the same in all these genes whether they code an acidic or a basic enzyme. The lengths of the intron in different β-1,3-glucanase genes can vary. The introns usually range in size from 400-600 base pairs, but some β-1,3-glucanase genes have shorter or longer introns. For example, introns in three peach β-1,3-glucanase genes are 160, 256 and 677 bp (Thimmapuram *et al.*, 2001; Ko *et al.*, 2003). In comparison, the curled-leaved tobacco β-1,3-glucanase gene has a 975-bp intron (Gheysen *et al.*, 1990). This gene encodes 370 amino acids.

The sequence results obtained indicates major variations among the intron sequence rather than the exons. The observed variations in the

intron/exon sequences and the 'CT' repeats in the introns does not show a uniform pattern between the relatively tolerand and susceptible clones. Thus in the present study this preliminary level attempts could not explain the tolerance and susceptibility of the different *Hevea* clones based on the differences in the DNA sequence observed.

Therefore, it is necessary to go deep into the functions of introns to see whether they play any major role in defence mechanism in plants mainly, the gain/ loss of gene function due to the presence of microsatellite insertions/deletions. Earlier reports show inhibition of gene expression by the presence of dinucleotide repeats in the promoter region of nucleolin gene and inhibition of gene expression by dinucleotide repeats in the introns of the tilapia prolactin 1 (Rothenburg *et al.*, 2001; Streelman *et al.*, 2002). CA repeat deletions in the intron 1 of HSD11B2 led to a significant decrease (40 %) in the expression of this gene in human cortical collecting duct cells and its influence on expression was dependent upon the location and orientation of the CA repeat (Agarwal, 2001). Since there is no report suggesting the deletion of introns associated with the tolerance and susceptibility of eukaryotic plant towards a pathogen attack, studies should be carried out to understand the role of the intron sequences in gene expression. The nucleotide substitutions, deletions and additions seen among the various tolerant and susceptible clones of *Hevea* can be a causative factor for the differential gene expression. This may or may not ultimately lead to functional instability of the protein due to shift in the three dimensional structure.

Since the promoter sequence contribute significantly towards gene expression, the characterization of promoter region of β-1,3-glucanase gene is essential to find out whether any sequence variation in the promoter region affects the binding of transcription factors and thereby affecting the expression level of a particular gene. The possibility that variation in their promoter sequences leading to differential gene expression also cannot be ruled out.

Since the nucleotide variations present in the clones could not explain the real cause for the differential expression of β-1,3-glucanase gene and

existence of tolerance/susceptibility of the major *Hevea* clones towards the ALF disease, studies were conducted to understand the regulation of β-1,3-glucanase gene and also to find out whether the differential expression of the gene observed in the earlier studies in tolerant and susceptible clones of *Hevea* is due to the difference in the regulatory regions (promoter sequence) of the gene. To study the intricate mechanism within the expression of this gene, it is essential to isolate and characterize the regulatory regions *viz* the promoter region of the gene which is harbouring the important regulatory elements, the cis-elements.

In general, the plant promoter architecture is important for understanding the evolution of promoters, where the important regulatory factors/elements involved in gene expression are located. The plant gene promoters are relatively compact, in which most regulatory elements tend to be clustered near the transcriptional start site and in some cases promoter sequence of 1000 bps or even less than that could drive proper regulated patterns of transcription (Guo and Moose, 2003). Understanding the structure of the promoter allows predictions related to promoter positions and expression profiles, and also sheds light on hidden transcriptional networks. Our current knowledge about plant promoter structure, especially with respect to the core promoter, is insufficient. A promoter region is typically a few thousand base pairs long and contains many different binding sites with often complex interactions. These binding sites contain about 10-15 base pairs relevant for the binding of specific transcription factors. The regulation of genes depends on the binding of transcription factors to the specific sites called cis - acting elements located in the regulatory region of the gene. Transcription factors and their binding sites emerge as a suitable starting point for quantitative studies of gene regulation. At the same time the core elements are shorter with about 5-8 relevant base pairs (Berg *et al.*, 2004). They identified that these sites are often organized in modules interspersed between regions containing no sites.

Under uninfected conditions, the β-1,3-glucanase gene is having a very low level expression in the leaf and high level expression in the latex showing

its tissue specificity as indicated by the earlier studies (Thanseem, PhD thesis, 2004). Studies have also shown that the protein is an induced one and is one of the major defence protein that accumulates intracellulary as well as extracellularly. In the present study we have identified different promoters of β-1,3-glucanase gene from the *Hevea* clone RRII 105 followed by sequencing of the PCR product. The inverse PCR technique used was proved successful. The inverse PCR gave rise to four amplification products as bands of different sizes in the agarose gel (1.2 kb, 0.8 kb, 0.76 kb and 0.4 kb). These fragments cover the upstream region of the gene of interest. The sequence revealed the presence of 913, 582, 553 and 198 bp promoter fragments in the corresponding bands amplified.

The four sequences upon analysis showed that the known regions belongs to the β-1,3-glucanase gene. The promoters are entirely different with each other towards their distal ends rather than the region near to the 'ATG' initiation codon. The one with the 198 bp promoter sequence was showing similarity with the earlier reported sequence of β-1,3-glucanase gene (Acc.No. AY325498) from the *Hevea* clone RRII 105. The 5'UTR region also showed minor variations with each other. The gene sequence following the forward primer showed 99 % similarity among the four isolated fragments. These similarities in the known gene portions of the inverse PCR product shows that the four isolated fragments represent multiple forms of promoters which regulates four different forms of β-1,3-glucanase gene. The deduced promoter sequence contains the core promoter elements, TATA boxes and the CAAT boxes positioned in relation to translation initiation codon. Characterizing the complex biological processes need to understand how the regulation of the gene networks is orchestrated. Genes expressed in the same tissue under similar conditions often share a common organization of at least some of these regulatory binding elements. *In silico* analysis has been used with success in the identification of putative regulatory elements in plant promoters (Wei-Min *et al.*, 2005; Pujade-Renaud *et al.*, 2005). Thus the proximal region of β-1,3-glucanase contains the elements shown to be necessary for an accurate initiation of basal transcription in promoters.

For precise and regulated transcriptional initiation, several functional elements have been identified as promoter constituents like the TATA box, Initiator (Inr) motif, Downstream Promoter Element (DPE, found from *drosophila*), TFIIB-Recognition Element (BRE), and the so-called cis-regulatory elements (Butler and Kadonaga, 2002; Smale and Kadonaga, 2003). In addition, some of the plant promoters similar to that of the animal promoters are found to be associated with CpG islands (Antequera and Bird, 1993) and have some relationship with gene regulation by DNA-methylation. Mostly in the plant promoters, the transcription start sites (TSS) have a CG-compositional strand bias, or GC-skew, where C is more frequently observed in the (+) strand than G (Tatarinova *et al.*, 2003 and Fujimori *et al.*, 2005). Some of the above features have been utilized for promoter prediction too (Bajic *et al.*, 2004 & 2006, Sonnenburg *et al.*, 2006). In addition to the identification of targets, effective manipulation of the complex metabolic processes which are targeted will require highly specific promoters able to regulate gene expression in a highly controlled manner, to ensure that spatial and temporal constraints are met. Transactivating proteins constitute a whole realm of molecules in the field of gene regulation and deserve a separate analysis. Here, the identified promoter region also contained other important regulatory elements like WRKY, W box, GT1 consensus, Dof core, Amy box etc. However, the positions and the frequency of occurrence of these core promoter elements differ in each of the sequence identified.

It is the architecture of the promoters that makes the differences in the expression patterns of pathogen-responsive genes. Pathogen-responsive promoters usually are dissimilar in number, order and type of regulatory elements present. This places the promoters at the end of one or more signal transduction pathways as the plant responds to pathogen attack. Despite the differences in architecture, functional dissection of the promoter regions of some of these genes is starting to uncover common *cis*-acting elements and has provided us with the tools to identify distinct DNA-binding proteins required to modulate transcription. It is interesting to observe that some of the cognate factors, for example EREBP and WRKY proteins, appear unique to plants whereas others such as bZIP and MYB proteins also have counterparts

in animals (Rushton and Somssich, 1998). This reflects the fact that plants have developed a unique defence system.

Some of the potential cis-acting elements have been identified as highly conserved sequences in the promoters of specific class of plant genes, such as W box and WRKY sequences in pathogen responsive genes, I box in light inducible gene promoters and GT1 box in promoters of genes responsive to stress (Jaiswal *et al.*, 2007). These elements are also present in the isolated promoter regions of β-1,3-glucanase gene in the present study. The WRKY transcription factors have been shown to induce gene expression in response to wounding (Eulgem *et al.*, 1999). WRKY factors show high binding affinity to DNA sequences designated as W box (C/T)TGC(T/C). The *wrky* gene family has been found in many plants but has not been identified to date in yeast or animal genomes (Zhang and Wang, 2005). WRKY homologues constitute a large family of DNA-binding proteins in plants that are involved in several key cellular functions, including disease resistance, stress response, dormancy and development (Pan *et al.*, 2009). The antifungal activity of β-1,3-glucanase gene supports the authenticity of this observation. WRKY proteins also play a regulatory role in plant development and dormancy (Eulgem *et al.*, 2000; Johnson *et al.*, 2002). WRKY factors act by directing the temporal and spatial expression of specific genes thereby ensuring proper cellular response to internal and external stimuli (Ulker and Somssich, 2004). In *Arabidopsis thaliana* more than 70 members of the WRKY family are present which have diverse roles in response to various pathogen and environmental conditions (Eulgem *et al.*, 2000) and the mRNA levels of these *wrky* genes also increased in response to these changing environments (Ulker and Somssich, 2004; Dong *et al.*, 2003). In parsley, three WRKY proteins, WRKY1, -2 and -3, bind specifically to functional W Boxes in the *PR1-1* and *PR1-2* promoters (Rushton *et al.*, 1996).

A number of defence related genes in plants have multiple copies of W-boxes in their promoters that are recognized by WRKY proteins and are necessary for the inducible expression of these genes (Droge-Laser *et al.*, 1997; Kawagoe and Murai, 1996) and most of the *wrky* genes in plants have

W boxes in their own promoters, suggesting a positive or negative auto-regulation of the *wrky* genes (Droge- Laser *et al.*, 1997; de Pater *et al.*, 1996). These factors have been identified as downstream components of a defence- and pathogen induced mitogen-activated protein kinase (MAPK 2) signaling pathway in plants (Martin and Paz-Ares, 1997). The transcription of WRKY genes is strongly and rapidly up regulated in response to wounding, pathogen infection or abiotic stresses in numerous plant species. WRKY proteins are also involved in other plant specific processes such as trichome development, senescence and the biosynthesis of secondary metabolites (Eulgem *et al.*, 2000). The antifungal activity of β-1,3-glucanase gene supports the authenticity of this observation. Much variation in number and type of cis-acting elements was observed between these promoters. WRKY factors, being transcriptional regulators, can act by directing the temporal and spatial expression of specific genes thereby ensuring proper cellular response to internal and external stimuli (Ulker and Somssich, 2004). These elements invariably contain TGAC core, which is essential to function by WRKY factor binding.

The WRKY domain structure composes about 60 amino acid residues and has a conserved WRKYGQK sequence which is also followed by a zinc finger-like motif at the carboxy terminal side (Rushton *et al.*, 1996; de Pater, 1996). Zinc-fingers are protein regions containing cysteine and/or histidine residues which are predicted to form a finger structure by binding a single zinc atom. In several cases, it has been demonstrated that the zinc-finger region and zinc are required for DNA binding of the protein. In other cases, however, the zinc-finger constitutes an interface for interaction with other specific proteins (Mackey and Crossley, 1998). All known WRKY proteins contain either one or two WRKY domains. There are three groups of WRKY proteins which are classified on the basis of both the number of WRKY domains and the features of their zinc finger-like motifs. All of these WRKY domains have similar sequence specificities for DNA binding (Eulgem *et al.*, 2000). Here all the promoter sequences identified for the β-1,3-glucanase gene possess varying number of WRKY elements. Four WRKY elements are present in the

promoter with 553 bps, three with that of 582 bp promoter two with the 913 bp promoter and one with the 198 bp promoter.

W Boxes were present in all the isolated promoter fragments of the glucanase gene in the present study. It is also reported in 80% of genes identified as pathogen inducible (Chen *et al.*, 2002). Among the two most studied types of W Box (Rushton *et al.*, 1996) elements; the first contains a hexamer sequence 'TTGACC' whilst the second consists of the two tetramer half sites TGAC-Nx-GTCA (Rushton & Somssich, 1988). A W Box in the parsley PR-10 class gene, *PR1-1*, is the site of a fungal elicitor-inducible DNA-binding activity (Meier *et al.*, 1991). A similar activity also binds to a W Box in the promoter of the tobacco chitinase gene *CHN50* (Fukuda, 1997). Earlier reports have shown not only that W boxes are functional elicitor response elements in the parsley *PR1* genes (Rushton *et al.*, 1996), the maize PR-1 class gene *PRms* (Raventos *et al.*, 1995), and the tobacco class I chitinase gene *CHN50* (Fukuda, 1997; Warner *et al.*, 1993) but also that they are sufficient to direct elicitor responsive expression in transient expression systems (Rushton *et al.*, 1996, Raventos *et al.*, 1995). Other pathogen-responsive promoters contain W box sequences within functional areas of the promoters. These include the potato glutathione *S*-transferase *gst1* (*prp1*) (Hahn and Strittmatter 1994), PR10 genes from asparagus (*AoPR1*) (Warner *et al.*, 1993) and potato (*PR-10a*) (Matton *et al.*, 1993), and the stilbene synthase gene, *Vst1*, from grapevine encoding an enzyme of phytoalexin biosynthesis (Schubert, 1997). Thus, W Boxes may be a general feature of a large subset of pathogen-responsive gene promoters. W Boxes are binding sites for the WRKY family of DNA-binding proteins (Rushton *et al.*, 1996; de Pater, 1996).

Several Myb consensus elements were observed in the isolated promoter sequences. Myb proteins have been known to play important roles in various developmental processes such as controlling secondary metabolism, regulating cellular morphogenesis and serving in the signal transduction pathways responding to plant growth regulators. Some plant Myb elements regulate the expression of PR proteins (Jin and Martin, 2000). In cysteine

proteinase gene promoter of barley, Myb elements are reported as involving in gibberellic acid induction. The tobacco *MYB1* gene is induced by TMV during the hypersensitive response and the development of systemic acquired resistance.

The combination of different cis-elements and trans-acting factors as observed in these isolated promoter fragments, may produce the diversity and specificity required for the regulation of gene expression. There may be one or more sites for the same factor in one promoter region. At the same time, the sequences of binding sites are fuzzy, i.e. different sites for the same factor differ by about 20-30 percent of the bases relevant for binding (Berg *et al.*, 2004). This makes the identification of sites a difficult bioinformatics problem. Several such elements are screened from the isolated glucanase gene promoters. One of the frequent elements observed in the promoter sequences other than the WRKY and W boxes was the 'Dof protein binding site'. This seems to be a key factor for the response to light in leaves. Dof domains act as transcription activation domains *in vivo* (Yanagisawa, 1997). There are 5-12 such elements observed in these promoter sequences of the β-1,3-glucanase gene. The presence of several other light regulatory elements including I-box, GT1 and Dof, suggests that light may have role in regulation of the gene expression. Variation in number and type of cis acting elements was observed between the promoters which can be correlated to the length of the promoter region identified.

It was also identified that the 'TAAGAGCCGCC' motifs (GCC-boxes) which are found to be present mostly in the pathogen inducible promoters, were absent in all the four promoter sequences. It has been reported that the transcriptional elements responsive to salicylic acid (SA), ethylene and TMV are present in the -0.45 to 1.5 kb region of the 1.5 kb tobacco *Gglb*50 promoter. The enhancer activity and the ethylene responsiveness depend on the integrity of two copies of the AGC box, AGCCGCC, present in the promoters of several ethylene responsive genes. It is obvious that the differences in the number, order, type and occurrence of the regulatory elements in these isolated promoter fragments of β-1,3-glucanase gene can

also cause difference in their expression patterns towards various biotic/ abiotis stresses and also in various tissues. The cloned gene promoters from *Hevea* would be useful for future investigations on the disease regulation of *Hevea brasiliensis* against a variety of fungi. It is suspected that the low level of glucanase gene expression in the leaf may be a limiting factor in tolerating the fungal attack especially in the susceptible clones of *Hevea* towards the abnormal leaf fall disease. This is the first report showing the presence of different forms of β-1,3-glucanase present in a single host of *Hevea brasiliensis.*

There are promoters controlling gene expression in a tissue-dependent manner and according to the developmental stage of the plant. In order to have a minimal effect on overall growth and development of transgenic plants with novel phenotype, it is desirable to use tissue specific promoters rather than constitutive promoters. It is advantageous in the respect that the transgenes which are driven by these type of promoters will only be expressed in tissues where the transgene product is desired, leaving the rest of the tissues in the plant unmodified by transgene expression. These tissue-specific promoters may be induced by either endogenous or exogenous factors, so they can be classified as inducible promoters as well. Rushton and Somssich (1998) suggests that the expression of pathogen-responsive genes show both a temporal and spatial hierarchy. Some genes undergo a rapid localised activation at the site of infection, whereas others are more slowly activated either locally and/or systemically. It is ideal and important to target the foreign gene expression precisely or if possible specifically where it is needed. This approach is dependent on availability of promoters specific enough to limit the expression of transgene particularly in the organ or tissues of interest. Still there are relatively few alternatives or adjuncts of such promoters, yet such promoters are needed for the delivery of multiple transgenic traits and help to avoid the risk of transgene silencing by the repeated use of the same promoter. The CaMV 35S promoter has been widely applied in various plant species as a powerful regulator that can provide a constitutive gene expression. Several investigators have proposed potential hazards associated with the use of

CaMV promoter in genetically engineered crops (Kohli, 1999). Transcription regulatory regions in higher eukaryotes, represented by cis-regulatory modules are responsible for specific spatial and temporal gene expression patterns. Unlike constitutive expression of genes, tissue-specific expression is the result of several interacting levels of gene regulation. As such, it is preferable to use promoters from homologous or closely related plant species to achieve efficient and reliable expression of transgenes in particular tissues. This is one of the main use for the β-1,3-glucanase gene promoters from *Hevea brasiliensis*.

Eukaryotic genomes are packaged into nucleosome particles that prevent the DNA from interacting with other DNA-binding proteins. The isolated promoter regions of β-1,3-glucanase gene forms showed high nucleosome forming potential in some regions, typical to the promoter sequences as they exhibit intermittent high and low nucleosome forming tendencies. Here the nucleosome packaging density was analyzed with the different functional types of glucanase gene promoters through the RECON programme. The nucleosome forming potential of the isolated promoter fragments revealed regions with greater nucleosome potentials in all the promoter fragments isolated. It was found earlier that in promoters of tissue-specific genes, the nucleosome formation potential was essentially higher than with the genes expressed in many tissues, or the housekeeping genes. Hence, the capability of nucleosome positioning in the promoter region may serve as a factor regulating gene expression (Levitsky *et al.*, 2001). The gene transcription is dependent usually on cis-element positioning and chromatin structure. Hence this nucleosome positioning may act either positively or negatively on the gene regulation by these newly isolated promoters.

Richmond and Davi in 2003 identified the 1.9-Å-resolution crystal structure of the nucleosome core particle containing 147 DNA base pairs revealing the conformation of nucleosomal DNA with unprecedented accuracy. The DNA structure is remarkably different from that in oligonucleotides and non-histone protein–DNA complexes. The DNA base-pair-step geometry has, overall, twice the curvature necessary to accommodate

the DNA superhelical path in the nucleosome. DNA segments that bent into the minor groove are either kinked or alternately shifted. The unusual DNA conformational parameters induced by the binding of histone protein have implications for sequence-dependent protein recognition and nucleosome positioning and mobility. There is a dynamic balance between packaging regulatory sequences into chromatin and allowing transcriptional regulators to access these sequences so as to regulate the gene transcription. Often the access to the cis-elements by the transcription factors is restricted by the nucleosomes, but these can be repositioned or ejected by enzymes known as nucleosome-remodellers. In turn, the DNA sequence can also impart stiffness or curvature to the DNA, thereby affecting the position of nucleosomes on the DNA, influencing particular promoter 'architectures' (Cairns, 2009). These facts can also be applied to the different β-1,3-glucanase gene promoters which in turn can cause different regulatory patterns of the gene. Experimental evidence shows that DNA packaging can control accessibility of specific sequences by blocking access to irrelevant nonfunctional sites (Sekinger *et al.*, 2005; Yuan *et al.*, 2005). Recently in the genome-wide studies in yeast, it was found that constitutive and regulated genes have architectures that differ in terms of nucleosome position, turnover, remodelling requirements and transcriptional noise. The rather high values of nucleosome potential in the promoter region may be related to the fact that expression of this region is inducible and tissue-specific. The nucleosome structure is formed by the binding of DNA by the histones. The core of the nucleosome contains an octamer protein structure consisting of 2 subunits each of H2A, H2B, H3 and H4. The internucleosomal DNA is occupied by the Histone H1 which is identified as the linker histone. The core of the nucleosome contains approximately 150 bp of DNA. The length of the linker DNA between each nucleosome can vary from 20 to more than 200 bp. These nucleosomal core structures would provide a "beads-on-a-string" structure if the DNA were pulled into a linear structure and observed under an electron microscope.

Fig: 117 shows the diagrammatic representation of the nucleosome.

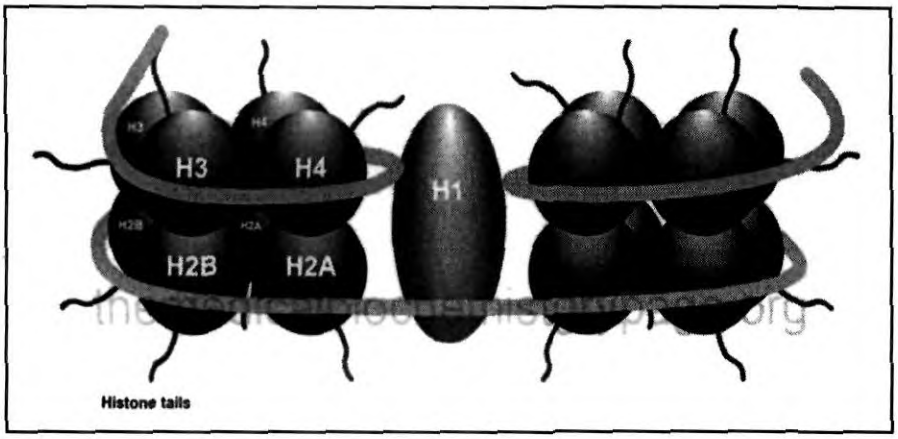


Fig. 117. Diagrammatic representation of the nucleosome
(http://themedicalbiochemistrypage.org/dna.php)

Additional information could be extracted by predicting acetylation and methylation of nucleosomes to identify sequences with regulatory activity (Heintzman *et al.*, 2007). There are evidences that promoter lengths include functional information and several categories of genes have been shown to have promoters longer or shorter than promoters of the other genes in the genome. The sequence-directed nucleosome positioning selectively expose functionally important DNA sequences by constraining their locations to the linker region or rapid accessibility to functionally important sequences by constraining their location within the core particle (Svejstrup, 2003). An increase in nucleosome density has been demonstrated in promoters of genes requiring fine-tuning (Jaiswal *et al.*, 2007). Hence the high nucleosome formation tendency showed by the promoter regions of β-1,3-glucanase gene shows that these genes are tightly regulated.

The promoters isolated also vary in the presence of CpG islands which usually prevents the methylation. Genes which are constantly switched on-the "house keeping genes" usually contain CpG islands. These genes usually have a broad tissue pattern of expression. Genes which are relatively tissue specific are also regulated by CpG island methylation (Antequera and Bird, 1993). Here, the isolated promoter fragments of 913 and 553 bps showed the presence of 1-2 number of CpG islands. The sequence with the 847, 582 and 198 bps promoter was not showing any presence of the CpG islands in the

isolated regions. It is interesting to observe that eventhough there is very high sequence similarity in the promoter region containing the 847 and 913 bps, no CpG islands were observed in the 847 bp promoter whereas 2 CpG islands were observed in the 913 bp promoter. This further proves that the gene with the identified 913 bp and the 553 bp promoter fragments may be constantly switched on in the host plant. Since CpG is one of the main sites of methylation in plant genomes, the potential methylation level of each of these gene is to be evaluated and compared with each other. For this, further upstream regions of these individual promoters are also required.

In most of the cases these CpG sites are not methylated if the gene is actively expressed and methylated in cells with little or no transcription of the gene (Bird, 1995). It is also very important to note that non-methylated CpG island within the promoter region is not always associated with actively transcribed gene. For the transcription of the gene the lack of methylation of the CpG island within the promoter region of the gene is required. This modulatory role of methylation is reflected by the fact that chemically induced de-methylation of CpG islands associated with inactivated genes leads to their partial reactivation (Singer-Sam and Riggs, 1993). The CpG dinucleotides within the promoters without CpG island are usually methylated in a tissue-specific manner and can reflect the transcriptional status of the genes. In the case of CpG island containing promoters, the lack of methylation is usually associated with the chromatin pattern of actively transcribed genes, as characterized by an opened nucleosome configuration, reduced amount of histone H1 and presence of acetylated histones (Tazi and Bird, 1990). Thus the presence of nucleosomes and CpG islands are inter-related.

The PR (Pathogenesis-related) proteins comprises a vast and heterogenous group of proteins which are induced in plants by pathogen infection and exogenous chemicals. These proteins usually take part in the systemic acquired resistance (SAR) that develops in a resistant plant upon infection with a pathogen. As these proteins are responsive not only to pathogens but also to chemicals, the promoter sequences of these proteins have become attractive as inducible expression systems in plants. For this

purpose, promoter sequences from diverse PR proteins have been isolated from plants such as *Arabidopsis* and maize. Recently, these systems have been developed for human cells as well. The differences in the promoter sequence may contribute to the constitutive, induced and differential expression of the protein in *Hevea brasiliensis*. It was found that among the β-1,3-glucanase isoforms present in soybean hypocotyls and leaves, some isoforms may be constitutively expressed in plant tissues, but certain isoforms may be induced and accumulated either in all plant organs or only in a particular organ after pathogen infection and chemical stress. Some isoforms exhibit high levels of activity throughout the pathogenesis, where as other isoforms contributes to symptom expression only at the late stage of infection (Yi and Hwang, 1996). The promoter – gene fusion studies are required to learn the activity of the different promoters upon induction and non – induction of the pathogen. Promoters of hevein (Pujade-renaude *et al.*, 2005; Saleena *et al.*, 2006), REF (rubber elongation factor) (Acc. No. AY712939), β-1,3- glucanase (AY325498), farnesyl diphosphate (EU090239), 3-hydroxyl-3-methyl-glutaryl-coenzyme A reductase I (HMGR1) (EU159430) genes were reported recently from *Hevea brasiliensis*.

It is worth mentioning that all the isolated β-1,3-glucanase gene promoters are highly conserved in the 5'UTR regions, core promoter region, upto TATA box, whereas the homology decrease towards the upstream 5` end. This highlights the probable difference in the expression profile of the coding gene downstream to them due to the variation in the number and order of cis-regulatory elements residing in upstream region of the promoter.

In the study, as the isolated promoter sequences are expected to share small conserved sequence motifs, which may not figure in a BLAST search. The availability of large number of sequences, as phylogenetic foot printing has become an important tool to identify cis-regulatory elements in the promoter sequences. Although not complete, the availability of cis-acting regulatory databases and tools of bioinformatics help to predict the transcriptional properties of new-entry sequences with considerable accuracy.

The authenticity and certainty of a newly identified conserved motifs increase as the number of species providing nucleotide sequence increases in the analysis. The similarity shown by a small portion of the *Hevea* 913 bp promoter region of glucanase with that of the Zebra fish sequence is found interesting. Earlier several categories of genes have been identified with longer or shorter promoters than the rest of the genes in the *S. cerevisiae* genome (Kristiansson *et al.*, 2009). Furthermore, it has been shown that differences are conserved in several other species which suggests that evolution adapts promoter length according to gene function.

It is reported that the promoter length and gene function are related in yeast, fungi, and plants. In particular, the promoters for stress-responsive genes are in general longer than those of other genes. Essential genes have, on the otherhand, relatively short promoters. The findings can be utilized to identify the relevant cis-regulatory elements in a set of coexpressed genes. Our results suggest that genes with complex transcriptional regulation tend to have longer promoters than genes responding to few signals. The core type of a promoter determines not only transcriptional characteristics but also evolutional tendency. Regulatory regions are often found upstream of the core promoter region. The structure of a promoter is recognized by the presence of known promoter elements. Therefore, accurate recognition of the promoter structure of β-1,3-glucanase gene promoters relies on the comprehensive list of promoter elements within the isolated regions as well as in the non-identified regions.

The isolation of different promoter regions of β-1,3-glucanase gene speculate the existence of multiple coding regions for the gene. So later the attempts were carried out to isolate the downstream regions of the identified promoter regions. From a biotechnology standpoint, these discoveries may provide valuable informations to adopt gene transfer strategies aimed at improving plant tolerance to pathogens.

In the present study six new genomic sequences of β-1,3-glucanases has been obtained. The sequences revealed that they belong to different forms

of β-1,3-glucanase genes regulated by different promoter sequences. The biological significance of β-1,3-glucanases gene multiplicity and the relationship between sequence and function, however, remains unclear. The distribution of β-1,3-glucanase genes may protect against catastrophic losses of these genes by unequal crossing over or gene slippage that might occur if β-1,3-glucanase genes were clustered at a single locus (Dover, 1993). In peach two β-1,3-glucanasase have been identified PpGns 1 and PpGns2. PpGns2 product displays only two differences from PpGns1 in the signal peptide and one in the (partial) mature protein (141 amino acids). The 5'-flanking promoter regions of these two genes share 90% identity in nucleotide sequences interrupted by five major gaps (4-109 nt long). The promoter region of these two contained various sequences similar to cis-regulatory elements present in different stress-induced plant genes (Thimmapuram *et al.*, 2001).

There are many isoforms of β-1,3-glucanases reported in a variety of crops. The diversity of β-1,3- glucanases function is mirrored by its large gene family size (Jin *et al.*, 1999). Three peptide sequences were obtained from β-1,3-glucanase that had been purified from eggs of the sea urchin *Strongylocentrotus purpuratus* (Bachman and McClay, 1996). A number of distinct sequence classes have been defined in tobacco (Linthorst *et al.*, 1990), barley (Hoj and Fincher, 1995), soybean (Jin *et al.*, 1999) and arabidopsis (Dong *et al.*, 1991; Hird *et al.*, 1993; Delp and Palva, 1999). Studies aimed to analyse the gene mapping in soybean showed the presence of 12 soybean classes of β-1,3-glucanase genes comprising >40 genes (Jin *et al.*, 1999). Sequence and phylogenetic analyses of these β-1,3-glucanase genes showed that members of the same β-1,3-glucanase class, as well as closely related classes, often mapped to the same linkage group or to duplicated regions of linkage groups. Seven β-1,3-glucanase genes were located on the long arm of chromosome 3 in barley (Li *et al.,* 1996). Though the genes on the same chromosome seem to be closely related to one another they are substantially different from those on other chromosomes (Muthukrishnan *et al.* 1984). About five distinct classes and a total of 12 β-1,3-glucanase genes have been identified in tobacco (Felix and Meins 1986; Linthorst *et al.* 1990; Ori *et al.* 1990; Payne *et al.* 1990; Bucciaglia and Smith, 1994). This suggests that the

duplications of large chromosomal domains followed by extensive gene duplication and divergence and also the duplication of individual genes are involved during the evolution of the β-1,3-glucanase gene families.

In the present study, initially four genomic forms of β-1,3-glucanase gene isoforms were identified while checking whether the different promoter sequences belonged to the different forms of glucanase genes. For that specific primers were chosen from the promoter regions of each of the different forms of glucanase and the reverse primer was chosen from the conserved region which includes the stop codon also. All the forms except Gln 4 (the one with the 582 bp promoter) were amplified with these primer combinations. Gln 4 has been amplified in full length later when the reverse primer region was changed from the genomic region towards the 3'UTR region. This is due to the sequence variations in the reverse primer region upon which the concerned primer could not bind during the PCR. These sequence results also revealed that the four isolated promoter sequences belonged to four different forms of β-1,3-glucanases. On comparison of the nucleotide sequences, minor variations were observed in the exon region, with significant variation in the intron length particularly 'CT' repeats. While amplifying the promoter region of Gln 6 gene which harboured the 913 bp promoter sequence, a new form of β-1,3-glucanase gene (Gln 5) with identical promoter (847 bps) except for the absence of some of the internal sequence of the promoter present in the Gln 6 gene was obtained. A new form of β-1,3-glucanase has been obtained through the PCR amplification from the tolerant *Hevea* hybrid (towards ALF) FX 516 which was not amplified in RRII 105 (moderately tolerant towards ALF) and RRIM 600 (susceptible towards ALF under Indian conditions). This form of glucanase designated as Gln 7 was more similar in its genomic sequence with the Gln 2 and the earlier report – AY325498 (alignment score-98). The presence of additional glucanase might confer a synergistic glucanase activity in this host plant. The DNA structural changes revealed by the isochore patterns of all these different forms did not show significant variations due to the differences in the 'GC' content. These variations also influence the frequency, the extend of transcription, and the time to generate the final translated product. It is already suspected that

differences in 'GC' content could differentially modulate nucleosome assembly in the exons and introns. Exons are differentially marked from the introns both in terms of nucleosome occupancy and in terms of specific histone modifications. The basis for this different chromatin landscape lies within the DNA sequence itself. This implies that splicing signals, which were previously thought to act only at the RNA level, may also be meaningful at the DNA level and may mediate the observed differences in the chromatin landscapes of exons and introns. The differences in 'GC' content could differentially modulate nucleosome assembly in exons and introns. There is a preferential binding of the nucleosomes to the exons rather than with the introns. It was also observed that the GC content in exons was significantly higher than in the flanking introns, the coupling between chromatin structure, transcription and splicing (Schwartz *et al.*, 2009).

Usually the introns are characterized by very low 'GC' values and by a slope close to that of coding sequences, thereby showing a very low nucleosome forming potential. Here also the single intron present in the β-1,3-glucanase genes isolated showed very low nucleosome forming potential as observed from the isochore patterns. It was observed that in the gene itself the nucleosome position usually varies according to the type of nucleotide sequence present in a gene. This drives to the fact that the nucleosome potential as well as the chromatin structure can be varied depending on the presence of the 'GC' content and the repetitive sequences occurring in the exons as well as the introns. The exons and splice junction sequences often reside in nucleosomes (Denisov *et al.*, 1997; Kogan and Trifonov, 2005). The intron can act as the interspace region between the nucleosome. In the present study also there is a chance of the intron occupying the interspace region (117 bp intron nucleotide) which is also showing a less potentiality in the isochore patterns. With reference to all these aspects it can be hypothesized that deletion of intron nucleotides can favour speedy transcription to take place rather than with large intron with additional nucleotides.

Many reports state the prompt response of some of the glucanase isoforms upon induction with the biotic/ abiotic stress with increased activity.

(Anuratha *et al.*, 1996; Masoud *et al.*, 1996). This can be due to the structure of the gene which governs the chromatin structure, transcription, splicing, the regulatory elements in the promoter, methylation and also the final confirmation of the protein which is decided by the amino acid sequence. The variations in the intronic nucleotides found within these isoforms can contribute to the functional regulation by chromatin structuring to some extend. Though isochore patterns is usually implied upon long stretches of DNA sometimes small segments of DNA also give interesting results. Studies in *Arabidopsis thaliana* shows that the differences in 'GC' content among isochores are mainly due to the 'GC' content variation of introns, the third codon positions and intergenic regions (Zhang and Zhang, 2004). All these factors are considered so important because these are very critical for a gene to be transcribed to a final functional protein. A single nucleotide difference within DNA can cause a single change in the amino acid sequence of a protein. Because protein structures are the result of their amino acid sequences, some changes can dramatically change the properties of a protein by destabilizing the structure or changing the surface of the protein in a way that changes its interaction with other proteins and molecules. Often, a single amino acid change within the sequence of a protein does not change its behavior and interactions with other molecules, but it does destabilize the structure.

The promoter of Gln 5 (847 bp promoter) was similar to the Gln 6 (913 bp promoter) and the gene sequence of Gln 5 was found similar to the report by Thanseem and Thulaseedharan (2003) (NCBI accession: AY325498) with an alignment score of 98. The reported sequence of β-1,3-glucanase by Sponer *et al.* (2005) and Yeang and Chow (2000) is exactly matching by 100%. Likewise the isolated new form of glucanase, Gln2 with the isolated 553 bp promoter is exactly matching with the 3EM5 sequence which is the crystallographic structural sequence of a β-1,3-glucanase from *Hevea*. Within the isolated forms, Gln 4 is showing maximum variations with the other forms including variation in the stop codon. The Gln4 sequence is having 373 amino acids while all the other forms had 374 amino acids. The amino acid at the 21st position (methionine) is absent in Gln 4. The molecular weight of all these

forms are around 41 kDa except for Gln 4 (40 kDa). The pI varies slightly and ranges between 8-9 with all the forms. The maximum bascicity observed was with Gln 2, Gln 4 and Gln 7 among the seven forms. The hydropathy plots showed approximately 17 potential hydrophilic peaks in the Hopp-woods scale which is an indicator of the antigenic potential regions of these peptides. In Gln 3 only 16 antigenic potential sites are there. It has been reported that isoforms differs in the transcript termination, alternative splicing and alternative promoter usage (Bemmo *et al.*, 2008).

The presence of the signal peptide was analysed through Signal P analysis. 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 which 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 (Jannick *et al.*, 2004). The predicted signal peptide of the isolated sequences showed a signal peptide probability of 0.9 with cleavage sites between positions 36 and 37 in all the forms except with Gln 4 in which the cleavage site was in between the 35^{th} and 36^{th} nucleotide. The region is similar to the earlier reports of β-1,3-glucanaes from *Hevea*. The PlantMPLoc prediction showed that all the new forms isolated is directed towards the vacuole. The 3'UTR analysis proved that these different forms differ in their downstream 3'UTR regions as indicated by the variability in the binding of the three sets of UTR specific reverse primers. The 3'UTR primers were synthesised based on a reported mRNA sequence by Chye and Cheung (1996) (NCBI Accession No – U22147). The degree of binding of these primers varied in Gln 2, 3, 4, 5, and 6 as indicated by the non-amplified bands in some forms. The Gln 3 got amplification with all the three primers used indicating the close similarity of the 3'UTR region with that of the earlier report. With the different forms of a single gene of other reports whose the 3'UTR region is known, it can be seen that they differ in their 3'UTR length and also in the number of poly A in the poly A tail. The location of the poly A tail also differs in the isoforms. The

Hevea cis-prenyl transferases are an example for this. When the effect of the 3'-UTR on both poly (A) and poly(A)$^+$ mRNA was examined, by increasing the length of the 3'-untranslated region (UTR) on expression of luciferase mRNA in transiently transfected carrot protoplasts it was observed that for poly(A)- mRNA, there was a consistent increase in expression when the length of the 3'-UTR was increased from 4 to 104 bases. For poly (A)$^+$ mRNA, expression increased 18-fold when the length of the 3'-UTR was increased from 7 to 27 bases. Further increase in the length of the 3' UTR did not affect expression. The increase in expression was largely due to an increase in translational efficiency. These data suggest that the length of the 3'-UTR plays an important role in determining the extent to which a poly (A) tail can stimulate the translation of an mRNA (Tanguay and Galley, 1996).

The Clustal W multiple sequence alignment and the phylogenetic tree analysis of the amino acid sequence also follows the statement for the existence of multiple genomic forms of β-1,3-glucanase in a single clone of *Hevea brasileinsis*. There are differences between the earlier reported forms and also with the new forms of β-1,3-glucanases. It is reported that the multigene families encoding the β-1,3-glucanases are large and all the member genes clearly have a common ancestral origin. It is to be noted that the amino acid sequences of the isolated β-1,3- glucanase genes and the earlier reports of the same gene showed 82-99 % identity with each other showing that they have undergone considerable divergence. In tobacco, three classes of β-1,3-glucanase cDNA clones differ only at 18 positions in 1055 bases of overlapping sequence (Shinshi *et al.*, 1988). This high level homology is maintained by the inter genomic DNA exchange between the genes (Sperisen *et al.*, 1991). In soybean, 2 classes of β-1,3-glucanase genes (*SGlu8* and *SGlu9*) differ at only six positions in 700 bases. The reason for this high level homology of these different forms of β-1,3-glucanase gene in *Hevea* remains fascinating.

Using the bioinformatic approaches which are standardized and optimized, it can be used for the swift identification of potential antigenic regions. Till now there are no reports explaining the details of the antigenic

epitopes of β-1,3-glucanase enzyme. In our study we made an attempt to analyze the comparative abundance and distribution of antigenic epitopes in the β-1,3-glucanase sequences obtained. Antigenic epitopes identified through the EMBOSS-Antigenic software in this study showed the abundance of hydrophilic residues in the β-1,3-glucanase protein motif. There were 17 antigenic epitope sites which are more or less conserved in all the sequences despite a very small differences caused by a shift of one or two amino acid positions due to the shift in the ORF. It was also observed that in the Gln 3 peptide sequence only 16 antigenic epitopes are present apart from the 17 epitopes in others, which can provide less antigenicity. Eventhough the epitope variation is there it still conserves the structure. This indicates that providing minor variations in the glucanase protein one could alter the antigenicity of the protein without much affecting the protein structure and the function.

The majority of cell surface and secreted proteins are glycosylated, with carbohydrates covalently attached through either a nitrogen atom (supplied by the amino acid asparagine) or an oxygen atom (supplied by serine or threonine). N-linked glycans are attached to a nitrogen of asparagine or arginine side chains of amino acid at the N- terminal end of a protein as a part of the protein modification step. The glycosylation machinery in eukaryotic cells is available to all proteins that enter the secretory pathway. In plants, most proteins of 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 (Kukuruzinska and Lennon, 1998). N-glycosylation usually occurs in the asparagines which occur in the Asn-Xaa-Ser/Thr stretch (where Xaa is any amino acid except Proline). When proline is present it could mask the glycosylation site preventing the glycosylation. While this consensus tripeptide (also called the N-glycosylation sequon) may be a requirement, it is not always sufficient for the asparagine to be glycosylated. These oligosaccharides modify the intrinsic properties of

proteins to which they are attached by altering the stability, protease resistance, or quaternary structure.

The site-specific glycosylation of a protein also suggests that the 3D structure of the protein plays a role in determining the extent and type of its own glycosylation (Dwek, 1996). The carbohydrate moiety of a glycoprotein may contribute directly in the recognition events (Drickamer & Taylor, 1993) and also modify the properties of the protein (Rademacher *et al.*, 1988; Varki, 1993). Reports say that the large size of carbohydrates is probably the most significant factor in modifying the properties of proteins to which they are attached which may influence the rate of processes and conformational changes in proteins. NetNGlyc software attempts to distinguish glycosylated sequons from non-glycosylated ones. The software predicts a high potential for glycosylation at position 101 in all the glucanases identified except in Gln 4 which is having the glycosylation site at 100^{th} asparagine residue. Out of this Gln 2 showed another glycosylation site at the 63^{rd} asparagine also.

The structural analysis of the 3D models showed similar pattern of the arrangement of α- helices and β-sheets both *Hevea* as well as barley. Though amino acid sequence of both barley (PDB ID -1GHS) and *Hevea* β-1,3-glucanase (3EM5) differs upon the alignment with an alignment score of 50, it was very interesting to find out that both the glucanases of this distantly related groups shows a similar structural pattern. They showed upto 100% structural alignment indicating that these enzymes are closely related in evolutionary terms. The location of catalytic residues forming the catalytic cleft (substrate binding groove) in the final tertiary structure of the protein is also exactly aligning though there is positional difference in the primary peptide sequence of both. In barley the catalytic cleft resides within 231E-288E (E= glutamic acid), while in *Hevea* 3EM5, it resides within 240E- 297E. However, the number of amino acid residues (57) within the catalytic cleft is found to be conserved in both. Like wise the superimposed modelled structures of the report AY325498 and 3EM5 also shows similar results. In all the sequences including the earlier reports the site of the catalytic cleft is found to be similar with less than 3 amino acid variations in the catalytic cleft.

The catalytic clefts of Gln 2 and 3EM5 is very similar. Likewise the catalytic residues of Gln5 is 100 % similar to Gln 6. Usually the catalytic amino acid residues are located within deep grooves which extend across the enzymes and which probably bind the substrates. Eventhough the polypeptide backbones of these enzymes are structurally very similar, the differences in their substrate specificities, and hence their widely divergent functions, can be acquired primarily by these small amino acid substitutions within the groove. Thus the structural similarities of these enzymes provide us an opportunity to explore the enzyme activity, stability and substrate specificities which are linked to the enzymic function.Varghese *et al.*, (1994) noticed that without any major changes in the polypeptide backbone of β-glucanases, difference in substrate specificities for these enzymes could be achieved by small differences in amino acids residing within in the catalytic clefts as shown by the differences in substrate specifity by two barley glucanases.

The new forms of β-1,3-glucanase are basic as indicated by the isoelecric points. The highest basiscity was shown by Gln2 followed by Gln 3 and Gln 7 (pI- 9). It is very interesting to find the structural conservation of glucanases despite the amino acid sequence variations in the closely/ distantly related plant members. Even the human glioma pathogenesis-related protein (GliPR) which is highly expressed in the brain tumor glioblastoma multiforme is exhibiting 35% amino acid sequence identity with the tomato pathogenesis-related (PR) protein P14a, which has an important role for the plant defence system (Szyperski *et al.*, 1998).

The nucleotide similarity of the new form of glucanase with the other earlier reported forms of glucanases from distantly related sources like *P. sativum*, *H. vulgare*, *S. cerevisiae*, *V. vinifera*, *N. tabaccum* etc. and also with the acidic glucanases of *M. sativa* shows relationships among various biological species. The occurrence of the conserved sequences in these diverse groups is suggestive of some wider significance. The nucleotide and amino acid sequence comparisons together with the phylogenetic analysis based on sequence provides a means to infer the molecular evolution of this gene family. It does reflect the functional divergence of the protein also. The

global phylogeny based on the conserved regions strongly suggest that the glucanases are a very ancient family of genes.

Through the acquisition or loss of regulatory cis-elements, the expression profiles of these β-1,3-glucanase genes may diverge independently of coding sequence allowing genes to acquire new or modified functions. The results in the study also demonstrate the possibility that the regulatory effects of genetic variation of β-1,3-glucanase gene is far more complex than previously observed. This extra layer of molecular diversity within the β-1,3-glucanase gene in *Hevea* may account for natural phenotypic variation and disease susceptibility also. An understanding of the nature, distribution and function of each of these proteins is very important in the management of biotechnology-based crop improvement. The existence of multiple β-1,3-glucanase genes in *Hevea* might also promote the development and maintenance of structural and functional diversity in the β-1,3-glucanase gene families. The expression of the promoter could be studied in a heterologous system with the help of a reporter gene fused with the foreign promoter *via* transformation. As mentioned earlier, the promoter in our study is having major responsive elements for RNA polymerase and various *cis* elements for its functional regulation. The WRKY box in the promoter region respond to different biotic and abiotic stresses. The promoter sequences isolated from *Hevea* can be used to monitor the defence gene expression in *Hevea.*

For functional validation of the different promoter forms isolated, promoter: GUS fusion binary vectors were developed and studied in *Hevea* callus and in the heterologous system, tobacco through *Agrobacterium* mediated genetic transformation. *Agrobacterium* mediated plant genetic transformation outshines all the currently known gene transfer techniques for the production of transgenic plants. The major advantages offered by the *Agrobacterium* mediated gene transfer mechanism are; natural gene transfer system, stable integration of foreign DNA, integration of low number of gene copies into plant chromosomes, transfer of relatively large segments of DNA, little rearrangement of the insert, high frequency of transformation, easy and efficient regeneration of transformed plants and no requirement of any high-

tech equipments or skills. *Agrobacterium*-mediated transient expression is a simple and efficient method for *in vivo* assays of plant promoters and transcription factors. Using this method, earlier two promoters of pathogenesis-related (PR) genes have been tested and thereby successfully identified the cis-regulatory regions in these promoters that are responsive to salicylic acid treatment or tobacco mosaic virus infection (Yang *et al.*, 2000).

pCAMBIA1381Z binary vector is designed for promoter testing *in planta*, these vector feature a promoter-less version of *gus*A (N358Q) with the catalase intron immediately downstream of a truncated *lacZa* containing either the pUC8 or pUC9 polylinker. The vector pCAMBIA 1381Z has hygromycin as the plant selection gene and bacterial selection is available with kanamycin. The truncated *lacZa* is functional for blue/white screening of clones in suitable *E.coli* host strains. pCAMBIA1381 Z TDNA vector contains a fully functional *gus*A reporter construct for simple and sensitive analysis of gene function or presence in regenerated plants by GUS assay. The construct uses *E.coli gusA* (N358Q - to avoid N-linked glycosylation) with an intron (from the castor bean catalase gene) inside the coding sequence to ensure that expression of glucuronidase activity is derived from eukaryotic cells, not from expression by residual *A.tumefaciens* cells. The *gusA* reporter gene is cloned in new modular format. These plasmids are suitable for insertion of other genes of interest containing their own promoter and terminator. In the present study one of the pCAMBIA vector, pCAMBIA1381Z, is taken as the parent vector for developing the new vector back bone. Here we describe the construction of plasmid vectors for transient gene expression using *Agrobacterium*, infiltrated into *Nicotiana benthamiana* leaves and *Hevea brasiliensis* callus tissue. We have generated a series of pCAMBIA 1381Z TDNA vector ideally suited to transient gene expression necessary for stable transformation.

In the present study, the functional validation of the promoter of *Hevea* β-1,3-glucanase gene isoforms in transgenic tobacco and *Hevea* callus has been carried out. Eleven binary constructs has been prepared including five basic constructs and six deletion constructs. The recombinant binary vector transformed *Agrobacterium* colonies were screened by colony PCR

using the insert, GUS and hygromycin gene specific primers. The positive colony was chosen to transform tobacco leaves and *Hevea* callus. After two days, transient asaay for the GUS activity has been performed with the *Hevea* callus and tobacco leaves. *Hevea* callus showed positive GUS activity whereas tobacco leaves showed no GUS gene expression. The mature transformed tobacco leaves also showed no GUS gene activity in the leaves during the non-induced and the induced state with 0.1 % salicylic acid. In tobacco, it was identified that a region from –1452 to –1193 in a glucanase gene promoter containing two copies of the heptanucleotide 'AGCCGCC', which is also highly conserved in plant-stress and defence-related genes, is necessary for high level expression in leaves (Vögeli-Lange *et al.*, 1994b). This region was absent in all the five basic constructs. Probably this region may be present with the further upstream region of the promoter. This may be a reason for the absence of GUS gene expression driven by the glucanase gene promoters in the transgenic tobacco leaves or due to the less sufficient upstream sequence necessary for the multiple cis- regulatory elements to drive the expression. An increased level of GUS activity was very well seen with the callus transformed with binary constructs with the higher number of nucleotides especially with the 913 bp promoter. This can be due to the occurrence of multiple cis-regulatory elements as the number of base pair increases. Expression of the GUS reporter gene in the other basic constructs and deletion constructs showed more or less a very similar pattern of regulation in *Hevea* callus. The results of deletion analyses suggest that multiple positive and negative elements in the glucanase gene promoter regulate its activity.

Most *in vivo* expression studies indicate that some of the cis-elements in the promoter regions cannot act on their own but require the presence of additional cis acting elements for their function (eg. G-box elements in the GalUR promoter for fruit expression in strawberry (Foster *et al.*, 1994; Martı´nez-Herna´ndez. *et al.*, 2002)). The expression of the promoters in the callus may be due to the identical tissue homogeneity of the *Hevea* callus or due to the presence of the trans-elements in *Hevea* genome driving the regulation of these promoters. Callus is a representative of cells that is intended to become an adult plant. The high level of expression of GUS gene

driven by the 913 bp promoter in the *Hevea* callus indicates that the cis-elements necessary for the fair expression of the promoter resides within the upstream sequence of the promoter. The failure of GUS gene expression in the tobacco leaves may be due to the absence of the enhancer elements (residing in the upstream region of the promoter) / trans elements in tobacco or due to the inability to compete for a limiting amount of its cognate transcription factor(s), or related proteins. The most efficient promoters in tobacco cells were not the same as those in rice cells, suggesting differences in the specificity of gene expression between plant species and/or between dicotyledonous and monocotyledonous plants.

Earlier, van de Rhee *et al.,* (1993) observed that the basic glucanase promoter with 1476 bps were able to drive expression in response to TMV infection and ethylene treatment, but no response was found to incision wounding. Further, high GUS activity was found in lower leaves and roots of healthy transgenic plants, carrying the 1476 bp basic glucanase promoter/GUS construct. When the promoter was truncated up to position - 446 all activity was lost, indicating that the region between -1476 and - 446 of the basic glucanase promoter is necessary for organ-specific and developmentally regulated expression as well as for induced expression in response to infection and other stress treatments. It was observed earlier that the cis- element GT-1 and sequences related to GT-1 can also act as negative regulators in different genes (Faktor *et al.*, 1996; Zhou, 1999). Recently, it has been demonstrated that I-box-like elements down-regulate gene expression in melon fruit, suggesting a new function of the I-box-like element as a negative regulator (Yamagata *et al.*, 2002). This could be another case for the I-box elements and the GT-1 sequences found with the β-1,3-glucanase gene promoters for its functioning in tobacco leaves. A chimeric gene composed of 2998 bp of the promoter sequence of *Solanum tuberosum* from the *gluB* gene was fused to the β-glucuronidase (GUS) coding region and used to transform potato and tobacco plants. Transcriptional activation of the *gluB* promoter was investigated in response to inoculation with *Phytophthora infestans* (*Pi*) or tobacco mosaic virus (TMV). In pathogen inoculated transgenic plants, GUS

activity was strongly induced locally around necrotic lesions (Mac *et al.*, 2004).

Alleles of trans-acting factors in the *Hevea* callus and tobacco could have different affinities to DNA binding sites. In addition, dimerization or heterodimerization of *trans*acting factors could be important for transcriptional activation (Pysh *et al.*, 1993; Grotewold *et al.*, 2000). The experiment further demonstrates that the trans-activation profile for these basic and deletion promoters was specific. Qi-La *et al.*, (2008) reported a very low GUS activity in the leaves of *Nicotiana* when transformed with the 1157 bp promoter of a *Gastrodia* antifungal protein.

It is often advantageous for plants to express the introduced transgenes in specific tissues or under specific conditions. High expression of transgenes may be required under certain circumstances. The nature of the specificity of transcription is the first step in gene expression. In general, a promoter has to switch to an active state of transcription so that upon the synthesis of the necessary trans-acting factors the transcription machinery can act on the promoter of a gene. Therefore, gene expression is also a measure of the activity of *trans*-acting factors as well (Wu and Messing, 2009). One can envision that β-1,3-glucanase genes are controlled by a number of transcription factors to coordinate their timely and tissue-specific expression. Recent studies have shown that target genes need to undergo tissue-specific chromatin modification at the DNA binding site to function, suggesting that the tissue specificity of genes is also regulated at the chromatin level (Locatelli *et al.*, 2009), which would also suggest that chromatin remodeling has to occur before transcriptional activation can begin. Previous studies with other transgenes have also shown that some sort of presetting occurs at the promoter site because the epigene promoter had a chromatin structure that was resistant to tissue-specific modification. This absence of tissue specific modification may be another factor for the lack of GUS gene expression to be driven by the inserted *Hevea* β-1,3-glucanase gene promoters in heterologous tobacco.

Agrobacterium-mediated transient gene expression system in intact plant leaves is a rapid and useful method of analysis of gene expression. Transient gene expression system offers a number of advantages over stable expression.By this method many different heterologous proteins can be produced without the need to generate transgenic plants, which is difficult for many plant species (Fischer *et al.,* 1999; 2004; Horn *et al.,* 2004). One of the most important advantages of the transient gene expression technique as compared to the stable gene expression is its simplicity and easy performance. Transient gene expression can be assayed directly post-agro-infiltration (Kapila *et al.,* 1997). Usually *Agrobacterium*-mediated transient expression of a transgene achieves the highest level, 2–3 days following argo-infiltration, after which the expression level decreases rapidly.

In plants like *Hevea brasiliensis*, tissue specific and inducible promoters are very important for the development of transgenic plants. The activity of a promoter in transient assays reflects its activity in different homologous and heterologous transgenic tissues which showed a loss-of function and gain-of-function in the different forms of transgenic tissues. The β-1,3-glucanase gene involved in the defence responses is mainly an induced one where as it will be expressed constitutively in the latex vessels. For any transgenic approach for enhancement of defence mechanism, defence gene specific promoters are very essential. Investigation of this work demonstrated that the prototypic β-1,3-glucanase promoter of the *Hevea brasiliensis* is, in fact, modular and comprised of several cis elements, each of which confers cell-type-specific gene expression in different tissues. The apparent constitutive/ induced expression of the β-1,3-glucanase promoter results from the combinatorial and synergistic interaction of the various cis-elements (Benfey *et al.*, 1990). The isolated promoters of β-1,3-glucanase gene to monitor defence gene expression and tissue specificity in *Hevea* and other species of plants after isolation of more promoter regions and identification of the multiple cis-acting elements.

Transcription is usually regulated by a combinatorial control, meaning that several different proteins must bind to specific elements in DNA in a coordinated manner to achieve appropriate expression of the target gene. Transcriptional regulation therefore consists of transcription factor (s) (trans-acting factor) and the corresponding binding site(s) in the DNA (c/.v-element) (Wolberger, 1999). Not much is known about the transcriptional regulation of stress related plant genes. Some regulatory elements and *trans*-acting factors are, however, known and may be of importance in analyzing the regulation of the plant gene expression.

Better understanding of the role of β-1,3-glucanase gene isoforms characterized in this study during the host–pathogen interaction requires isolation and analysis of the properties of the respective β-1,3-glucanase isozymes. The identification of the right form of β-1,3-glucanase with enhanced defence activity towards the pathogen causing ALF should be undertaken. This will help in the utilization of the right form for enhanced disease tolerance in the transformation experiments.

β-1,3-glucanase enzyme has been purified and the genes encoding them have been isolated and sequenced in several higher plants (Simmons, 1994). Abeles *et al.*, (1971) suggested that the β-1,3-glucanases and chitinases in the glucano hydrolases family might function as defence against fungal pathogens. At present compelling evidences are there for β-glucanases acting alone or in combiantion with other PR proteins especially chitinase that can help defend plants against the fungal infections. These hydrolases act in two different ways; 1) directly by degrading the cell wall and 2) indirectly by promoting the release of cell wall derived materials that can act as the elicitors of the defence reactions. β-1,3-gluanases hydrolyses the β-1,3-linked glucans which forms the callose. β-1,3-glucan is the main structural component of the cell wall of many pathogenic fungi. The fungal hyphal tip is thought to be particularly susceptible to lysis, by thinning the cell wall at the hyphal tip that causes the swelling and ultimate bursting of the hyphal tip. The fungal cell wall synthesis involves a delicate balance between β-glucan hydrolysis and synthesis, which could be disrupted by the plant β-glu activity (Simmons,

1994; Arlorio *et al.*, 1992). Various research works on the plant-pathogen interactions and successful production of transgenic plants have proved that plant β-glu could be an effective tool in controlling many fungal pathogens by enhancing the disease tolerance of the host plants.

Since the early 1980's, yeasts have been used for the large scale production of intracellular and extracellular proteins of human, animal and plant origins (Romanos, 1995). Since the promoters controlling the expression of these genes are among the strongest and most strictly regulated yeast promoters, yeast is widely used as a superior host for the production of a broad range of recombinant proteins (Zhang *et al.*, 2005). Recently, *Pichia pastoris*, a methylotrophic yeast has become an outstanding expression system for fundamental study and expression of recombinant proteins. *P. pastoris* was initially chosen for the production of single-cell protein (SCP) due to its ability to achieve high cell density and product yield (Siegel and Brierley, 1989). It has been developed into a highly successful system for the expression of heterologous genes but yet, they have less-developed vector systems and strains and are more costly than bacterial methods.

In order to find out a direct evidence on inhibition of pathogenic fungal growth by the host β-glu, a functional gDNA clone for each of the six different forms of β-1,3-glucanases was constructed in pPICZ A vector that could express the *Hevea* β-glu in the new generation yeast, *Pichia pastoris*, and the recombinant protein was purified and tested for the anti-fungal properties.Once transformed into the yeast host, the free DNA ends promote homologous recombination into the host AOX1 region. Since the pPICZ A vector lacks an yeast origin of replication, transformants can only be isolated if recombination occurs between the expression construct and the *P. pastoris* genome. Methanol at 0.5 % concentration was found to be optimum for maximal expression for pPICZ vector (non- secretory vector) transformed to the X-33 strain of *Pichia* (a wild type strain- Mut+). The fusion protein expressed by pPICZ A vector could be used for subsequent analyses because of the success in purifying it as a single band.

When total proteins from the induced *Pichia* colonies with recombinant pPICZ A vector were analysed by SDS-PAGE, the band size of ≈ 43.5 kDa of the predicted fusion protein (*Hevea* glucanase) was obtained. For Gln 4, ≈ 42.5 kDa protein has been obtained (the size of the his- tag and *c-myc* epitope is added to the original protein size) The recombinant fusion protein could be purified as a single band by passing through an affinity column for the fusion tag at the N-terminal region. To test whether the protein is a glycosylated one or not it requires other steps. There is every possibility to get the signal peptide cleaved off from the protein and the protein to get glycosylated after, thus increasing the molecular weight of the protein again.

The glycosylation favors the protein for its conformational stability and its functional activity. The recombinant fusion protein retained functionality of the glucanase enzyme as shown by the activity staining. The result of the activity staining indicates that these proteins were conformationally folded and retained their native structure. This was demonstrated by the use of laminarin that served as the glucanase substrate in the glucanase staining. In 2009, Fuentes-Silva *et al.*, identified the crystal structure of a native endo-β-1,3-glucanase (Hev b 2), and reported as an allergen from *Hevea brasiliensis* (PDB ID- 3EM5). However they have not studied the antifungal activity of the enzyme. In the present study it is revealed that the 3EM5 sequence belongs to Gln 2 which is showing the highest antifungal activity in comparison with others. Earlier, a recombinant latex class I chitinase was produced using pMAL expression vectors and its immunoglobulin binding reactivity was studied by immunoblot experiments using sera from latex-allergic patients (Rihs *et al.*, 2003).

The purified recombinant *Hevea* β-glu was assayed for its anti-fungal activity against ALF causing *P. meadii*. The proliferation of the fungal mycelium got retarded near the filter paper discs of Gln 2, Gln 5, Gln 6 and Gln 7. The Gln 2 showed maximum antifungal activity. This result clearly indicates the inhibitory action of the purified enzymes from *Pichia*. The fungi over grew these clear inhibition zones one week after inoculation. The

differences in their activity may be due to minor amino acid variations within the catalytic clefts of the protein. In 1994, Varghese *et al.,* noted that a difference in substrate specificity and function can be achieved in barley glucanases without much change in its poly peptide backbone. They also noted that a relatively small number of changes in the amino acid in the substrate binding groove of two barley enzymes are responsible for the distinct substrate specificity of the enzyme. Yu *et al.,* (2003) also report bacterial expression of a barley glucanase, which is functionally active.

In the present study, 12 µg of purified protein of β-1,3-gluanases could sufficiently inhibit the growth of *P. meadii.* However, the potential concentration needed for complete inhibition was not estimated. Ole e 9 (46 kDa) is a 1,3-β-glucanase and major allergen from olive pollen, which is a principal cause of allergy in Mediterranean countries. Its C-terminal segment (101 amino acid residues) has been produced as a recombinant polypeptide in the yeast *Pichia pastoris* (Palomares, 2003). Gao *et al.,*(2008), cloned a novel β-1,3-glucanase gene (glu) from *Trichoderma viridae* LTR-2 cDNA in *Pichia pastoris.*

When 10 µg of the purified rice chitinase was applied on sterile paper discs, the growth of the rice sheath blight pathogen *Rhizoctonia solani* (Velazhahan, *et al.*, 2000) was inhibited. A basic β-glu induced in pepper (*Capsicum annum*) had antifungal activity against *P. capsici* at 100 µg/ml (Kim and Hwang, 1997). Tonon *et al.,* (2002) reported complete inhibition of sporangia in the germination of *P. infestans* with 0.3 µg/ml of purified enzymes from the infected tissues of potato. Mauch *et al.,* (1988a) observed that 65 µg/ml β-glu purified from pea pods inhibited only *Fusarium solani* among the 8 fungi tested, including three representatives of the oomycetes when applied individually. Majority of the reports shows use of combinations of β-glu and chitinase to get better *in vitro* anti-fungal properties. In combination, their inhibitory effect seems to be several-fold higher than when these enzymes are used individually. Hu *et al.,* (1999), expressed three anti-fungal proteins; trichosanthin, tobacco class I chitinase and tobacco class I glucanase in *E.coli* and the recombinant proteins were tested for anti-fungal

activity. All the three kinds of proteins showed inhibitory activities, but in combinations of two, the activity was found to enhance several folds. When all of them are combined, a very high anti-fungal activity was observed. However, in the present study, since the pathogen is a chitin less fungus, only β-glu might be playing a major role in hydrolyzing the fungal cell wall.

Since β-glu can exist in many structural isoforms that differ in size, primary structure, isoelectric point, cellular localization and pattern of regulation, proper selection of glucanase genes is very important for the development of transgenic plants with enhanced disease resistance. A soybean endo-β-1,3-glucanase gene (PROKLA-Eg) multiplied in *Agrobacterium* strain LBA 4404, was simultaneously introduced into single buds of *in vitro* grown banana cultivar, rastali (AAB) against *Fusarium oxysporum* wilt disease. The transformed plantlets showed increased glucanase activity than the non-transformed ones (Maziah *et al.*, 2007).

Several glucanase isoforms have been isolated, which are having major roles in various physiological and developmental processes, but less important in disease resistance. In addition to their possible role in defence, glucanases may have functions in a number of developmental processes in plants as well. Glucanases are very likely involved in cell wall metabolism and cell expansion in seedlings (Wong and MacLachlan, 1980) and in microsporogenesis (Worall *et al.*, 1992). In several studies, different β-glu isoforms have been tested for *in vitro* antifungal activity (Mauch *et al.*, 1988b; Sela-Buurlage *et al.*, 1993). Only class I basic vacuolar isoforms of β-glu were found to be effective in promoting the lysis of hyphal tips and inhibiting the growth of several fungi (Lawrence *et al.*, 1996; Anfoka and Buchenauer, 1997). However, the class II chitinases showed limited growth inhibitory activity against *Fusarium solani* in combination with higher amounts of class I β-1,3-glucanase in tobacco (Sela-Buurlage *et al.*, 1993). The class II glucanases showed no inhibitory activity. An exception to this was reported by Tonon *et al.*, (2002) where they could isolate and purify an acidic isoform of glucanase, with *in vitro* antifungal properties. The extra cellular class II acidic glucanases with weak anti-fungal activity may be involved in the releasing of

elicitors from fungal cell wall that activate the host defence reactions. Present study also confirms the role of class I basic vacuolar isoforms of glucanase in fungal resistance. In a previous study (Andreu *et al.*, 1998), it was shown that preparations of partially purified glucanase from potato tubers were able to degrade the glucans purified from *P. infestans* cell walls. This result and the direct fungicidal action reported here in, suggest that this hydrolytic enzyme could act directly by inhibiting the growth of the invading fungi. Yu *et al.*, (2003) also report bacterial expression of a barley glucanase, which is functionally active.

The present study focused on the analysis of the relative antifungal activity of each of the different forms of β-1,3-glucanases isolated. The role of β-1,3-glucanase in defending the fungal diseases, especially abnormal leaf fall in *Hevea*, has been confirmed through earlier studies. The present study will highlight the need to select the right candidiate gene for the transgenic approach in rubber crop improvement for disease resistance. Such a transgenic tree, which over-expresses the right anti-fungal protein, with built-in resistance to many fungal diseases, will be beneficial to the rubber plantation industry world wide. This approach is also highly relevant to other economically important crops like pepper, coconut, arecanut, potato, etc. among which different species of *Phytophthora* cause serious menace. The development of a functional gDNA clone in a non-pathogenic microorganism (endophytes) with rubber β-1,3-glucanase may be helpful in the bio-control of many *Phytophthora* mediated diseases in several crop plants.

Though the plant immune system shows some mechanistic similarities and apparent common origin with the immune systems of insects and mammals, it exhibits many plant-specific characteristics. Plant disease resistance derives both from pre-formed defences and from infection-induced responses mediated by the plant immune system. The PR proteins are the most widely used marker of acquired resistance in plants. In plants most often the disease resistance is brought about by constitutive and induced mechanisms.

PR protein accumulation is one of the most common indicator for active, induced plant defence. The proteins with β-glu activity have been well investigated in several plant species (Simmons, 1994; Leubner-Metzger and Meins, 1999). There are several reports on the induction of β-glu during pathogen infection in different plant species like tobacco (Vogeli-Lange *et al.*, 1988), potato (Kombrink *et al.*, 1988), tomato (Joosten and De Wit, 1989), soybean (Yi and Hwang, 1996), bean (Xue *et al.*, (1998), pepper (Egea *et al.*, 1999), wheat (Kemp *et al.*, 1999), peach (Zemanek *et al.*, 2002), barley, sorghum etc. β-Glu gene from a woody plant species was isolated for the first time from *Hevea* by Chye and Cheung, (1995).

Thanseem *et al.*, (2005b) identified and isolated a pathogen inducible form of basic β-1,3-glucanse from *Hevea* clone RRII 105 which was designated as Gln 1 when different isoforms were isolated in the present study. In that earlier study it was found that although Gln 1 is present in tolerant and susceptible clones of *Hevea*, the expression of Gln 1 is found to prolong in the tolerant clone while in the susceptible clone the expression is getting diminished after 48 hrs. It is not sure that the activity observed by Thanseem *et al.* (2005a) is from Gln 1 or sum of all the glucanase isoforms together which were not identified in that study. Later in the present study, different glucanases have been obtained which are found to be regulated by different promoters. The exon sequences of these genes showed more or less a conserved pattern with minor variations. Due to the highly conserved nature of these sequences, the expression studies to select the right candidates from a set of genes is relatively a difficult task. The primer could cross- hybridize and it will not be able to judge whether the amplified product is the right one or not. In the present study, the observation of the constitutive expression of Gln 4 in two *Hevea* clones support the hypothesis that not all the glucanases may be involved in the reaction against fungal infection alone.

With Gln 4 specific primers a product of approximately 180 bp PCR productwas amplified from the fungal induced leaf cDNA samples and the band intensity was same in the tolerant (RRII 105) and the susceptible clone

(RRIM 600) at the different time intervals confirming a constitutive expression of Gln 4. In the susceptible clone RRIM 600, at 96 hrs the leaves got decayed and abscissed. This factor may have caused the diminished expression of the Gln 4 and actin at 96 hrs due to the degeneration of mRNA transcripts at the particular time. The tissue specific expression analysis of Gln 4 through RT-PCR showed that the latex and callus exhibited the high level of expression while the leaf samples showed least level of expression for Gln 4. This study is in agreement to the previous reports which showed that β-1,3-glucanases show constitutive high level of expression in the latex and low level of expression in the non-induced leaves of *Hevea.* The quantification of RNA transcript levels that can be performed conventionally using RT-PCR has many advantages, such as being able to detect even lower levels of gene expression. But, even though RT-PCR is more rapid and specific, quantification can be difficult since there may be many sources of variation, such as template concentration and amplification efficiency. One approach to overcome this problem is to include a gene of interest with an internal control and quantify the gene of interest relative to the internal control. RT-PCR requires only small amounts of DNA, takes less time to complete, is less expensive than northern blot analysis and requires only standard PCR techniques. Here at the end of 32 cycles, a good level of expression has been obtained with Gln 4 in latex and callus while a low level of expression in the leaf. The use of primers to amplify the actin gene fragment in each sample helped to account for variation in template concentration and amplification efficiency. Actin is supposed to be a better internal control than 18S RNA in RT-PCR because, recognized that 18S RNA sequences are highly conserved, therefore, the internal control was amplifying both plant and fungal 18S RNA from the fungal infected tissues, leading to an under estimation of plant gene expression (Burleigh, 2001).

Many plant species examined thus far show the presence of multiple β-1,3-glucanase isozymes. These isozymes can be constitutive or induced. Further, the β-1,3-glucanase isozyme patterns in infected plants are usually different from uninfected plants. Most studies showed that the quantity of isozymes (based on band intensity on native polyacrylamide gels) increased

after infection with pathogens (Pan *et al.*, 1991; Shimoni *et al.*, 1992; Churngchow *et al.*, 1995; Roulin and Buchala, 1995; Lawrence *et al.*, 1996; Roulin *et al.*, 1997; Burketova *et al.*, 1999; Egea *et al.*, 1999; Urquhart and Punja, 2002; Shinya *et al.*, 2004). In some cases, new isozymes appear in infected plants. For example, a study of tobacco plants showed that normal tobacco leaves contained one major β-1,3-glucanase isozyme, but an additional isozyme was detected in the tobacco mosaic virus-infected leaves. Furthermore, the intensities of both isozyme bands were considerably greater than those of the uninfected tobacco leaves (Pan *et al.*, 1991). In the case of barley (*Hordeum vulgare L.*), the predominant isoforms in the normal leaves were the isozyme GI and to a lesser extent, the isozyme GIII. After infection with *Rhynchosporium secalis*, GI remained approximately constant, while the level of GIII increased. However, the most noticeable response to infection was the induction of a high level of the isozyme GII (Roulin *et al.*, 1997). Changes in isozyme patterns were also observed when plants were treated with other stimuli. Sela-Buurlage *et al.* (1993) explained that only specific chitinases and glucanases exhibit the antifungal activity with the experiments in tobacco.

Present study reports the presence of a non-inducible β-1,3-glucanase - Gln 4 in *Hevea* (both in tolerant and susceptible clones of *Hevea)*. The level and onset of Gln 4 expression is not found to be positively correlated to the level of resistance to the pathogen as in previous studies done in *Hevea* (Thanseem *et al.*, 2005). In muskmelon and tomatoes infected with *Fusarium oxysporium* (Ferraris *et al.*, 1987), resistant melon cultivars infected with cucurbit powdery mildew fungus (Rivera *et al.*, 2002), black pepper infected with *Phytophthora* (Jebakumar *et al.*, 2002) etc, the resistant ones showed more glucanase activity than the susceptible ones. But the results of Gln 4 in the present study is not in accordance with the above mentioned findings as Gln 4 is showing or keeping a steady state level of expression in both the tolerant and the susceptible clones of *Hevea* upon induction and non-induction. The antifungal study of this particular form also showed nil activity towards the *P.meadii* mycelial proliferation.

In this study, the primers used for the RT-PCR experiments, correspond to this particular basic isoform of glucanase. As the different glucanase isoforms share a high degree of homology, the RT-PCR product as well as the signals generated in northern may not be fully attributed to the expression of a single isoform alone. In such cases the results obtained cannot be attributed to the function of a single form alone. Therefore, studies like this should be conducted in such a way so as to obtain the result of a single form of glucanase alone. Along with that immunocyto-localization studies are necessary to determine the sub cellular localizations of β-glu isoforms specifically induced in tolerant and susceptible *Hevea* clones in response to *Phytophthora* infection.

Earlier it was observed that β-glu was constitutively expressing in the latex of rubber tree. Kush *et al.*, (1990) reported that the transcription levels of plant defence or stress induced genes were 10 – 50 fold higher in laticifers compared to leaves. Tapping is a process of controlled wounding to collect the latex. Various enzymes stored in the laticifers may be providing an important contribution to the plant defence mechanism by sanitizing and sealing the wounded areas on the tree as reported in the case of *Carica papaya* (El Moussaoue *et al.,* 2001). Our results also indicate that out of the many isoforms some forms like Gln 4 is constitutively expressing in the latex as well as in the callus but showing a low level of expression in the leaves. This is in accordance with the result of Chye and Cheung, (1995) where higher expression of β-1,3-glucanase gene was reported in the laticifers than leaves. The constitutive nature of gene expression was further confirmed using the latex collected from a freshly opened tree. Constitutive expression of other defence related genes like chitinase and lysozymes has also been reported in the latex of *Hevea* (Martin, 1991). Earlier Thanseem *et al.*, (2005) studied the constitutive expression of β-glu in the latex of six clones using northern hybridisation. In that it was observed that the band intensity was more in the case of tolerant clones particularly for FX 516, which is a hybrid of *Hevea brasiliensis* with a wild relative *H. benthamiana*. However, a susceptible

clone RRIM 600 also recorded higher levels of gene expression in the latex, comparable to that of tolerant clones.

The present study indicates that β-glu- Gln 4 is a non- pathogen inducible form showing a constitutive level of expression in the tolerant and the susceptible clones. It may be playing some other roles in *Hevea.* This novel enzyme may play an indirect role in plant defence by causing the formation of oligosaccharide elicitors, which elicit the production of other PR proteins or low molecular weight antifungal compounds, such as phytoalexins as reported by Keen and Yoshikawa (1983) ; Ham *et al.*, (1991) ; Klarzynski *et al.*, (2000). It is reported in tobacco that only specific *(Nicotiana tabacum)* chitinases and β-1,3-glucanases exhibit antifungal activity (Sela- Buurlage *et al.*, 1993). However in the present concept, a more detailed study is further needed to learn the expression of different forms of β-1,3-glucanase gene with real time PCR using the more specific locked nucleic acid probes.

To the agriculture world, plant protection against various kinds of biotic/ abiotic stresses is a major challenge. With the beginning of the molecular era of plant biology in the early 1980's, a major area of research has been to identify, clone and characterize various genes involved in disease resistance. It is anticipated that biotechnological approaches can engineer durable disease resistance in *Hevea* by engineering plants to express resistant proteins that can recognize pathogen molecules essential for pathogenicity. Numerous plant R-genes have been used with varying degree of success in crop improvement programmes in the past and many of them are being continuously exploited. The enhancement of the host resistance to the fungal attack can be prevented by i) the incorporation of resistant genes through conventional breeding methods ii) transforming plants to express the genes of choice and iii) inducing natural resisatnce by applying the biotic or abiotic inducers. With the onset of recent genomic, bioinformatics and molecular biology techniques, it is quite possible to select the right R-gene for efficiently controlling the plant diseases caused by pathogens.

Chapter VI

Summary and Conclusion

Over many years, the plants have been bred to resist pests and diseases. Significant advances have been made worldwide in controlling many of the important parasites that affect economically important crop plants. Though plant diseases can be controlled by means of conventional breeding and production of resistant cultivars, the process is extremely time consuming. Moreover, the use of agrochemicals for control of the disease is costly and deleterious to the environment. It also eventually becomes less efficient due to evolution of new resistant and virulent pathogens. Plants are engaged in a continuous co-evolutionary struggle for dominance over their pathogens. The production of disease resistant transgenic plants could alleviate this problem to some extent. With the advent of modern molecular biology techniques, deeper insights into plant-pathogen interactions is possible than ever before. The vast molecular level studies in the field of plant-pathogen interactions form the basis for novel transgenic approaches towards crop protection. Together with cell-culture technology, genetic engineering of crop plants with enhanced resistance to diseases is now a reality producing disease-resistant plant genotypes. The unraveling of plant defense mechanisms, dissection of signal transduction components of hypersensitive responses, systemic acquired resistance pathways and cloning of resistance genes are now opening new avenues for genetic engineering of crop plants with enhanced resistance to diseases. Establishment of the role of the candidate gene in defense responses would be essential for engineering effective and durable resistance to pathogens in the field.

Hevea brasiliensis is the major commercially cultivated species for natural rubber (NR). NR from *Hevea brasiliensis* (Para rubber tree) is a high molecular mass polymer of isoprene units (C_5H_8) with *cis*-configuration (*cis*-1,4-polyisoprene). The NR is used in the manufacture of more than 35,000

products. Among the various fungal diseases affecting rubber plantations, the abnormal leaf fall disease (ALF) or *Phytophthora* leaf fall (PLF) is a serious threat to *Hevea brasiliensis* (rubber tree). The disease is caused by *Phytophthora spp*, an oomycete fungi which causes heavy yield loss in rubber plantations. *Phytophthora meadii* is the causative organism in South India. Although, many promising clones with a fair degree of tolerance to ALF disease could be evolved through conventional breeding programmes, a fully tolerant clone of *Hevea* has so far not been developed. In a perennial tree crop like *Hevea*, conventional breeding programmes are elaborate, labour intensive and time consuming. An understanding of the function of a well-known defense-related R-gene protein like β-1,3-glucanase (β–glu, (1,3-β-D-glucan 3-glucanohydrolase, (EC 3.2.1.39)), a member of the PR-2 family of PR (pathogenesis related) proteins that catalyse endotype hydrolytic cleavage of 1,3-β-D glycosidic linkages in β-1,3-glucans present in the cell wall of many oomycete fungi, could help as a viable alternative approach for the development of more sophisticated molecular tools to combat ALF disease in *Hevea*. Earlier studies held at the Rubber Research Institute of India led to the identification of a *Phytophthora* inducible β-1,3-glucanase gene in both tolerant and susceptible clones of *Hevea*. In tolerant and susceptible clones, the gene expression was found to be induced on infection with the fungus. However, there was a prolonged expression beyond 48 hrs of the post-infection period only in tolerant clones. It was also found that the heterologously expressed purified protein (*E. coli*) could effectively inhibit mycelial proliferation of *Phytophthora* in the *in vitro* system.

The present work was undertaken to study the mechanism behind the differential expression of β-1,3-glucanase gene in the tolerant and susceptible clones. Attempts were made to 1) find out sequence variations, if any, in the β-1,3-glucanase gene in different clones with difference in the relative level of disease tolerance/ susceptibility to abnormal leaf fall disease caused by *Phytophthora spp*, 2) identify the sequence/ isoforms showing maximum antifungal activity, 3) identify the *cis*-acting elements in the promoter region of β-1,3-glucanase gene in tolerant and susceptible clones, 4) functional

validation of the identified promoter in transgenic tobacco using promoter : GUS fusion binary vector after induction with salicylic acid and 5) *In vitro* anti-fungal properties of the proteins derived from β-1,3-glucanase isoforms.

Initially, PCR amplification of β-1,3-glucanase gene from selected relatively tolerant and susceptible clones of *Hevea* using the primers designed from the earlier reported sequence was done and the nucleotide sequence was characterized. Approximately 1.3 kb genomic sequence was amplified from different clones. On nucleotide sequencing and comparison with repeated sequences revealed 1229 - 1262 bps with one intron in different clones. 'CT' repeats were also observed in the intron. Variations within the intronic 'CT' repeat sequences were also found among the clones studied. The intron was located at +103 position from the translation initiation codon (TIC). Minor nucleotide variations were observed in the exons of different clones. However, these sequence variations in different clones could not be correlated with disease tolerance/ susceptibility. Therefore attempts were made to characterize the 5'regulatory sequences of the β-1,3-glucanase promoter region upstream to the already reported sequence of β-1,3-glucanase gene (with 198 bp) through inverse PCR to understand variation if any with the cis-elements present between the clones which control the differential gene expression. The results revealed the existence of five forms of β-1,3-glucanase gene promoter sequences in addition to the earlier reported sequence in a single *Hevea* clone RRII 105. Partial promoter fragments of 913, 847, 582, 553, and 198 bps were seen in the amplified products. Out of these five forms, the smallest fragment (198 bp promoter) was exactly aligning with the earlier reported promoter sequence by Thanseem *et al.*, (2003) (AY325498). The promoter sequences contained cis-elements usually present in a defense gene associated promoters. However the GCC box present in the promoter region of many pathogen responsive genes was not found in the isolated regions of the β-1,3-glucanase gene promoters. The presence of CpG islands were observed in two promoters (913 and 553 bp promoter) and their chances to present in the unidentified regions of the promoter sequences obtained cannot be ruled.

In order to understand whether the coding DNA sequence is also different, different forms of β-1,3-glucanase gene promoters along with their coding sequence were amplified and characterized from the *Hevea* clone RRII 105. It was observed that each promoter form has separate coding sequences showing the existence of multiple forms of β-1,3-glucanase gene in *H. brasiliensis*. These different gene isoforms were designated as Gln 2 (with the 553 bp promoter sequence amplified), Gln 3 (198 bp promoter), Gln 4 (582 bp promoter), Gln 5 (847 bp promoter), and Gln 6 (913 bp promoter) respectively. This indicates the presence of at least six β-1,3-glucanase isoforms in a single *Hevea* clone RRII 105. Another form of glucanase has also been obtained from the most tolerant inter-specific hybrid of *Hevea* 'FX 516' (*H. benthamiana* × *H. brasiliensis*) towards abnormal leaf fall disease (Gln 7). The multiple forms might aid in synergestic activity of the glucanases in this host plant. The deduced ORF of these gene isoforms showed significant variations which can affect the structural as well as their functional integrity. Differences in the 3'UTR nucleotide sequence was observed after spanning a considerable length (that is similar in all forms) from the stop codon. The differences exist in the downstream UTR region as indicated by variations in the reverse primer binding affinities (designed from the 3'UTR region) of these different forms. This may be correlated to the differential stability and translocation of the various forms of glucanase mRNA to a certain extent. Out of the newly isolated six forms of glucanase, the one designated as Gln 4 shows more nucleotide/ deduced amino acid sequence variations from the other forms. This particular form is having a different stop codon, 'TAA' (amber codon) rather than the 'TGA' (ochre codon) seen in other forms. RT-PCR analysis showed mRNA abundance of this particular isoform in the latex as well as in callus, but low level in the leaf. The pathogen induction studies also revealed a constitutive level of expression for this particular glucanase gene form in the tolerant (RRII 105) and susceptible (RRIM 600) *Hevea* clone towards ALF indicating no significant role in disease tolerance mechanism. However, more in depth study is required with real time PCR experiments upon induction and without induction using more specific locked nucleic acid probes.

The presence of signal peptide was confirmed through 'Signal P' analysis in all the six glucanases. The deduced amino acid sequence showed the presence of 374 amino acids in all the forms except in Gln 4 that showed 373 amino acids. The deduced isoelectric point (pI) showed that all these are basic glucanases showing minor variations in their iso-electic points. The NetNGlyc prediction showed that all the six protein forms of the glucanse have potential glycosylation sites. This is in agreement with the findings that β-1,3-glucanase is a glycosylated protein. Hydropathy plots revealed the hydrophobic (Kyte-doolittle hydropathy plot) and the hydrophilic characters (Hopp-woods scale) of the glucanases isolated. The Hopp-woods scale showed presence of at least 17 potential hydrophilic peaks which has been detected as the 17 antigenic epitopes by EMBOSS-antigenic software. Alterations in the antigenic epitope can either cause enhanced or decreased antigenic activity.

The protein modeling studies showed that the β-1,3-glucanase form, Gln2 exhibits 100 % similarity towards a protein crystallographic structure of a β-1,3- glucanase isolated previously isolated from *Hevea* (PDB ID- 3EM5 A). By superimposing the 3D structures of barley β-1,3-glucanase (1GHS) and the *Hevea* β-1,3-glucanase (3EM5 A), an interesting feature observed was despite their significant amino acid sequence variations, the 3D structures of glucanases from both these distant groups were perfectly aligning with each other showing the evolutionary structural conservation of the β-1,3-glucanase protein in plants. The catalytic clefts residing within the groove were also found to superimpose perfectly with each other. The protein alignment of the reported *Hevea* glucanases with the newly isolated forms showed few variations among them. However, the positions of the glutamic acid residues flanking either side of the catalytic cleft were found to be conserved in all the glucanases. Little variations (2-3 amino acid sequence variations) were observed in the catalytic clefts of these peptides. There are reports stating that the differences in the amino acids residing in the catalytic cleft can contribute to differences in the functional activity of the protein. The catalytic clefts of Gln 2 and 3EM5 are exactly similar. Another striking feature found was the

entire amino acid sequence of Gln 2 is aligned 100 % with the 3EM5 sequence, thereby forming a completely overlapping structure.

For the functional validation of the promoter sequences identified for the different forms of glucanases, binary vectors have been constructed using a promoter-less version of pCAMBIA 1381Z TDNA vector. Total 11 constructs with different promoter insert sizes for the comparison of promoter activity have been prepared (5 basic constructs + 6 deletion constructs). The vectors have been transformed to the *Agrobacterium* strain EHA 105. Tobacco leaf discs and *Hevea* callus were infiltrated with transformed *Agrobacterium tumefaciens* strain EHA 105. The GUS activity through transient assay showed a fair level of the reporter gene expression in the *Hevea* callus, where as in the tobacco leaf discs, no GUS expression was observed. The construct with 913 bp promoter showed increased level of GUS activity within the *Hevea* callus indicating the need for multiple regulatory elements for efficient gene expression of the β-1,3-glucanase gene in *Hevea*. The induction experiments with 0.1% salicyillic acid also did not show any detectable levels of GUS activity in the regenerated transgenic tobacco plants. This difference in the expression pattern in tobacco and *Hevea* callus may be either due to the tissue specificity of the *Hevea* β-1,3-glucanase gene promoters, presence of trans-elements in the *Hevea* callus necessary for the regulation of the promoter or due to the absence of essential enhancer elements in the isolated region of these promoter sequences. Based on the present sequence data provided in this report, further upstream elements of all these glucanase gene promoters can be identified through RAGE (Random amplification of Genomic DNA Ends), inverse PCR or overlap extension PCR so that the promoters can be used for developing disease resisitance through transgenic approaches or molecular marker assisted breeding.

The antifungal assay using six different forms of β-1,3-glucanase gene have also been conducted. Since the individual cDNAs of these different forms of glucanase cannot be amplified specifically from a common gene pool due to the occurrence of cross-hybridization of the PCR primers, the gDNA have been PCR amplified from cloned vectors and transformed to the new

generation yeast *Pichia pastoris*, the eukaryotic host where the splicing machinery is active. The non-secretory vector pPICZ A was used for the transformation. To confirm the gene integration into *Pichia* genome, southern blotting analysis using one of the forms of glucanase - Gln 2 had been performed. The southern blot showed positive integration of the gene in *Pichia* genome where as no integration of the gene in the negative control (*Pichia* DNA integrated with the insert-less vector) was observed. The time interval for the expression of the protein had also been standardized and it was found that the highest induced expression of the protein occurred at 96 hrs. The protein isoforms were purified with his-tag purification column. The presence of purified protein was also analyzed through western blot analysis using anti his-tag antibody. To confirm that the protein isolated was functionally active, activity staining of the purified proteins were performed using laminarin as the substrate for the glucanase enzyme. The results were found to be positive with all the forms having only a little relative difference in the band intensities. The *in vitro* antagonistic activity of the six glucanase isoforms against *P. meadii* have been performed in PDA plates using the disc diffusion method with 12 µg of the purified protein. In the antifungal assay, it was observed that Gln 2 exhibits the maximum antifungal activity compared to the other forms Gln 5, Gln 6 and Gln 7 where as Gln 3 and Gln 4 does not exhibit any antifungal activity. The activity can be expressed as Gln2> Gln5> Gln6> Gln7> Gln3=Gln4. This difference in the anti fungal activity may be due to the difference in the amino acids residing within the catalytic clefts. This information is very much important for determining the members of glucanase family efficiently performing the role of antifungal activity in *Hevea*.

This is the first report on molecular characterization of different forms of β-1,3-glucanase genes from a single clone of *Hevea brasiliensis* (RRII 105). β-1,3-glucanases have been reported as a part of the plant's general or horizontal resistance, which is more durable, and hence commercially more attractive, compared to the race-specific resistance mediated by the *avr-R* gene interaction that will breakdown rapidly whenever a new race of virulent pathogen evolves. It is now understood that plant species produce a broad

range of β-glu differing in molecular weight primary structure, cellular localization & catalytic activity and iso-electric points performing multifaceted actions. There are several acidic and basic glucanases distributed through out the plant kingdom. The participation of glucanases to counteract plant pathogens especially the oomycete fungi has been confirmed through their enzymatic induction during pathogenesis, *in vitro* anti-fungal properties, and finally through the development of transgenic plants with enhanced resistance. Considerable progress in understanding the structure and regulation of plant β-1,3-glucanases has been made in the previous years.

The results obtained in this study could be utilized to explore the possibility of a transgenic approach to control fungal pathogens with the right form of β-1,3-glucanase providing disease resistance in future crop improvement programmes. Since efficient transgenic approaches with considerable progress already exist in *Hevea*, the development of a transgenic plant with a highly efficient disease resistant transgene would not be a big constrain in future transformation experiments. Following a detailed investigation on the presence of still more number of isoforms and their functional efficiencies, the pooled information can be utilized for the marker assisted selection (MAS) of parents for controlled breeding for disease resistance in *Hevea*.

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