MOLECULAR CLONING AND CHARACTERIZATION OF THE GENE ENCODING β-1,3-GLUCANASE IN *Trichoderma* spp.

By

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(2006-11-109)

THESIS

Submitted in partial fulfillment of the

requirement for the degree of

Master of Science in Agriculture

(PLANT BIOTECHNOLOGY)

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2008

DECLARATION

I hereby declare that the thesis entitled **"Molecular cloning and characterization of the gene encoding β-1,3-glucanase in *Trichoderma* spp."** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, or other similar title, of any other university or society.

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CERTIFICATE

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Acknowledgement

I humbly bow my head before the lord ***Almighty*** *who blessed me with the power and courage to complete this endeavour successfully in spite of the most difficult times face by me during the period of my study.*

Words cannot express my deep sense of gratitude and indebtedness to ***Dr. Sally K Mathew****, Professor and Head, Department of Plant Pathology and chairperson of my Advisory Committee. I wish to place my heartfelt thanks to her for her inspiring guidance, untiring help, patience, encouragement, constructive criticism and valuable suggestions during the period of the investigation and preparation of the thesis. My sincere and heartfelt gratitude ever remains with her.*

I owe a great debt of gratitude to ***Dr. P.A. Nazeem****, Professor and Head, CPBMB and member of my advisory committee for her unwavering support and enthusiasm, relevant suggestions and whole hearted cooperation during my academic career.*

I am ever grateful to ***Dr. D. Girija****, Professor, CPBMB and member of my advisory committee for her invaluable help, guidance and critical assessment throughout the period of the work. I thank her for all the help and cooperation she has extended to me.*

I express my sincere gratitude to ***Dr. R. Keshavachandran,*** *Professor, CPBMB and member of my advisory committee for his valuable suggestions and guidance rendered me for the completion of the research programme and preparation of the thesis.*

I also avail this opportunity to pay my sincere obligations and heartfelt thanks to ***Valsala*** *madam,* ***Augustin*** *sir,* ***Rajendran*** *sir and* ***Sujatha*** *madam of CPBMB for their encouragement and kind help offered at different stages of the study.*

I thank ***Mr. P. K. Sreekumar****, Farm Assistant, CPBMB, for the help in photography.*

I wish to express my sincere thanks to all the non-teaching staff members and labourers of CPBMB for their whole hearted cooperation and timely assistance.

Special thanks go to ***Santhosh chettan****, Students Computer Club, COH, for rendering necessary help whenever needed.*

I express my deep sense of gratitude to research workers of Dept. of Plant Pathology, CPBMB and Bioinformatics Centre, ***Gleena chechi, Suma chechi, Amrathaa chechi, Anu chechi, sunanda chechi, Shruthi chechi, Alisha chechi, Reena chechi, Shailaja chechi, Jini chechi, Shiba chechi, Soumya chechi, Sherin chechi, Simi chechi, Firoz chettan, Sunil chettan*** *and* ***Sharjahan,*** *who helped me in several ways for the completion of this venture. Words of gratitude fall short of expressing the care and concern rendered to me by my most esteemed sister* ***Praveena chechi*** *and* ***Rekha chechi****.*

Words fall short as I place on record my indebtedness to my seniors and class mates, ***Neema chechi, Likhitha chechi, Sameera chechi, Anjali chechi, Geena chechi, Ramya chechi, Saisree chechi, Liffey chettan, Fayas chettan, Shiji, Asha, Jusna, Ragina, Renu, Rashmi, Sindhu, Kimi, Hema, Sangeetha, Thenmozhi, Swapna, Julie, Nisha, Sathish, Thiagarajan, Rahul, Dinesh, Jaba, Alok, Suja, Kiran*** *and* ***Shivaji*** *for their prompt help and cooperation for the entire period of study.*

I am in dearth of words to thank my friends ***Tusar, Sagarika, Swarnalata, Swetaleena, Shrabani, Sujata, Tanushree, Sarika, Usha*** *for their encouragement and lively support.*

With gratitude and affection, I recall the boundless affection, constant encouragement, warm blessings and motivations from my parents, sisters and brothers without which this endeavour would never have become a reality.

Niharika Nath


Dedicated to
My Loving
Family

ABBREVIATIONS

A	Adenine
AASTATS	Amino acid statistics
ARI	Agarkar Research Institute
bp	Base pair
β	Beta
BLAST	Basic local alignment search tool
cm	Centimeter
cfu	Colony forming unit
CPBMB	Centre for Plant Biotechnology and Molecular Biology
°C	Degree Celsius
DNA	Deoxyribo Nucleic Acid
DNSA	3,5 – dinitrosalicylicacid
EDTA	Ethylene Diamine Tetra Acetic acid
G	Guanine
g	Gram
h	Hour (s)
IARI	Indian Agricultural Research Institute
IISR	Indian Institute of Spices Research
IPTG	Isopropylthio-β-D-galactoside
KAU	Kerala Agricultural University
kb	Kilo base pairs
kDa	Kilo Dalton
l	Litre
LB	Luria Broth
LBA	Luria Bertani Agar
ml	Millilitre
mM	Millimolar
µg	Microgram
µl	Microlitre
µM	Micromolar
min	Minute(s)
NASTATS	Nucleic Acid Statistics
NCBI	National Centre for Biotechnology Information

NCFT	National centre for Fungal Taxonomy
ng	Nanogram
nm	Nanometer
OD	Optical Density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
pH	Hydrogen ion concentration
%	Percentage
pI	Isoelectric point
RNA	Ribo Nucleic Acid
RNase	Ribonuclease
rpm	Rotations per minute
SDS	Sodium Dodecyl Sulphate
sec	Second (s)
T	Thymine
TAE	Tris Acetate EDTA
TE	Tris EDTA
TS	*Trichoderma* selective
TSB	*Trichoderma* special broth
U	Unit
UV	Ultra violet
V	Volts
v/v	Volume by volume
w/v	Weight by volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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Introduction

1. INTRODUCTION

Plant pathogens continue to reduce the availability of food resources on a global scale as well as to diminish the economic potential of green house and nursery industries (Pinstrup-Anderson, 2000; Oerke and Dehne, 2004). As fungi are one of the major causal agents of plant diseases, modern agriculture still highly depends on the use of fungicides to control plant diseases. Over dependence on chemical pesticides has become untenable in view of the health hazards and environmental pollution leading to elimination of several non target beneficial fauna like natural enemies, bees, pollinators, birds, reptiles, small mammals and micro-organisms. Numerous advantages have been described for the development of biocontrol agents for fungal diseases including being environmentally compatible, presenting the potential for a sustainable long-term diverse disease control, promoting nursery and field work safety and reducing the development of pest resistance (Mathre *et al*., 1999; Alabouvette *et al*., 2006).

Species of the genus *Trichoderma* have been used world-wide as effective biocontrol agent for a wide range of economically important plant pathogens with more than 50 registered *Trichoderma* based bioproducts available in the market (Woo *et al*., 2006). Even though *Trichoderma* spp. are usually considered free living saprophytes in soil, they are known to be opportunistic, avirulent plant symbionts as well as being parasites of other phytopathogenic fungi (Harman *et al*., 2004). Potential native *Trichoderma* spp. antagonistic against major soil borne pathogens of Kerala have been isolated and reported by many workers. The mechanisms that have been described to account for biocontrol of plant disease by *Trichoderma* spp. include mycoparasitism, induced resistance in the plant host, competition for the substrate and space and antibiosis (Howell, 2003). Mycoparasitic activity of *Trichoderma* is due to the production of several cell wall degrading enzymes such as glucanases, chitinases, cellulases and proteases (Cortes *et al*., 1998; Sanz *et al*., 2004; Steyaert *et al*., 2004). A considerable amount of research has been aimed at elucidating the β-1,6-glucanase system of *Trichoderma* spp., mainly *T. harzianum.* A search on literature revealed many reports regarding the research on

chitinase activity of *Trichoderma* in India. However, the reports on the glucanase activity are meagre and scanty.

β-1,3-glucan is one of the main structural component of the cell wall of oomycetes fungi. Thus, β-1,3-glucanases (EC 3.2.1.39) protein secreted by *Trichoderma* spp. have been suggested as the key enzymes in the lysis of phytopathogenic fungal cell walls during mycoparasitic action (Elad *et al*., 1982; Sivan and Chet, 1989). β-1,3-glucanases have also been reported to be pathogenesis related proteins in plants and proposed to have a major role in the defense reaction against pathogens (Bowler, 1990; Broglie *et al*., 1991). The antifungal activity is synergistically enhanced when different *Trichoderma* cell wall degrading enzymes act together or in combination with PR proteins, commercial fungicides, cell membrane affecting toxins or biocontrol bacteria (Lorito *et al*., 1993, 1996, 1998). For these reason, the genome of mycoparasites, which has evolved specifically to attack other fungi but not plants, represents a potential source of powerful antifungal gene.

Genetic transformation with the gene in plant has been reported to impart resistant to different fungal pathogen. Also the over expression of this gene in antagonistic *Trichoderma* spp. has resulted in increased biocontrol activity (Migheli *et al*., 1998; Djonovic *et al*., 2007). Gene encoding β-1,3-glucanase enzyme have been isolated from many plants. Any work regarding the molecular characterization of glucanase gene in *Trichoderma* has not been reported so far from Kerala. Hence the present study entitled 'Molecular cloning and characterization of the gene encoding β-1,3-glucanase in *Trichoderma* spp.' was undertaken with the following objectives:

1. Biochemical characterization for determining the glucanase activity of potential antagonistic *Trichoderma* isolates.
2. Isolation & characterization of β-1,3-glucanase gene in *Trichoderma* spp. which exhibit maximum enzyme activity.

Review of Literature

2. REVIEW OF LITERATURE

India, acknowledged as a growing economic giant with a growth rate of 12.5 per cent in the year 2006-07 is poised for a quantum leap into the global market. With a target of 16 per cent growth in its GDP, India is set to shine brighter in the coming year (Rabindra, 2006).

Modern agriculture is highly dependent on chemical pesticides. India accounts for one-third of the total pesticides poisoning cases in the world. During the onset of green revolution period, the consumption of pesticides went up to 289 g/ha in 1974-78 while it was just 16 g/ha in 1950-51 and in 1992-92, it became 431 g/ha. However the consumption declined to 288 g/ha in 1999-2000 due to the increasing awareness of the negative impact of use of pesticides on environment and health (Ramkumar and Chavan, 2004).

Even though chemical control is a promising method under intensive cropping programme, their use poses problems of residue left over on crop which seriously cause many health hazards. Combined with the continued public concern regarding the safety of pesticides and the effect of these compounds on the environment, a tremendous opportunity exists to develop biocontrol agents as direct substitutes for chemicals or as key components in integrated management systems that are more biointensive or ecologically based (Martin, 2003; Fravel, 2005). Chemicals used to control soil borne diseases are uneconomical, less effective and leave residues in soil and plants. So in recent years, scientist's attention has been shifted to the control of diseases using biocontrol agent, which are environmentally safe and promising alternatives to chemical pesticides.

During the past few years, the development of plant genetic engineering as a tool for crop improvement has led to the development of crop plants resistant to fungal pathogen using different molecular tools and transformation techniques.

Trichoderma has been demonstrated to be a potential biocontrol agent against several phytopathogenic fungi. Mycoparasitism has been described as the main process involved in the antagonistic action of *Trichoderma.* It is a complex process that includes release of lytic enzymes like chitinase, glucanase, cellulose and protease by *Trichoderma* for degradation of cell wall of fungal pathogen. β-1,3-glucan is a cell wall component of most fungi and β-1,3-glucanases have been found to be directly involved in the mycoparasitic interaction between *Trichoderma* species and its host.

A comprehensive review of the previous research studies related to the topic had been done in accordance with the objectives of the present study.

2.1. BIOLOGICAL CONTROL

According to Baker and Cook (1974), biological control is defined as "the reduction of inoculum density or disease producing activities of a pathogen or parasite in its active or dormant state, by one or more organism, accomplished naturally or through manipulation of environmental, host or antagonist or by mass introduction of one or more antagonists". Cook (1987) defined "Biological control" as the use of natural or modified organisms, genes or gene products to reduce the effects of undesirable organisms (pests) and to favour desirable organism such as crops, trees, animals, beneficial insects and micro-organisms. Biological control, using micro organisms against the plant pathogens is an effective and alternative tool in managing the disease and is gaining importance in recent years.

It aims at biological destruction of soil borne pathogens without impairing ecological balance. Although concerted efforts on biocontrol of pathogens were made since 1930's (Weindling, 1932), pragmatic approach to tackle the problem was made only in recent years. The interactions between biocontrol and pathogens have been studied intensively and the application of biocontrol agents in the protection of some commercially important crops is promising (Papavizas, 1985; Weller, 1988). A number of fungi belonging to the genera *Gliocladium, Trichoderma, Coniothyrium, Latesaria, Sporodesmium, Aspergillus* and *Fusarium*

and several bacteria and actinomycetes are known for their potential biocontrol activities against soil borne pathogens (Majezak, 1983; Adams, 1990; Naik and Sen, 1992).

Biological control, especially using fungal antagonists against fungal pathogens has gained considerable attention and appears to be promising as viable supplement or alternative to chemical control (Natarajan and Manibhushanrao, 1996). Dube (2001) described biological control as a mysterious natural phenomenon which operates in disease suppressive soil and is not easy to comprehend and initiate with success.

2.2. *Trichoderma* spp.

Originally, the taxonomy and identification of *Trichoderma* was introduced by Persoon (1794). *Trichoderma* pers. Extr., a genus under Deuteromycotina, Hyphomycetes, Phialospora, Hyphales, Dematiaceae has gained immense importance since last few decades due to its biological control ability against several plant pathogens (Liu and Baker, 1980; Papavizas, 1985; Liu *et al.*, 1995; Mukhopadhyay and Mukherjee, 1996; Hjeljored and Tronsmo, 1998; Kubicek and Harman, 1998).

The anamorphic fungal genus *Trichoderma* contains cosmopolitan soil borne fungi frequently and found on decaying wood as a saprophytic fungus (Samuels, 1996; Klein and Eveleigh, 1998). The researchers are interested on this genus because of its novel biological properties and biotechnological application. Benefits of *Trichoderma* includes their significance as economically important producers of industrial enzymes and antibiotics, usage in fabric detergent, animal feed and fuel production, alternative to conventional bleaching, effluent treatment, degradation of organochloric pesticides and more importantly as biocontrol agent and is harmful as parasite in mushroom cultivation and as pathogen in immunocompromised patients (Samuels, 1996; Kubicek and Pentila, 1998; Sivasithamparam and Ghisalberti, 1998; Hjeljored and Tronsmo, 1998).

Trichoderma has been recognized to comprise a significant amount of fungal biomass in soil (Widden and Abitol, 1980; Nelson, 1982) and is frequently present as indoor contaminant (Tharne *et al*., 2001). More recently, *T. longibrachiatum* has also become known as opportunistic pathogen of immuno compromised mammals including humans (Kredier *et al*., 2003).

Strains of different taxons from the *Trichoderma* genus (Hermosa *et al*., 2000) have invited agronomic interest not only because of their potential use as biocontrol agents against several fungi (Howell, 2003), but also a plant avirulent symbionts that can trigger plant induced resistance and promote plant growth (Harman *et al*., 2004).

Martinez *et al.* (2008) assembled 89 scaffolds to generate 34 Mbp of nearly contiguous *T. reesei* genome sequence comprising 9,129 predicted gene models. *Trichoderma reesei* is the main industrial source of cellulases and hemicellulases used to depolymerize biomass to simple sugars that are converted to chemical intermediates and biofuels, such as ethanol.

2.3. *Trichoderma* as a potential biocontrol agent

The most exhaustively studied microorganism as a biocontrol agent is *Trichoderma* sp. The antagonistic potential of this fungus was first demonstrated by Weindling (1932), who demonstrated the antagonistic action of *T. viride* on *Rhizoctonia solani*.

Some species of *Trichoderma* have been extensity tested and described as biological control agents against wider range of plant pathogens *viz. Rhizoctonia, Fusarium, Sclerotium, Macrophomina, Sclerotinia, Pythium, Phytophthora, Ralstonia* spp. etc. (Papavizas, 1985; Chet, 1987; Hornby, 1990; Deacon, 1991).

Wells *et al*. (1972) first demonstrated antagonistic action of *T. harzianum* against *Sclerotium rolfsii* under field conditions. The antagonism of *Trichoderma*

spp. against *Rhizoctonia solani* was observed by various workers (Hardar *et al*., 1979; Kim and Row, 1987; Hazarika and Das, 1998; Mathew and Gupta, 1998).

Hardar *et al*. (1979) observed that *T. harzianum* could directly attack *R. solani* and could control damping off of bean, tomato and egg plant seeding caused by this pathogen. The fermenter biomass production of *T. viride* applied as dust to the seed potatoes infected with sclerotia of *R. solani* before planting reduced disease incidence in field by 50 per cent (Beagle-Ristanio and Papavizas, 1985).

Critinzio (1987) noticed *in vitro* antagonistic effect of *Trichoderma* sp. on *Phytophthora capsici* in capsicum. Sarma *et al*. (1994) noticed predominance of *Trichoderma* spp. antagonistic against *P. capsici* of black pepper. Thomas *et al*. (1996) observed various antaganostic mechanisms of *Trichoderma* spp. and concluded *Trichoderma* as a potential bioagent against *Phytophthora* of small cardamom. Dipping of black scurf infected potato tuber in two per cent aqueous suspension of *T. viride* grown on wheat straw–wheat bran substrate significantly reduced black scurf incidence for three years in field experiments (Singh *et al*., 1997).

According to Mahanty *et al*. (2000), although biological control approach was not better than chemical control in terms of per cent disease incidence caused by *Phytophthora* in betel vine, the use of highly effective isolate of *T. harzianum* was much safer and gave equivalent yield in terms of number and weight of leaves produced.

Gupta *et al*. (2002) observed drastically inhibition on the growth of *Fusarium oxysporum* f.sp. *ciceri, S. rolfsii* and *R. solani* by *T. viride* under *in vitro* conditions. Vyas and Mathur (2002) also observed effective inhibition on the growth and sporulation of *F. oxysporum* with the effect of volatile and non-volatile antibiotics of *Trichoderma* spp. The cell lytic enzymes like β-1,3-glucanase and chitinase play a major role in inhibiting the cell wall synthesis of *F. culmorum* (Witkowska and Maj, 2002).

According to Rajan *et al*. (2002), *T. viride* and *T. harzianum* were found more effective to control the foot rot disease of black pepper. The isolates of *Trichoderma* viz., *T. viride, T. aureoviride, T. harzianum, T. pseudokoningii, T. polysporum, T. longibrachiatum* and *T. koningii* were able to inhibit, over grow and lyse the mycelia of *P. capsici* of black pepper to varying degree (Saju *et al*., 2002). He also reported that the percent inhibition of foot rot by different species of *Trichoderma* varied from 20-81.

2.4. Mechanism of antagonism

A number of parasitic fungi capable of penetrating thick walls of both chlamydospores and oospores have been identified (Snahe *et al*., 1977). The mechanisms by which *Trichoderma* spp. control diseases include their competitive saprophytic ability, antibiotic production, direct parasitism and lysis (Ayers and Adams, 1981; Bell *et al*., 1982). Initially the mycoparasite grows directly towards its host and often coils around it or attaches to it by forming hook like structures and appressoria (Elad *et al*., 1983). Elad *et al*. (1984) observed that *Trichoderma* spp. penetrating the host mycelium, by partially degrading its cell wall. Finally, he assumed that, *Trichoderma* spp. utilize the intracellular content of the host. From further works, it appears that *Trichoderma* mycoparasitism is a complex process involving several successive steps (Chet, 1987).

2.4.1. Induction of systemic resistance in plants by *Trichoderma*

Recent discoveries indicate that *Trichoderma* spp. can also induce systemic and localized resistance to a variety of plant pathogens. Certain strains also have substantial influence on plant growth and development both in axenic system (Lindsey and Baker, 1967; Yedidia *et al.,* 2001) and natural field soil (Chang *et al.*, 1992; Harman, 2000).

According to Harman (2000), *Trichoderma* spp. exhibited two types of mechanism like rhizosphere competence, which is the ability of the micro organism

to grow and function in the developing rhizosphere and induce systemic acquired resistance (SAR), which gave long term protection at a substantial distance from the infection court.

The first clear demonstration of induced resistance by *Trichoderma* was given by Bigirmana *et al.* in 1997. They showed that treating soil with *T. harzianum* strain, T-39, impart resistance in bean plants to *B. cinerea* and *Colletotrichum lindemuthianum,* even though T-39 was present only in the rhizosphere of the crop. Evans *et al.* (2003) reported that a strain of *T. stromaticum* (T-203) is endophytic in the vascular system in cocoa. Mathew (2006) reported two endophytic *Trichoderma*, *T. viride* and *T. pseudokoningii* from black pepper.

2.4.2. Lytic enzymes

Mycoparasitism by enzymatic lysis of pathogenic fungal hyphae through the production of enzymes like β-1,3-glucanase, chitinase, cellulase and protease has been reported by many scientists (Elad *et al*., 1983; Papavizas, 1985; Inbar and Chet, 1997; Cortes *et al*., 1998; Vazquez-Garciduenas *et al*., 1998; Zeilinger *et al*., 1999; Sanz *et al*., 2004; Steyaert *et al*., 2004).

Phaff *et al*. (1979) reported classification of β-glucanases as β-1,3-glucanase, β-1,6 and β-1,4-glucanase based on the type of glycosidic linkages cleaved by them. Fungal cell walls contain different kinds of β-glucans as structural component. These β-glucans are generally composed of a major component of predominantly β-1,3–linked glucans with branches of β-1,6–glycosidic linkages (Peberdy, 1990). However, these β-glucanases such as β-1,3-glucanase, β-1,6-glucanase enzymes may be involved in the effective and complete degradation of mycelial or conidial walls of phytopathogenic funi by *Trichoderma.* Cellulose, the homopolymer of 1,4- β-glucan, is used as an energy source for *Trichoderma* spp. The cellulolytic system of *Trichoderma* spp. consist of three classes of enzymes such as 1,4-β-D-glucan cellobiohydrolases, endo-1,4-D-glucanases and 1,4-β-D-glucosidases, which were hydrolyse cellulose. The cellulolytic system of *T. ressei*

RUT C30, which is known to be very high cellulase producing strains, was characterized by Migheli *et al.* (1998).

β-1,3-glucanases are widely distributed among bacteria, fungi, yeasts, actinomycetes, insects, fish and higher plant (Boller, 1985; Pan *et al*., 1989).They are classified as exo-β-1,3-glucanases [β-1,3-glucan glucohydrolase (EC 3.2.1.58)] and endo-β-1,3-glucanases [β-1,3-glucan glucanohydrolase (EC 3.2.1.6 or EC 3.2.1.39)]. These enzymes can hydrolyse the substrate by two possible mechanisms (Pitson *et al*., 1993). Those are, exo-β-1,3-glucanases hydrolyze β-glucans by sequentially cleaving glucose residues from the non-reducing end, and endo-β-1,3-glucanases cleave β-linkages at random sites along the polysaccharide chain, releasing smaller oligosaccharides and glucose.

2.4.3. Role of β-1,3-glucanase

Distinct physiological roles of β-1,3-glucanases in different organism has been proposed by different scientists.

2.4.3.1. In fungi

Peberdy (1990) reported that β-1,3-glucanases have physiological role in morphogenetic-morpholytic process during development and differentiation in fungi. He also found that chitin and β-1,3-glucan are the main structural components of fungal cell wall except the class oomycetes, which contain β-1,3-glucan and cellulose. Oomycetes like *Phytophthora infestans, P. capsici* and *Pythium* spp. have a cell wall that is comprised of 80-90 per cent β-D-1,3-glucan. It has been suggested that β-1,3-glucanases play a nutritional role in saprophytes and mycoparasites (Chet, 1987; Lorito *et al*., 1994).

According to Rapp (1992) and Stahmann *et al.* (1992), β-1,3-glucanases have been related to the mobilization of β-glucans under conditions of carbon and energy source exhaustion, functioning as autolytic enzymes. Schaeffer *et al*. (1994)

reported that β-1,3-glucanases are also involved in fungal pathogen-plant interactions, degrading callose (β-D-1,3-glucan) in host's vascular tissues during pathogen attack.

The fungal cell wall composed of chitin, β-D-1,3-glucans, β-D-1,6-glucans, lipids and peptides embedded in a protein matrix. It is important to note that fungi have significant internal turgor pressure so that even slight perturbation of the cell wall results in fungal cell lysis (Harold and Caldwell, 1990; Kaminsuyi *et al*., 1992; Money and Harold, 1993).

Thus, β-1,3-glucanases produced by *Trichoderma* spp. are key enzymes in the lysis of cell walls during their mycoparasitic action against phytopathogenic fungi (Shen *et al*., 1991; De la Cruz *et al*., 1995; Noronha *et al.,* 2000; Montero *et al.*, 2006).

2.4.3.2. In bacteria

Watanabe *et al*. (1993) reported that β-1,3-glucanases have metabolic function in bacteria. These enzymes are polysaccharide endohydrolases (Chen *et al*., 1993; Varghese *et al*., 1994). β-1, 3-glucanases hydrolyze 1,3-β-glucanase linkages, but they usually require a region of unsubstituted, contiguous 1,3-β-linked glucosyl residues. Henrissat and Bairoch (1996) found that the bacterial β-1,3-glucanases was included in the family 16 of glucosyl hydrolases.

2.4.3.3. In plant

β-D-1,3-glucan (called callose in plants) is also a cell wall component of certain cell types during specific developmental stages of plants (Abeles and Forrence, 1969; Kauss, 1987, 1992; Kotake *et al*., 1997).

As the substrate for β-1,3-glucanases exists in plants, these enzymes are involved in various physiological and developmental processes such as cell elongation (Masuda and Wada, 1967; Heyn, 1969), cell division (Fulcher *et al*.,

1976), fruit ripening by cell wall degradation leading to fruit softening (Hinton and Pressey, 1980), fertilization (Ori *et al.*, 1990), pollen germination and tube growth (Roggen and Stanley, 1969; Meikle *et al*., 1991), bud dormancy (removal of phloem callose) (Krabel *et al.*, 1993), microsporogenesis (dissolution of pollen tetrads into free microspores) (Bucciaglia and Smith, 1994), somatic embryogenesis (Helleboid *et al.,* 2000), seed germination (Morohashi and Matsushima, 2000; Buchner *et al.,* 2002), and flower formation (Neale *et al.*, 1990**;** Akiyama *et al.,* 2004).

Based on the hydrolytic activities of β-1, 3-glucanases and their relationships to pathogen infections, β-1, 3-glucanases have been suggested as an important component of plant defense mechanisms against pathogens (Kauffmann *et al.,* 1987; Mauch and Staehelin 1989; Linthorst, 1991; Cordero *et al.*, 1994). Plant β-1,3-glucanases are pathogenesis related proteins as synthesis of these enzymes can be induced by pathogen or other stimuli. In addition, these enzymes may play an indirect role in plant defense by causing the formation of oligosaccharide elicitors, which elicit the production of other PR proteins or low molecular weight antifungal compounds, such as phytoalexins (Keen and Yoshikawa, 1983; Ham *et al.,* 1991; Klarzynski *et al.,* 2000).

2.5. PR Proteins

Plants, when exposed to pathogens such as fungi and viruses produce low-molecular weight antimicrobial compounds called phytoalexins, antimicrobial peptides and small proteins and up-regulate a number of antimicrobial proteins. These plant proteins are called as pathogenesis related (PR) proteins (Van Loon, 1985).

PR proteins were originally identified in tobacco plants infected by tobacco mosaic virus (Van Loon and Van Kammen, 1970). These proteins were subsequently detected in a wide range of plant species. PR protein can be acidic or basic proteins depending on their isoelectric points (pI). Acidic PR proteins are predominantly located in the intercellular spaces. Basic PR proteins, while having similar functions to acidic PR proteins, are mainly located intracellularly in the

vacuole (Legrand *et al*., 1987; Niki *et al*., 1998; Van Loon and Van Strien, 1999). Most PR proteins are acid soluble, low molecular weight and protease-resistant proteins (Leubner-Metzger and Meins, 1999; Neuhans, 1999).

Based on amino acid sequence similarities, serologic relationships and/or enzymatic or biological activities, PR protein can be categorized into 17 families (Shi, 2005)

Table 1. Recognized families of pathogenesis-related 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 P_{69}	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

2.5.1. PR-2 proteins

The PR-2 proteins encoded by different β-1,3-glucanase gene show differences in size, isoelectric point (pI), primary structure, cellular localization and pattern of regulation (Meins *et al*., 1992). Based on these differences especially primary amino acid sequence differences, β-1,3-glucanases can be divided into four different classes (Payne *et al*., 1990; Ward *et al*., 1991; Leubner-Metzger and Meins, 1999). Class I β-1,3-glucanases are basic, vacuolor, pathogenesis inducible proteins

of ~33kDa (Castresana *et al*., 1990; Beerhues *et al*., 1994). Class II, Class III and Class IV are acidic, extra cellular proteins of about 36 kDa (Beffa *et al*., 1993; Bucciaglia and Smith, 1994). Class II and Class III isoforms are induced upon pathogen attack but class IV isoforms are non responsive.

Class I proteins are synthesized as preproteins that are processed prior to being enzymatically active. Premature class I β-1,3-glucanases contain a signal peptide and C-terminal extension. Uknes *et al*. (1992) found that two *Arabidopsis* β-1,3-glucanases, BG1 and BG3 showed structural homology to tobacco class I proteins which are basic vacuolar enzyme except for the absence of a C-terminal extension.

PR-2 proteins (β-1,3-glucanases) have been found in a wide variety of plants inducing tobacco (De Loose *et al*., 1988), soybean (Takeuchi *et al*., 1990), *A. thaliana* (Uknes *et al*., 1992), rubber tree (Chye *et al*., 1995), pepper (Kim and Hwang, 1997), banana (Peumans *et al*., 2000), rice (Yamaguchi *et al*., 2002), strawberry (Shi, 2005) etc.

There proteins are active *in vitro* at micromolar levels (450 µg/ml) against a wide number of phytopathogenic fungi. The antifungal activity of PR-2 proteins has been convincingly demonstrated by a number of *in vitro* enzyme and whole cell assays as well as in planta using transgenic plants over expressing a PR-2 protein (Stinzi *et al*., 1993; Jach *et al*., 1995; Zadoks and Waibel, 2000).

2.5.1.1. Induction of β-1,3-glucanases by pathogen and pathogen derived elicitors

Acidic and basic β-1,3-glucanases were induced in the presence of various polysaccharides.

De la Cruz *et al*. (1995) suggested that β-1,3-glucanase in *Trichoderma harzianum* was specifically induced by autoclaved yeast cell walls or filamentous fungi. Noronha and Ulhoa (2000) studied the activity β-1,3-glucanase of *T.*

harzianum by using different fungal cell walls from *R. solani, Sclerotium rolfsi* and *Pythium* spp. as inducer. They found high levels of β-1,3-glucanase activity in the culture medium containing purified cell walls of *Pythium* spp. β-1,3-glucanase activity was higher with laminarin (β-1,3-glucan) than with pastulan (β-1,6-glucan) or pullulan (α-1,6-glucan), suggesting that the induction patterns of the enzyme may vary in response to the glucan structure and type of linkage (Vazquez-Garciduenas *et al*., 1998). Montero and Ulhoa (2006) reported that the highest activity was obtained in *T. koningii* when cell wall of *R. solani* was used as inducer rather than chitin, chitosan, cellulose, starch and glucose.

Many studies have shown that β-1,3-glucanases are constitutively expressed at low levels in plants, but are dramatically increased when plants are infected by fungal, bacterial or viral pathogens. Van Kan *et al*. (1992) reported that the mRNA for a tomato acidic β-1,3-glucanase accumulated to a higher level in leaves infected by the fungal pathogen *Cladosporium fulvum*.

Different β-1,3-glucanases may be induced in different ways. It was studied in tobacco plant that class I β-1,3-glucanases were induced as a local response while classes II and III were induced both locally and systemically (Lusso and Kuc, 1995; Bol *et al*., 1996).

There are many findings which showed higher expression levels of these enzymes after infected 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).

Induction of β-1, 3-glucanases by pathogens also depends on the plant clones. The production of β-1, 3-glucanase upon infection with *Corynespora*

cassiicola was compared in different clones of *Hevea brasiliensis*. Considerable variability of the enzyme's activity was observed among different clones during pathogenesis. Increased enzyme activity was found in the tolerant clone, while a decrease was observed in the susceptible clone (Philip *et al.*, 2001).

Some components of pathogens or degraded components of pathogens can serve as elicitors of β-1,3-glucanases. These elicitors may be component of the cell surface of the pathogen that are released by host enzymes including fungal β-glucan, chitin, chitosan, glycoproteins and N-acetyl chito oligosaccharides (Chang *et al*., 1992; Kaku *et al*., 1997; Muench-Garthoff *et al*., 1997).

Three classes of biochemical elicitors, those are produced by *Trichoderma* strains and induce resistance in plants. These are proteins with enzymatic on other functions, homologues of protein encoded by the avirulence (*Avr)* genes and oligosaccharides and other low-molecular weight compounds, that are released from fungal or plant cell walls by the activity of *Trichoderma* enzyme (Hammand-Koshak *et al.*, 1995; Baker *et al.,* 1997).

A 22 kDa xylase, that is secreted by *Trichoderma* spp. was shown to induce ethylene production and plant defense responses (Lotan and Fluhr, 1990). It produced ony localized resistance and necrosis. Hanson and Howel (2004) reported that six peptides ranged from 6.2 to 42 kDa size of *T. virens* were shown to have elicitor activity. Woo *et al*. (2006) reported *Avr* gene from *T. atroviride* function as race or pathovar specific elicitor for inducing hypersensitive responses and defence reaction in plant corresponding to resistance gene.

2.5.1.2. Synergistic action

Lorito *et al*. (1994) reported that the antifungal activity is synergistically enhanced when different *Trichoderma* cell wall degrading enzymes are used together. It has also reported about mycoparastic activity of *Trichoderma* spp., that was increased when ß-1, 3-glucanase and other plant PR protein act synergistically (Lorito *et al*., 1996). There were many reports about the compatibility of biocontrol

agent *Trichoderma* with different fungicides, insecticides and chemical fertilizers (Sharma *et al*., 2001; Sarma, 2003; Jayaraj and Rambadran, 1998). Synergism among lytic enzymes and between enzymes and antibiotics suggests formulations to test mixtures of *Trichoderma* transformants that produce different enzymes, in order to improve the antagonistic effects of BCAs on phytopathogenic fungi (Benitez *et al.*, 1998). It has been shown that β-1,3-glucanases inhibit spore germination or the growth of pathogens in synergistic cooperation with chitinases (Benitez *et al.,* 1998; EI-Katany *et al.,* 2001) and antibiotics (Howell, 2003; Harman *et al.,* 2004). Chawla (2003) reported increased resistance to fungal pathogens by β-1,3-glucanase genes in association with chitinase genes in plants.

2.6. BIOCHEMICAL CHARACTERISATION

Tangarone *et al*. (1989) purified a laminarinase (endo-β-1,3-glucanase) from *T. longibrachiatum* and characterized through 12.5 per cent SDS-PAGE. He found the molecular weight of protein as 70 kDa and pI was 7.2.

Pan *et al*. (1991) found the elevation of ß-1, 3-glucanase activity in the systemically protected leaves of both *Peronospora tabaciana* and TMV induced tobacco plant. He also studied the localization of β-1,3-glucanase isoforms (G_1, G_2 and G_3) in 15% PAGE gel. A study on groundnut showed that three isozymes of β-1,3-glucanase existed in the plant and the activities of these isozymes increased after infection by *Cercospora arachidicola* (Roulin and Buchala, 1995).

De La Cruz *et al*. (1995) characterized the β-1,3-glucanase protein from *T. harzianum* by SDS-PAGE after purification. He also sequenced N-terminal and internal peptide of the enzyme from Western blots. Vazquez-Garciduenas *et al*. (1998) characterized β-1,3-glucanase in *T. harzianum*. He found that seven extracellular β-1,3-glucanase produced upon induction with laminarin, a soluble β-1,3-glucanase and the molecular weight ranged from 60 to 80 KDa with pI 5 to 6.8. He also estimated β- 1,3 – glucanase activity produced by *T. harzianum,* grown in

PDB supplemented with the mycelium of *Phytophthora citrophthora* as the sole carbon source and reported to be 700 mU/ mg of protein/ min.

Noronha and Ulhoa (2000) characterized a 29 KDa β-1,3-glucanase from *T. harzianum* by 10% SDS-PAGE after it was being purified in sequential steps by gel filtration, hydrophobic interaction and ion exchange chromatography. They estimated β-1,3–glucanase activity of *T. harzianum* grown in the culture medium containing cell walls of *Pythium* spp. and reported to be 14.62±0.06 U/ mg of protein/ min. They found the K_m and V_{max} values for ß-1,3-glucanase, using laminarin as substrate were 1.72 mg/ ml and 3.10 U/ml respectively and also suggested that the enzyme was strongly inhibited by $HgCl_2$.

Parab (2000) and Jabekumar *et al*. (2001) observed that β-1,3-glucanase activities were significantly higher in *P. capsici* infected black pepper leaf tissue. They compared their activities in healthy and infected plants by colourimetric assay and SDS-PAGE. They detected high levels of enzyme in tolerant cultivar.

β-1,3-glucanase from *T. koningii* induced by cell wall of *R. solani* was characterized. The molecular weight of the purified enzyme was 75 kDa was estimated by SDS-PAGE (Montero and Ulhoa, 2006).

Montero *et al*. (2007) characterized the three different isoform of β-1,6-glucanase (BGN16.1, BGN 16.2, BGN 16.3) of *T. harzianum* through SDS-PAGE and Western blotting .

2.7. MOLECULAR CHARACTERIZATION

The PCR process was originally developed to amplify short segments of a longer DNA molecule (Saiki *et al*., 1985). β-1,3-glucanase gene was isolated from different *Trichoderma* spp., fungi, bacteria and plants by PCR based methods. The details of β-glucanase gene isolated from some of the fungi are given in Table 2.

Table 2. Sequence information of β-glucanase gene isolated from some of the fungi

Sl. No.	Fungus name	Accession No.	Gene name	Source	Size (bp)	Authors
1	*Aspergillus fumigatus*	XM_747722	exo-beta-1,3-glucanase (Exg0)	mRNA	2844	Nierman *et al.*, 2005
2	*Trichoderma asperellum*	EU314718	beta -1,3 - exoglucanase	mRNA	3096	Bara *et al.*, 2003
3	*T. harzianum*	X84085	endo-1,3(4)-beta-glucanase	mRNA	2484	De la Cruz *et al.*, 1995
4	*T. reesei*	AF282243	beta-1,3-1,4-glucanase	mRNA	285	Xu *et al.*, 2000
5	*Hypocrea virens*	EF633613	endo-beta -1,3-glucanase (bgnI)	DNA	2345	Mohammadzadeh *et al.,* 2007
6	*T. viride*	EF176582	beta-1,3-glucanase (glu) gene	DNA	2410	Gao *et al.*, 2008
7	*T. longibrachiatum*	X60652	endo-1,4-beta-glucanase (egll)	DNA	1845	Perez-Gonzalez, 2006
8	*Piromyces communis*	EU314936	endo-1,3-1,4-beta-glucanase (licWF3)	mRNA	934	Duan *et al.*, 2008
9	*T. harzianum*	X79197	endo-beta-1,6-glucanase	mRNA	1452	Lora *et al.*, 1995
10	*Hypocrea virens*	EU149643	beta-1,6-glucanase	DNA	1351	Shaikh, 2007
11	*Hypocrea virens*	AF395756	beta-1,3-glucanase precursor (bgn2)	DNA	3027	Kim *et al.,*2002

The isolation and characterization of an endo-β-1,3-glucanase namely BGN 13.1 from the mycoparasitic fungus *T. harzianum* was carried out by synthetic oligonucleotide primer by PCR technique. The expression pattern was studied at cDNA and mRNA level through southern and northern blotting (De la Cruz *et al*., 1995).

In *Trichoderma* spp., a number of gene encoding bydrolytic enzymes were cloned and characterized viz. Proteinase gene (Prb1) from *T. harzianum* (Geremia *et al*., 1993), endochitinase gene (ThEn-42) from *T. harzianum* (Hayes *et al*., 1994), α-1,3-glucanase (AGN 13.2) from *T. asperellum* and aspartic protease (SA 76) from *T. harzianum.*

Liu *et al*. (2004) reported the cloning and expression of the endo-β-glucanase II (EG III) cDNA gene of *T. viride* through RT-PCR and yeast expression vector pYES2.

George (2005) isolated β-1,3-glucanase gene by PCR with gene specific primer from two species of *Piper* namely *P. nigrum* and *P. colubrium* and cloned. Djonovic *et al*. (2007) reported a gene (gluc 78) encoding β-1,3-glucanase from *T. atroviride*. Using degenerated primed PCR and cDNA library screening, the three isozyme of β-1,6-glucanase from *T. harzianum* were cloned and sequenced.

Gao *et al.* (2008) designed a pair of primers according to fungal glucanase genes obtained from GenBank and cloned a novel beta-1,3-glucanase gene (glu) from *Trichoderma viride* LTR-2 cDNA by PCR. Then they cloned the fragment in pMD18-T vector and sequenced.

2.7.1. Polymerase chain reaction (PCR)

The PCR process was originally developed to amplify short segments of a longer DNA molecule (Saiki *et al.*, 1985). According to Mullis *et al.* (1986), PCR

is a ubiquitous technique which amplifies a specific region of DNA, so that enough copies of that region is available to be adequately tested or sequenced.

In the original description of PCR method, Klenow DNA polymerase was used and because of heat-denaturation step, fresh enzymes had to be added during each cycle (Saiki *et al.*, 1985; Mullis *et al.,* 1986; Mullis and Faloona, 1987). A break through came with the introduction of *Taq* DNA polymerase from the thermophilic bacterium *Thermus aquaticus* (Lawyer *et al.,* 1987). The *Taq* DNA polymerase is resistant to high temperature and so does not need to be replenished during the PCR (Saiki *et al.*, 1988; Erlich, 1989)

Primer length and sequence are of critical importance in designing the parameters of a successful amplification. The melting temperature of a nucleotide increases both with its length and with increasing (G+C) content. The annealing temperature (Ta) should be about 5^{o}C below the lowest melting temperature (Tm) of the pair of primers to be used (Innis and Gelfard, 1990). Rychlik *et al*. (1990) suggested that if the Ta is increased by 1^{o}C every cycle, both the specificity of amplification and yield of products <1kb in length, were increased. While the PCR is a simple in concept, practically there are a large number of variables which can influence the outcome of reaction (Pavlov *et al.,* 2004).

2.7.1.1. Degenerate primers and primer designing

The degeneracy of the primer is the number of unique sequence combinations it contains. Lee *et al.* (1988) described the common use of degenerate oligonucleotides, when the amino acid sequence of a protein is known. It can be employed to search for novel members of known family of genes and homologous gene between species (Wilks, 1989; Nanberg *et al.,* 1989). Compton (1990) describes using 14-mer primer sets with 4 and 5 degeneracies as forward and reverse primers, respectively, for the amplification of glycoprotein B (gB) from related herpesviruses. A PCR primer sequence is called degenerate if some of its positions have several possible bases (Kwok *et al.,* 1994). De la Cruz *et al.*(1995) designed

the specific primer for β-1,3-glucanase on the basis of the amino acid sequence of the internal peptide of the protein β-1,3-glucanase (BGN 13.1) for PCR. Montero *et al.* (2007) designed degenerated primer from the sequences of glucanase from different *Trichoderma* spp. available in the database for amplification of β-1,6-glucanase (*bgn* 16.3) by degenerate primed PCR.

2.7.1.2. Cloning of PCR product

PCR products generated using a non proof reading DNA polymerase such as *Taq* DNA polymerase, which lacks 3′→5′ exonuclease activity, have a single template-independent nucleotide at the 3′ end of the DNA strand (Clark, 1988; Newton and Graham, 1994). This single-nucleotide overhang, which is most commonly an A residue, allows hybridization with and cloning into T vectors, which have a complementary 3′ single T overhang. PCR products generated using a proofreading DNA polymerase, such as *Pfu* DNA polymerase, have blunt ends and must be cloned into a blunt-ended vector or need a single 3′A overhang added to ligate into a T - vector (Knoche and Kephart, 1999).

2.7.2. Transformation in *E. coli*

Mandel and Higa (1970) found that treatment with $CaCl_2$ allowed *E. coli* cells to take up DNA from bacteriophage λ. Cohen *et al*. (1972) showed that Calcium chloride treated *E.coli* cells are effective recipients for plasmid DNA. During transformation, a restriction deficient strain of *E.coli* is used as a transformable host. It is found that *E.coli* cells and plasmid DNA effectively interact in an environment of calcium ions and low temperature (0 – 5°C). A subsequent heat shock (3°C – 45°C) is important. The calcium chloride influences the cell wall permeability, which plays an important role in binding of DNA to the cell surface (Hanahan, 1983). The actual uptake of DNA is stimulated by the brief heat shock (Old and Primrose, 1994).

Inoue *et al.* (1990) have optimized the conditions for the preparation of competent cells. They stored cells up to 40 days at -70°C while retaining efficiencies of 1-5 × 10^9cfu/µg, but competency was affected only minimally by salts in the DNA preparation. Liu and Rashdbaigi (1990) reported the optimum efficiencies of transformants are 10^7 to 10^9 cfu/µg.

Sambrook *et al*. (1989) reported restriction analysis of plasmid DNA as a method of finding the desired recombinants within a small number of randomly chosen transformed cells. Many of the cloning vectors carry a short segment of *E.coli* DNA that contains the regulatory sequences and the coding information for the first 146 amino acids of the β-galactosidase gene (*lac Z*). This coding region contains a polycloning site that does not disrupt the reading frame but results in the harmless interpolation of a small number of amino acids into amino terminal fragment of β-galactosidase. The carboxy terminal portion of β-galactosidase is coded by the host cells of this type of vectors. The host encoded and vector encoded proteins together constitute the active protein (Ullmann *et al*., 1967).

The recognition of lac^+ bacteria that result from α complementation is easy because they form blue colonies in presence of the chromogenic substrate X-gal (Horwitz *et al*., 1964). The insertion of a fragment of foreign DNA into the polycloning site of the plasmid results in the production of an amino terminal fragment that is not capable of complementation. White colonies are formed by the bacteria carrying recombinant plasmid while the non-recombinants produce blue colonies. Screening of these colonies can be done visually to recognize bacteria that carry putative recombinant plasmids.

2.8. Sequence analysis

The cloned cDNA of BGN 13.1 from *T. harzianum* has a size of 2484 bp and 2288 bp corresponding to open reading frame. The translation of ORF has 762 amino acids with predicted molecular weight of 81,246 Da (De la Cruz *et al*., 1995). The comparison of the nucleotide and amino acid sequence of BGN 13.1 has not

shown any significant homology to any α or β glucanases or other related protein families. Hence, it represented a new class of β-1,3-glucanase of glycosyl hydrolase 55 family.

Kim *et al*. (2002) cloned the β-1,3-glucanase of *T. virens*, sequence length 3027 bp named (bgn1). The protein is β-1,3-glucanase precursor belonged to the same family of BGN 13.1 i.e. glycosyl hydrolase of 55 family.

The sequence analysis of beta-1,3-glucanase gene (*glu*) from *Trichoderma viride* LTR-2 indicated that *glu* was composed of 2289 nucleotide residues and the fragment contained an Open Reading Frame coding 762 amino acids. By nucleotide blasting in NCBI glu showed high homology to β-1,3-glucanase genes from *Trichoderma* spp., especially with *T. harzianum* (bgn3.1) and *Hypocrea virens* (bgn13.1), which the homology reached 93%. The sequence was submitted to GenBank having Accession Number is EF176582 (Gao *et al.*, 2008).

2.9. Genetic transformation

Several studies have have demonstrated that the integration of multiple copies of genes encoding hydrolytic enzymes increases targeted enzyme activity and biocontrol activity of the transformed strain. Examples include integration of multiple copies of the Prb1 gene into the genome of *T. atroviride* (Flores *et al.,* 1997) and *T. virens* (Pozo *et al.,* 2004), endochitinase Chit33 in *T. harzianum* (Limon *et al*., 1999), and endochitinase Chit36 in *T. harzianum* (Viterbo *et al.*, 2001).

Lorito *et al*. (1998) transferred the gene encoding endochitinase (chit 42) from *T. harzianum* (P1) into tobacco and potato and demonstrate a high level and broad spectrum of resistance against a number of plant pathogen viz. *R. solani, A. solani, B. cinerea* and *A. alternata*.

Bolar *et al*. (2000) demonstrated enhanced resistance to apple scab incited by *Venturia inaequalis* in transgenic apple plants that had been transformed with genes encoding both endo and exo-chitinases from *T. atroviride* (P1).

Mora and Earle (2001) reported that transgenic broccoli plants expressing a *T. harzianum* endochitinase gene were obtained by *Agrobacterium tumefaciens* mediated transformation, showing resistance to *Alternaria brassicicola*.

Liu *et al*. (2004) showed the enhanced resistance to fungal pathogens by transformation in rice with the genes encoding cell wall degrading enzymes, endochitinase (*ech 42, nag 70*) and β-1,3-glucanase (*glu 78*) from *T. atroviride*.

Transgenic tobacco lines that over expressed the endochitinase CHIT 33 and CHIT 42 from *T. harzianum* and showed enhanced resistance to biotic and abiotic stress agents (Dana *et al*., 2006).

Djonovic *et al*. (2007) generated transformants of *T. virens* in which β-1,3-glucanase (TvBgn2) and β-1,6-glucanase (TvBgn3) were constitutively coexpressed in the same genetic *T. virens* GV29.8 wild type background. The higher enzymatic activity of double over-expression transformants positively correlated with observed *in vitro* inhibition of *P. ultimum* and *R. solani* mycelia and with enhanced bioprotection of cotton seedling against *P. ultimum, R. solani* and *Rhizopus oryzae*.

Materials and Methods

3. MATERIALS AND METHODS

The present study on "Molecular cloning and characterization of the gene encoding β-1,3-glucanase in *Trichoderma* spp." was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB) and Department of Plant Pathology, College of Horticulture, Vellanikkara during the period from 2006 to 2008. Description of the materials used and methodology adopted in the study has been furnished in this chapter.

3.1 MATERIALS

3.1.1 Chemical, glassware and plastic ware

The chemicals used for the study were of good quality (AR grade) from various agencies including MERCK, SRL and HIMEDIA. Molecular Biology Grade enzymes and buffers were supplied by Banglore Genei Ltd. All the plastic wares used were obtained from Axygen and Tarson India Ltd. The primers were synthesized from Integrated DNA Technologies, USA.

3.1.2 Equipments and machinery

The equipments available at Centre for Plant Biotechnology and Molecular Biology were used for the present study. Incubator Shaker (Dai Ki-S1010) was used for the incubation of cultures and centrifugation was done in KUBOTA centrifuge. DNA amplification was carried out in Eppendorf Master Cycler, Gradient (Eppendorf, Germany). UVP Geldoc IT- Imaging system (U.K.) was used for imaging the gel

3.2 MAINTENANCE OF *Trichoderma* CULTURES

Fifteen *Trichoderma* isolates maintained in the Department of Plant Pathology were used for the present study. Of these, twelve *Trichoderma* isolates viz. BPT -7, BPT – 8, BPT -9,CT-29, CT-30, CT -143, CT-145, GT-1, GT-2, GT–3,

GT – 4, VT -1 were collected from different crops and locations. All these isolates except BPT-8 and BPT-9 were isolated from the rhizosphere soils of various crops, whereas BPT-8 and BPT-9, the endophytic fungi were isolated from roots and stems of black pepper. In addition, reference cultures of *T. viride*, *T. virens* and *T. harzianum* were also used in this study (Plate 1). *Trichoderma* isolates selected were antagonistic to soil borne pathogens such as *Pythium aphanidermatum, Phytophthora capsici, P. meadii and Ralstonia solanacearum,* causing rhizome rot of ginger, *Phytophthora* rot of black pepper and vanilla and bacterial wilt of chilli and ginger respectively. Cultures kept in refrigerated conditions were further cultured on *Trichoderma* selective medium (Annexure I). It was then purified by hyphal tip method and subcultured on PDA slants (Annexure I). These pure cultures were used for further studies (Table 3).

3.2.1 CULTURAL CHARACTERS AND IDENTIFICATION OF *Trichoderma* ISOLATES

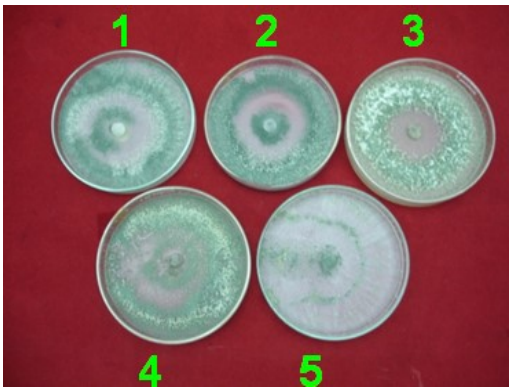
Cultural characters viz. growth, colour and pigmentation of various *Trichoderma* isolates on PDA medium were observed and recorded. For the confirmation of the isolates, cultures were sent to Agarkar Research Institute, Pune; Indian Agricultural Research Institute, New Delhi and National Centre for Fungal Taxonomy, New Delhi.

3.3 BIOCHEMICAL CHARACTERIZATION

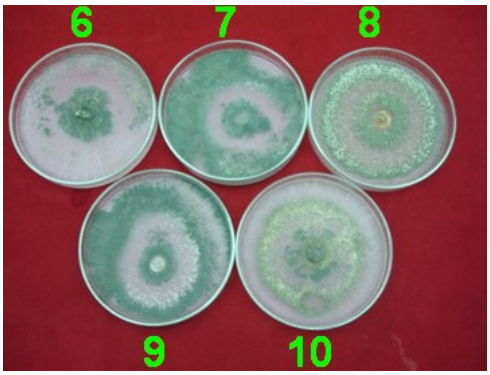
β-1, 3- glucan is one of the main structural component of the cell wall of oomycetes fungi like *Phytophthora* and *Pythium*. *Phytophthora capsici,* the major pathogen, causing *Phytophthora* rot of black pepper in Kerala is selected for this particular study.

Table 3. Details of *Trichoderma* isolates used for the present study

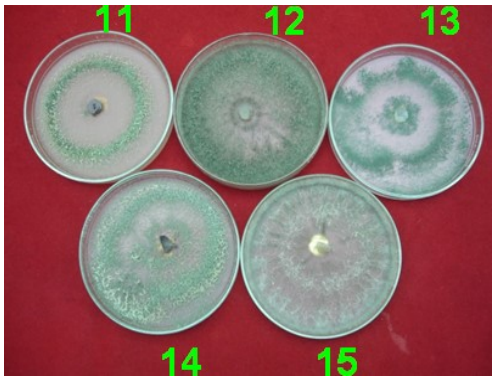
Sl No.	Isolate No.	Source	Locations
1	BPT – 7	Black pepper	Nilambur, Malappuram
2	BPT – 8	Black pepper	Cherumkuzhy, Thrissur
3	BPT – 9	Black pepper	Nilambur, Malappuram
4	CT – 29	Chilli	Vellanikkara, Thrissur
5	CT – 30	Chilli	Ozhalapathy, Palakkad
6	CT – 143	Forest soil	Nilambur, Malappuram
7	CT – 145	Forest soil	Nilambur, Malappuram
8	GT – 1	Ginger	Nenmara, Palakkad
9	GT – 2	Ginger	Nenmara, Palakkad
10	GT – 3	Ginger	Mudappallur, Palakkad
11	GT – 4	Ginger	Mudappallur, Palakkad
12	VT – 1	Vanilla	Vellanikkara, Thrissur
Reference cultures			
13	*T. viride*		KAU, Thrissur
14	*T. harzianum*		IISR, Calicut
15	*T. virens*		PDBC, Bangalore



1. *T. viride*-1 (KAU), 2. *T. harzianum* – 30, 3. *T. aureoviride*,
4. *T. viride* – 8, 5. *T. piluliferum*



6. *T. pseudokoningii* – 143, 7. *T. harzianum* – 1, 8. *T. harzianum* – 4,
9. *T. harzianum* (IISR), 10. *T. flavofuscum*



11. *T. virens* – 1, 12. *T. virens* (PDBC), 13. *T. harzianum* – 29,
14. *T. pseudokoningii* – 9, 15. *T. virens* – 2

Plate 1. *Trichoderma* isolates used for the study

3.3.1 Isolation of *P. capsici*

The pathogen, *P. capsici,* was isolated from *Phytophthora* infected black pepper leaves, adopting standard protocol (Zentmyer, 1960). The fungus isolated on carrot agar medium was purified, subcultured and maintained on PDA slants.

3.3.2 Preparation of *Phytophthora* cell wall extract

P. capsici was inoculated on PDA mediated Petriplates (Plate 2A). Four mycelial discs of size six mm from seven day old culture were inoculated to 50ml sterile carrot broth and incubated at room temperature with constant shaking at 150 rpm for seven days (Plate 2B). After the required incubation, the mycelial mat was removed by filtration, washed repeatedly with distilled water and then ground to a paste with mortar and pestle. This was treated with methanol and chloroform in the ratio of 1:1 and later with same volume of acetone and dried in oven at 50°C for 48 h. The dry weight was estimated and stored at 4°C.

3.3.2 Enzyme production in liquid culture

Trichoderma isolates were tested for glucan utilization in *Trichoderma* special broth (TSB) with *P. capsici* cell wall extract as sole carbon source instead of glucose. Five mycelial discs (six mm in diameter) were cut from the periphery of seven day old culture of the test fungus with the help of cork borer and then inoculated separately to 50ml of 0.2% *P. capsici* cell wall amended *Trichoderma* special liquid medium and incubated at room temperature with rotary shaking at 100 rpm for seven days (Plate 2C). After the incubation, the mycelial mat was removed by centrifugation and the supernatant was used as crude enzyme for the assay of glucanase activity.

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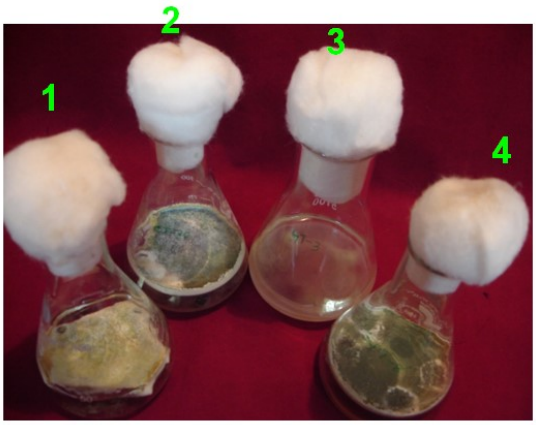
A. Growth of *P. capsici* on PDA medium

B. Growth of *P. capsici* in carrot broth

C. Growth of *Trichoderma* spp. in *P. capsici* cell wall amended TSB medium



D. Growth of *Trichoderma* spp. in minimal media for DNA isolation

1. *T. viride* – 1, 2. *T. harizianum* – 30, 3. *T. aureoviride,* 4. *T. viride* – 8

Plate 2. Cultures of *Phytophthora capsici* and *Trichoderma* spp. in different media

3.3.3 Assay of β-1, 3- glucanase enzyme

The glucanase activity was assayed colorimetrically as suggested by Pan *et al.*(1991). The principle of the assay is that, the enzyme, β-1,3-glucanase, released by *Trichoderma* binds to the substrate molecule (laminarin) and produce glucose which on react with colouring cum stopping reagent, 3,5-dinitrosalicylicacid (DNS), give reddish yellow colour (Annexure II). Then absorbance (A) was measured spectrophotometrically at 500 nm.

Protocol

1. For the glucanase assay, the assay mixture, contained 0.5 ml of 50 mM sodium acetate buffer (pH 5.2), 62.5 µl of 4% laminarin and 62.5 µl crude culture filtrate was prepared.
2. The reaction mixture was incubated for 10 min at 37°C
3. Then reaction was stopped by addition of 375 µl 3, 5-dinitrosalicylicacid (DNS) and the mixture was kept in boiling water bath for 5 min for stabilization.
4. Then the volume of the reaction mixture was made up to 5.5 ml of using double distilled water and mixed properly
5. Simultaneously blanks were prepared for each sample in which the samples were boiled to inactivate the enzyme activity and followed the same steps as mentioned earlier.
6. The absorbance was read at 500 nm.

The enzyme activity was calculated from the standard curve of glucose using laminarin as substrate (Fig 1). One unit of enzyme is defined as the amount of enzyme necessary to produce one micro gram of glucose released per 10 min.

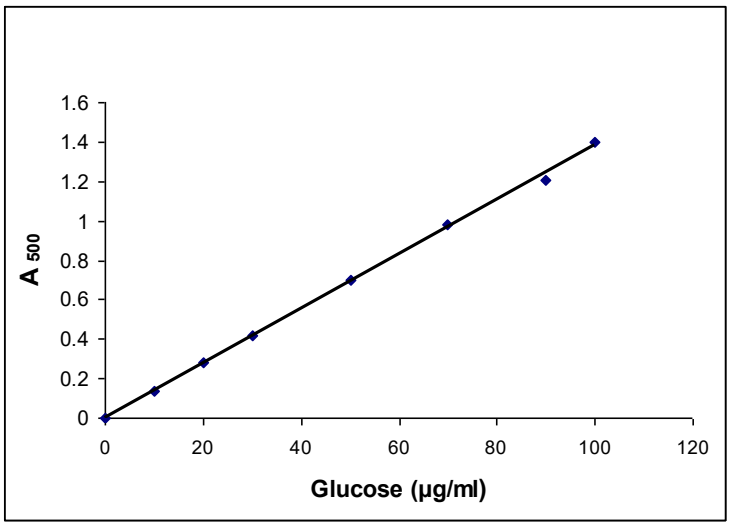


Fig.1. Standard curve of glucose

3.4 AMPLIFICATION OF β-1, 3- GLUCANASE GENE FROM *Trichoderma* spp.

Four *Trichoderma* isolates viz. *T. viride*-1, *T. harzianum*-30*, T. aureoviride* and *T. viride*-8 having high glucanase activity were selected for gene isolation. Total DNA was isolated from these selected *Trichodrma* spp., in order to amplify the β-1,3-glucanase genes. Specific primers for the amplification of β-1,3-glucanase genes were designed based on the conserved regions obtained after multiple sequence alignment. PCR was carried out using the specific primers at annealing temperatures specified for each primer.

3.4.1 Isolation of total genomic DNA from *Trichoderma* spp.

The total genomic DNA was isolated from *Trichoderma* isolates following the method as suggested by Cenis (1992), Cassago *et al.* (2002) with slight modification.

Reagents used

1. Extraction buffer
2. TE buffer

3. Chilled Isopropanol
4. 7.5M Ammonium acetae
5. Phenol:chloroform:isoamyl alcohol mixture (25:24:1, v/v/v)
6. RNase
7. 3M Sodium acetate
8. Ethanol (100% and 70%)

(Chemical composition of media and reagents used are given in Annexure III)

Protocol

1. For genomic DNA extraction, the selected four *Trichoderma* isolates were grown in minimal liquid medium for five days at room temperature (Plate 2D).
2. The mycelium was separated from the medium, washed with distilled water and dried.
3. One gram mycelium was ground in excess liquid nitrogen and six ml extraction buffer.
4. The ground tissue was transferred to 50 ml oakridge tube.
5. Phenol: chloroform: isoamyl alcohol mixture (25:24:1, v/v/v) was added and placed in a shaker in horizontal position at 200 rpm for 30 min at 37^0C.
6. The homogenate was then centrifuged immediately at 15,000 rpm for 5 min at 37^0C.
7. The upper aqueous phase was transferred to fresh oakridge tube after checking the volume.
8. To this, equal volume of chilled isopropanol and $1/10^{th}$ volume of 7.5M ammonium acetate were added.
9. The contents were mixed gently by inversion and then incubated at -20^0C for 30 min for complete precipitation of DNA.
10. The precipitated DNA was pelleted by centrifugation at 15,000 rpm for 15 min at 4^0C.

11. The supernatant was discarded and the pellet was washed with 70 per cent alcohol by giving a centrifugation at 10,000 rpm for 5 min at 4^0C. The supernatant was discarded.
12. The pellet was air dried and resuspended in 1 ml TE buffer.
13. 20 µl of RNase was added from a stock of 10 mg/ml and incubated at 37^0C for 30 min.
14. Equal volume of phenol: chloroform: isoamyl alcohol mixture (25:24:1, v/v/v) was added, mixed gently and centrifuged at 10,000 rpm for 10 min.
15. The upper aqueous phase was transferred to a fresh centrifuge tube and equal volumes of absolute alcohol and 1/10th volume of 3M sodium acetate were added.
16. Incubated at -20^0C for 30 min followed by centrifugation at 12,000 rpm for 15 min at 4^0C.
17. The pellet was washed with 70 per cent alcohol by giving a centrifugation at 10,000 rpm for 5 min at 4^0C. The supernatant was discarded.
18. The pellet was air dried and dissolved in 100-120 µl TE buffer.
19. The DNA sample was stored at -20^0C for further utilization.

3.4.2 ESTIMATION OF THE QUALITY OF DNA

3.4.2.1 Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed based on the method described by Sambrook *et al*. (1989) to check the quality of DNA and also to separate the amplified products.

Materials

1. Agarose (Bangalore Genei, Low EEO)
2. 50X TAE buffer (pH 8.0)
3. Electrophoresis unit (Biorad, USA), power pack, casting tray, comb
4. 6X Loading/ Tracking dye (Bangalore Genei, Bangalore)

5. Ethidium bromide solution (stock 10mg/ml; working concentration, 0.5µg/ml)

7. Gel documentation and analysis system (UVP Geldoc IT- Imaging system, U.K.)

3.4.2.2 Protocol

3.4.2.2.1 Preparation of agarose gel

1. 1X TAE buffer was prepared from 50 X TAE stock solution.
2. Agarose (1.0 per cent (w/v) for genomic DNA and 0.8 per cent (w/v) for PCR) was weighed and added to 1X TAE. It was boiled till the agarose dissolved completely and then cooled to lukewarm temperature.
3. Ethidium bromide was added to a final concentration of 0.5µg ml^{-1} as an intercalating dye of DNA and mixed well.
4. The open ends of the gel casting tray were sealed with a cellophane tape or kept in the respective stand and placed on a perfectly horizontal leveled platform.
5. The comb was placed properly and molten agarose was poured into the tray, allowing it to solidify.
6. After the gel was completely set (20 to 30 min at room temperature), the comb and cellophane tape were carefully removed.

3.4.2.2.2 Loading the DNA sample

1. The gel was placed in the electrophoresis tank with the wells near the cathode and submerged in 1 X TAE to a depth of 1cm.
2. A piece of cellophane tape was pressed on a solid surface and 1 µl 6X loading dye was dispensed in small quantity on the tape. A quantity of 3 to 5 µl of DNA was added to each slot (in the case of PCR products, 15.0 to 25.0 µl) mixed well by pipetting in and out for 2 to 3 times. Then the mixture was loaded in the wells, with the help of micropipette. λ DNA/*Eco* R1+ *Hind* III double digest (Bangalore Genei) was used as the molecular weight marker.

3. The cathode and anode were connected to the power pack and the gel was run at a constant voltage of 60 volts.
4. The power was turned off when the tracking dye reached at about 3cm from the anode end.

(Chemical composition of the buffer and dyes are given in Annexure IV).

3.4.2.2.3 Gel documentation

The DNA bands separated by electrophoresis were viewed and photographed and documented using UVP Geldoc IT- Imaging system.

3.4.3 QUANTIFICATION OF DNA

The quantity and purity of DNA was further analysed by using NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA). The absorbance of nucleic acid samples was measured at a wavelength of 260 nm and 280 nm. OD_{260}/OD_{280} ratio was used to assess the purity of nucleic acids. A ratio of 1.8 to 2 indicated good quality DNA.

3.4.4 PRIMER DESIGNING

The sequence information available in the public domain NCBI was accessed for collecting the recent information about β-1,3-glucanase genes. The following steps were used:

1. Complete nucleotide sequences encoding β-1,3-glucanase gene reported for different species of *Trichoderma* / *Hypocrea* were downloaded from NCBI Genbank (http://www.ncbi.nlm. nih.gov) and copied in FASTA format into a notepad. The accession numbers of selected sequences are provided in Table 4.
2. Multiple sequence alignment of nucleotide sequences downloaded was done using 'Clustal W1.83' (www.ebi.ac.uk./clustalw/; Thompson *et al.*, 1994).
3. Based on the homology, conserved boxes of 18 to 24 bases were selected throughout the sequence.

Table 4. Details of β-1, 3- glucanase gene sequences used for primer designing

Sl. No.	Fungal strain	Sequence details	Accession number in NCBI
1	*Hypocrea virens*	Endoglucanase (bgn13.1) gene, complete sequence	EF426721.1
2	*Hypocrea virens*	Endoglucanase (bgn13.1) mRNA, complete cds	EF426722.1
3	*Trichoderma harzianum*	mRNA for endo – 1,3 (4)-β -glucanase	X84085.1
4	*Trichoderma viride*	β-1,3- glucanase (glu) gene, complete cds	EF176582.1
5	*Hypocrea virens*	β-1,3- glucanase precursor (bgn2) gene, complete cds	AF395756.1

4. The forward and reverse primers were selected from conserved boxes in such a way that
   a) The conserved boxes should have GC content not less than 50 per cent
   b) Melting temperature (Tm = 4GC + 2AT; Suggs *et al*., 1989) should range between 60^0C and 70^0C.
   c) The distance between the primers should range from 500 to 1000 base pairs.
   d) It is preferable to have GC content at 3' end.
   e) There should not be any complementarity between forward and reverse primers.
   f) Repeats of single base should not appear within the primer sequence.
   g) The distance between forward and reverse primer should be greater than 500bp.
   h) Each primer should be 18 to 24bp long

5. For designing primers, the sequence of the forward primer was taken as such and for the reverse primer, the reverse complementary sequence was taken.

3.4.5 PCR Reaction

The total DNA extracted from *Trichoderma* isolates were amplified by polymerase chain reaction. PCR was carried out using β-1,3-glucanase gene specific primers designed during this study, at different annealing temperature in Eppendorf Master Cycler, Personal (Eppendorf, Germany).

3.4.5.1 Composition of the reaction mixture for PCR (25.0µl)

a) Genomic DNA (25ng) - 1.0µl
b) 10X Taq assay buffer - 2.5µl
c) d NTP mix (10mM) - 1.0µl
d) Forward primer (8pM) - 1.0µl
e) Reverse primer (8pM) - 1.0µl

f) Taq DNA polymerase (0.3 U)	-	2.0µl
g) Sterile distilled water	-	16.5µl
		25.0µl

The total volume for one PCR reaction was 25.0µl. The reaction was set in a 200µl microfuge tube chilled over ice flakes. A momentary spin was given for the reaction and set in thermal cycler for polymerase chain amplification under suitable programme. A negative control was kept using water as template.

3.4.5.2 Thermal cycler programme

The following programme was set in order to amplify β-1, 3- glucanase gene from the template DNA.

Step 1:	94^0C	for 2 min	- initial denaturation
Step 2:	94^0C	for 45 sec	- denaturation
Step 3:	55^0C	for 1 min	- annealing
Step 4:	72^0C	for 2 min	- extension
Step 5:	72^0C	for 10 min	- final extension
Step 6:	4^0C	for infinity to hold the sample	

30 cycles

The PCR product was checked on 1.0 per cent agarose gel and documented.

3.5 GEL ELUTION OF PCR AMPLIFIED FRAGMENTS

Products obtained in different PCR reactions were loaded separately on 1.0 per cent (w/v) agarose gel and desired band in each case was eluted using AxyPrep DNA Gel Extraction Kit (Axygen, Biosciences) as per manufacturer's guide lines.

1. DNA fragment of interest was excised from the gel using a sterile, sharp scalpel while avoiding much exposure to UV transilluminator.
2. Gel slice was weighed in a colourless 1.5 ml micro centrifuge tube.
3. 3X gel volume of solubilization buffer (w/v) was added.

4. The gel was resuspended in gel solubilization buffer by vortexing. It was heated at 75^0C until the gel was completely dissolved. Intermittent vortexing was given every 2 to 3 min to accelerate gel solubilization.
5. 0.5X gel solubilization volume of binding buffer was added and mixed properly.
6. Once the gel slice was completely dissolved, DNA fragments less than 400 bp was supplemented by adding 1X gel volume of isopropanol and mixed briefly by inversion.
7. A spin column was placed in a 2 ml collection tube.
8. Solubilized gel slice was transferred into the spin column that was assembled in the 2 ml collection tube and centrifuged at 12,000g for 1 min.
9. The filtrate was discarded. 500 µl of wash buffer was added to the spin column and centrifuged at 12, 000g for 30 sec.
10. The filtrate was discarded. 700 µl of desalting buffer was added.
11. Centrifugation was carried out at 12,000g for 30 sec.
12. As a second wash, 700 µl of desalting buffer was added
13. Centrifugation was carried out at 12,000g for 30 sec to ensure the complete removal of salt.
14. The filtrate was discarded. The spin column was again placed on collection tube.
15. Column was again centrifuged for 1 min at 12,000g to remove any residual buffer.
16. Spin column was transferred to a fresh 1.5 ml centrifuge tube. The eluent was prewarmed at 65^0C to improve the elution efficiency. To elute the DNA, 15 µl of eluent was added to the centre of the spin column. It was allowed to stand for 1 min at room temperature. Then it was centrifuged at 12,000g for 1 min.
17. Eluted DNA fragments were checked on 0.7 per cent (w/v) agarose gel and stored at - 20^0C.

3.6 CLONING OF GENE ENCODING GLUCANASE

3.6.1 Preparation of competent cells

Competent cells for plasmid transformation were prepared following the protocol of Mandel and Higa (1970). Both LB and LBA media (Annexure V) were used.

The steps followed for competent cell preparation were as follows:

Day 1:

- Single colony of 18 h old *Escherichia coli* JM 109 strain was inoculated to 3 ml LB medium in sterile condition and incubated overnight at 37^0C on a shaker set at 160 rpm.

Day 2:

- Three ml culture was transferred aseptically to 50 ml sterile LB broth and incubated for 4 h at 37^0C on a shaker set at 160 rpm until OD_{600} reached 0.4 to 0.5. The growth of culture was monitored at every 30 min.
- The cells were then aseptically transferred to a sterile disposable ice-cold 50 ml polypropylene tube.
- The culture was cooled on ice cubes for 20 min.
- The cell suspension was centrifuged at 3500 rpm for 10 min at 4^0C.
- The supernatant obtained was carefully discarded and the pellet was gently resuspended in 10 ml ice-cold, filter sterilized 0.1M $CaCl_2$.
- The tubes were kept on ice for 20 min and the cell suspension was centrifuged at 5000 rpm for 10 min at 4^0C.
- The supernatant was decanted and the pellet was resuspended in 2 ml of ice cold filter sterilized 0.1M $CaCl_2$. The tubes were kept on ice for 18 h.

Day 3:

- Four ml chilled glycerol was added to the cell suspension and mixed well using a sterile microtip.

- The competent cells prepared were stored at -80^{0}C as aliquots of 100 µl in chilled 1.5 ml microfuge tubes covered with aluminium foil for further use.

3.6.2 Screening of competent cells

Transformation of competent cells with a plasmid having ampicillin resistance (pUC18) was carried out to check the competence and purity of competent cells. The procedure followed for screening of plasmid is as follows,

- The competent cells stored at -80^{0}C were thawed over ice for 10 min.
- Plasmid DNA (10 µl) was added to 100 µl competent cells. Negative control was placed simultaneously without adding plasmid.
- The cells were kept on ice for 40 min. Heat shock was given at 42^{0}C for 90 sec in a water bath and plunged in ice for 5 min.
- LB medium (250 µl) was added to the cells and incubated at 37^{0}C for one hour on a shaker set at 120 rpm.
- The transformed cells (100 µl) were plated on LBA/ampicillin and incubated overnight at 37^{0}C in a shaker (100 rpm) and the recombinant clones were grown on ampicillin plate.

3.6.3 Ligation

The eluted product was ligated in pGEM-T vector (Fig. 2.) using pGEM-T Easy Vector System (Promega Corporation, USA) as per manufacturer's protocol.

1. Reaction mixture was prepared as described below:

2X rapid ligation buffer	-	5.0µl
pGEMT Easy Vector (50ng)	-	1.0µl
Eluted product	-	3.0µl
T4 DNA ligase (3 units/µl)	-	1.0µl

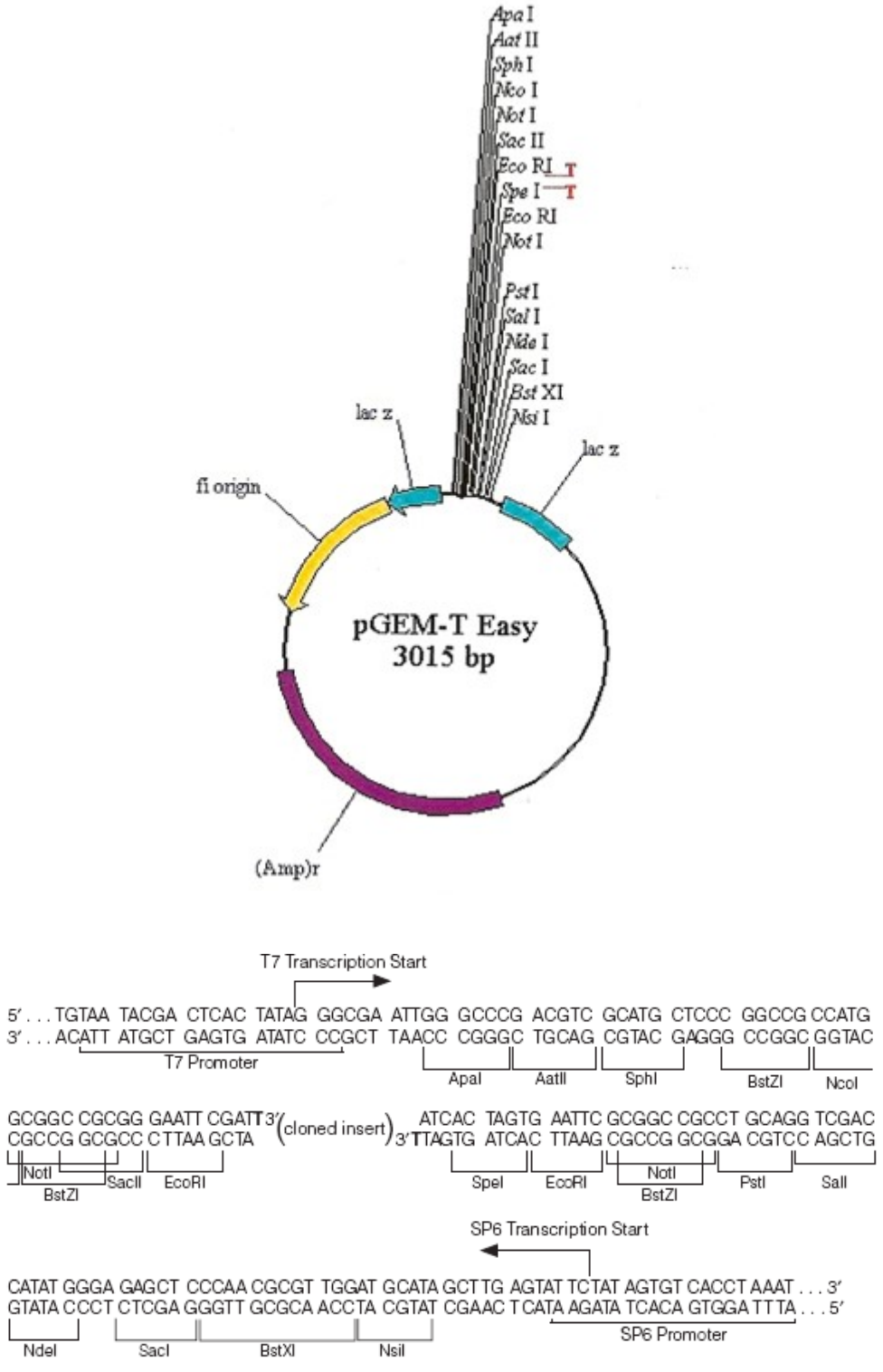


Fig. 2. pGEM-T Easy Vector (Promega) used for cloning PCR product. The *lacZ* region, promoter and multiple cloning sites are shown in the figure. The top strands of the sequence shown corresponds to the RNA synthesized by T7 RNA polymerase and bottom strands corresponds to the RNA synthesized by SP6 RNA polymerase.

2. The reaction mixture was incubated for one hour at room temperature. Then it was kept at 4^0C overnight. Next day it was taken for transformation of competent cells of *E. coli* JM 109.

3.6.4 Cloning of ligated product into competent cells

Reagents prepared:

1. Ampicillin – 5mg/ml in water
2. IPTG – 200mg/ml in water
3. X-gal – 10mg/ml in DMSO

The procedure followed for DNA transformation and blue white screening is as follows:

1. The vial containing competent cells was thawed on ice.
2. The ligated product was added to the competent cells, contents mixed gently and kept on ice for 40 min.
3. The tube was rapidly taken from ice; heat shock was given at 42^0C exactly for 90 sec without shaking and placed back on ice for 5 min.
4. 250µl of LB broth was added and the tube was inverted twice.
5. The tube was incubated at 37^0C for one hour with shaking.
6. 100 µl and 250 µl aliquots of the transformed cells were plated on LB/ ampicillin plates layered with IPTG (6 µl) and X-gal (60 µl) and incubated overnight at 37^0C.
7. The recombinant clones were selected based on blue-white screening.

3.7 ISOLATION OF RECOMBINANT PLASMID DNA

Plasmid DNA was isolated from single white colonies using alkaline mini- prep procedure as given by Birnboim and Doly (1979). Composition of the reagents used, are given in Annexure V.

3.7.1 Procedure for plasmid isolation

1. Cells were pelleted from overnight grown recombinant *E. coli* JM 109 culture containing plasmid DNA by centrifugation at 12,000 rpm for one minute at 4^0C.
2. 100 µl of ice cold solution I was added to the bacterial pellet and resuspended.
3. 200 µl of freshly prepared lysis buffer was added, mixed gently by inverting the tube for five times.
4. 150 µl of ice cold solution III was added to the tubes, mixed well and kept on ice cubes for five minutes.
5. The contents were centrifuged at 12,000 rpm for 5 min at 4^0C and the pellet was discarded.
6. To the supernatant, 0.6 volume of ice cold isopropanol was added and kept at room temperature for 5 min.
7. The contents were centrifuged at 12,000 rpm for 5 min at 4^0C and the supernatant was discarded.
8. The pellet was rinsed with 1 ml of 70 per cent (v/v) ethanol and mixed gently.
9. The tube was centrifuged at 12,000 rpm for 10 min at 4^0C.
10. The supernatant was discarded and the pellet air dried for 10 min.
11. Pellet was finally dissolved in 30 µl TE buffer.
12. Plasmids isolated were checked by electrophoresis on 0.8 per cent agarose gel and documented.

3.8 CONFIRMATION OF PRESENCE OF INSERT

3.8.1 PCR amplification of recombinant plasmid DNA

Polymerase chain reaction was carried out as described in section 3.4.5., using the designed gene specific primer except that the recombinant plasmid DNA isolated by alkali lysis method was used as template in place of fungal DNA.

The recombinant plasmid DNA isolated by alkali lysis method was amplified by PCR as described in section 3.4.5., using T7 forward and SP6 reverse universal primer but the annealing temperature was set at 41^0C in thermal cycler programme.

The PCR products were analysed on 1.0 per cent (w/v) agarose gel as described in Section 3.4.2.

3.9 MAINTENANCE OF CLONES

3.9.1. Preparation of pure culture of recombinant bacteria

Materials prepared

LBA medium and Ampicillin (5 per cent)

In a laminar flow, single white colony from the transformed plate was taken by using flame-sterilized bacterial loop. This was streaked on LBA plate containing ampicillin (50 mg/l). The plate was incubated overnight at 370C and further stored at 40C.

3.9.2. Preparation of stabs

Materials prepared

LBA medium and ampicillin (5 per cent)

The LBA medium containing antibiotic ampicillin (50 mg/l) was melted and poured into storage vial, aseptically and allowed to solidify. Single colony of recombinant bacterial colony was carefully lifted with a sterile bacterial loop. The loop loaded with bacteria was plunged into the solid medium and incubated at 370C overnight in the culture tube. The stabs showing good growth of bacteria were further stored in refrigerator at 40C.

3.9.3. Glycerol culture

Materials prepared

LB liquid and ampicillin (5 per cent)

In a laminar flow, recombinant colony from the transformed plate was taken using flame-sterilized loop. This was plunged into LB medium containing the antibiotic, ampicillin (50mg/l). It was incubated at 370C in a shaker at 120rpm overnight. On the next day aliquots (600µl) of cell culture was added to 100 per cent glycerol (400µl) aseptically and stored at –800C.

3.10 SEQUENCING OF CLONES

The recombinant clones from the isolates GT3-1, GT-322, BPT-822, T-112 and CT-3012 were sent to Banglore, Genei for sequencing. Sequencing was carried out using T7 forward primer to obtain 5'-3'sequence information of the insert from the forward region, in automated sequencer (ABI–31100 Genetic Analyzer, USA).

3.11 THEORETICAL ANALYSIS OF SEQUENCE

Theoretical analysis of the sequence was done using different bioinformatics tools to know the details of the sequence obtained.

3.11.1 Vector screening

The sequence obtained was subjected to vector screening using Vecscreen programme of NCBI to remove vector regions from the clones.

3.11.2 Nucleic acid sequence analysis

3.11.2.1 Homology search

The nucleotide sequences 'GT-311, GT-322 and BPT-822' were compared with the sequences available in the database using BLAST tool offered by NCBI after removing the primer sequence. Nucleotide-nucleotide blast (blastn) was carried out for homology search. The BLAST programme 'blastn' (Altschul *et al*., 1997) provided by NCBI (www.ncbi.nlm.nih.gov/Blast/Blast.cgi) was utilized for the purpose. The nucleotide sequence of the insert was pasted in the BLAST web

page and the programme was run at default settings. The best sequence alignment of the search results were noted and saved.

3.11.2.2 Detection of Open Reading Frame (ORF)

The programme 'ORF finder' (www.ncbi.nlm.nih.gov/gorf/gorf.html) of NCBI was used to find the open reading frame of the insert nucleotide sequence. The nucleotide sequence was copied and pasted in the displayed box and clicked on 'OrfFind'. The displayed web page showed ORF sequence in all reading frames. Open reading frames available in the entire region were noted and saved. The displayed amino acid sequence of the reading frames were pasted in a notepad and BLASTp search was performed. The results obtained were saved for further interpretation.

3.11.2.3 Detection of nucleotide statistics

Nucleotide composition of the sequence obtained was determined by nucleotide statistics tool provided by Biology Workbench (http://seqtool.sdsc.edu/). Initially, the nucleotide sequence of the insert was saved in the workbench. The site was entered and the nucleotide tool NASTATS was selected to obtain the details of nitrogen bases in the DNA fragment.

3.11.2.4 Restriction analysis

Restriction sites available in the DNA fragment for the restriction enzymes were detected by restriction analysis tool offered by NEB cutter (http;//tools.neb.com/NEB cutter 2). Important sites available for manipulation were selected and interpreted.

3.11.3 Amino acid analysis

Physical and chemical properties of the given protein from the deduced amino acid sequence were determined using amino acid statistics tool (AASTATS)

offered by Biology Workbench. The site was entered and selected for protein tools. Deduced amino acid sequence of the DNA sequence was saved in this Biology Workbench. The sequence was selected and appropriate tools were chosen as per instructions in the site. Protein sequence homology was analysed through protein blast tool (Blastp) from the NCBI.

Other analyses carried out were Kyte and Doolittle (Kyte and Doolittle, 1987) hydropathy plot using Hydrophobicity plot tool of Molecular tool kit (www.vivo.colostate.edu/molkit.html) and secondary structure prediction using SOPMA programme (Geourjon and Deleage, 1995) of ExPASY tool (www.expasy.org/tools).

Results

4. RESULTS

The results of the present study on 'Molecular cloning and characterization of the gene encoding β-1,3- glucanase in *Trichoderma* spp.' undertaken during the period 2006-2008 at the Centre for Plant Biotechnology and Molecular Biology and Department of Plant Pathology, College of Horticulture, Vellanikkara are presented below.

4.1 CHARACTERIZATION AND IDENTIFICATION OF Trichoderma ISOLATES

4.1.1 Cultural characterization

Cultural characters of *Trichoderma* isolates, such as colony colour, growth rate and pigmentation, observed are furnished below:

T. aureoviride – Colonies were observed to grow slowly on PDA medium as watery white mycelial mat with very few aerial hyphae. Conidial area at first, were white, gradually the centre became whitish green and ultimately the whole area appeared as dull grass green. The reverse side of the colony was dark brownish.

T. flavofuscum – Colonies grew rapidly, 6.5 to 8 cm in diameter in four days. The colony colour was white or greyish at first and changed to yellowish after conidiation.

T. harzianum – Colonies grew rapidly and reached upto 9 cm in four days at room temperature. Smooth surfaced watery white mycelial mat was observed first. The colour of the colony changed from whitish green to dull green or yellowish green with the development of conidia. They formed distinct ring like conidial zone. The reverse of the colony remain uncoloured.

T. piluliferum - Colonies grew slowly and reached upto 4 cm in four days. Smooth, translucent mycelial mat was formed on the surface of the medium. The reverse of the colony remain uncoloured.

T. pseudokoningii – Colonies grew fairly rapidly at room temperature covering 9 cm in five days. The surface of the colony was smooth and translucent. The colour changed from white to greenish white and then to bright green and the reverse of the colony became yellowish.

T. virens - Colonies grew rapidly, covering 9 cm in four days. The aerial mycelium appeared floccose, white to green. The colour of the colony changed from dark green to dung green shades after conidiation. The reverse of the colony was brownish.

T. viride – Colonies grew rapidly, reached upto nine cm in four days. The mycelium was smooth, watery white and became hairy due to the formation of loose scanty aerial hyphae. The colonies became dark green to dark bluish green in maturity and reverse remain uncoloured.

Identification reports of various *Trichoderma* isolates obtained from Agarkar Research Institute, Pune; Indian Agricultural Research Institute, New Delhi and National Centre for Fungal Taxonomy, New Delhi are presented in Table 5.

4.1.2 Biochemical characterization

For the induction of glucanase enzyme in *Trichoderma*, it was grown in *Phytophthora capsici* cell wall amended medium for seven days. The crude culture filtrate was used as the enzyme source and incubated 10 min with laminarin at 37^{o}C for releasing glucose from the substrate. β-1,3- glucanase enzyme specific activity of different *Trichoderma* isolates estimated is presented in Table 6. From the table, it is observed that, the 15 isolates have a wide range of glucanase activity ranged from 1.3U to 205.3U per 50ml culture filtrate. Among the fifteen isolates, seven isolates showed high enzyme activity of more than 100U and the maximum enzyme activity was observed *T. viride* – 1 (205.3U) followed by *T. harzianum* – 30 (157.8U), *T. aureoviride* (139.2U), *T. viride* - 8 (121.9U) and lowest enzyme activity of 1.3U was observed in *T. virens* – 2. The four isolates which showed maximum glucanase activity were selected for gene isolation.

Table 5. Identification report of *Trichoderma* isolates used in the study

Sl No.	Isolate No.	Identification No. of isolates	*Trichoderma* species identified
1	BPT – 7	ITCC-6436.06	*Trichoderma piluliferum*
2	BPT – 8	NCFT-1302.07	*T. viride* (Endophytic fungus)
3	BPT – 9	ITCC-6437.06	*T. pseudokoningii* (EF)
4	CT – 29	NCFT-1297.07	*T. harzianum*
5	CT – 30	ARI-7	*T. harzianum*
6	CT – 143	ARI-8	*T. pseudokoningii*
7	CT – 145	ITCC-6435.06	*T. flavofuscum*
8	GT – 1	NCFT-1292.07	*T. virens*
9	GT – 2	NCFT-1293.07	*T. virens*
10	GT – 3	ITCC-6433.06	*T. aureoviride*
11	GT – 4	ITCC-6434.06	*T. harzianum*
12	VT – 1	ARI-11	*T. harzianum*

Table 6. Estimation of glucanase activity in various *Trichoderma* isolates

Isolates No.	*Trichoderma* isolates	Enzyme activity (U/50ml/10min)
T-1	*T. viride* - 1	205.3
CT-30	*T. harzianum* - 30	157.8
GT-3	*T. aureoviride*	139.2
BPT-8	*T. viride* - 8	121.9
BPT-7	*T. piluliferum*	116
CT-143	*T. pseudokoningii*- 143	104.5
VT-1	*T. harzianum* -1	103.3
GT-4	*T. harzianum* - 4	37.2
T-2	*T. harzianum* (IISR)	21
CT-145	*T. flavofuscum*	18.7
GT-1	*T. virens* - 1	17.5
T-3	*T. virens* (PDBC)	14
CT-29	*T. harzianum* -29	10.5
BPT-9	*T. pseudokoningii*- 9	3.6
GT-2	*T. virens* - 2	1.3

4.1.3 Molecular characterization

4.1.3.1. Isolation and quantification of genomic DNA from *Trichoderma* isolates

The protocols suggested by Cenis (1992) and Cassago *et al*. (2002) were adopted with slight modifications for the isolation of genomic DNA from selected *Trichoderma* spp. The quality of DNA isolated was tested using agarose gel electrophoresis. The electrophoretic profile on 0.8 per cent agarose gel revealed uniform discrete bands from all the samples indicating good quality DNA free from RNA and other contaminants (Plate 3).

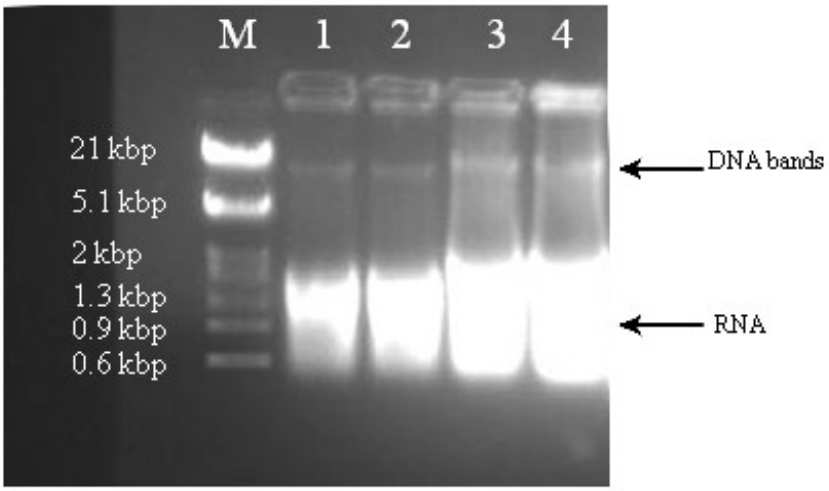
The quality of DNA present in the sample was determined using NanoDrop® ND-1000. The ratio of optical density value at 260nm to that at 280nm ranged from 1.77 to 1.81 indicating relatively pure DNA in the samples without protein contamination. The ratio OD_{260}/OD_{280} was highest for the isolate *T. viride* - 1 and least for the isolate *T. aureoviride*. The details of OD_{260}/OD_{280} value and quantity of DNA isolated from each isolates are given in Table 7.

The quantity of DNA as assessed by spectrophotometry using nanodrop varied from 164.82 µg g^{-1} to 204.08 µg g^{-1} of mycelia for various isolates. The quantity of DNA was found to be high in isolate *T. viride* - 1 which recorded 204.08 µg g^{-1} of mycelia where as *T. viride* - 8 recorded the lowest value 164.82 µg g^{-1} of mycelia.

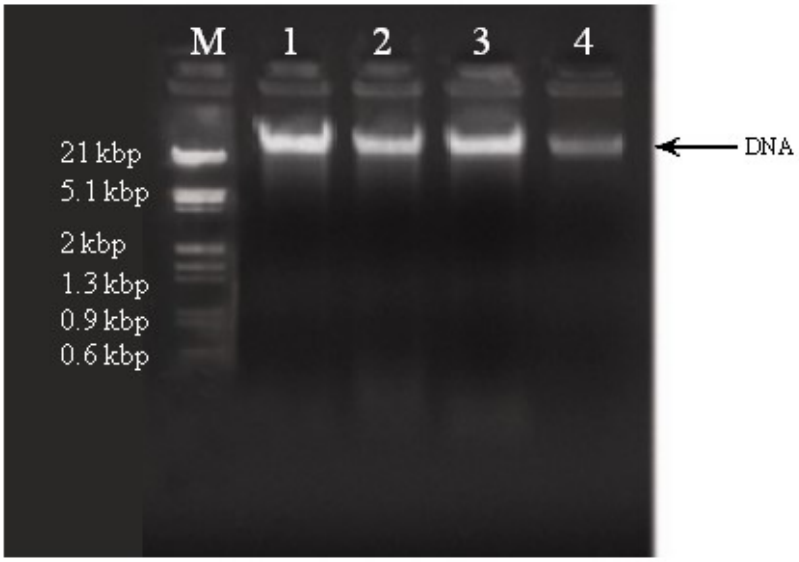
4.1.3.2. Primer designing

Based on the parameters described in Section 3.4.4, three pairs of primers were designed for β-1,3- glucanase gene.

Multiple sequence alignment of the nucleotide sequences (Table 4) was done using ClustalW 1.83 available from European Bioinformatics Institute (www.ebi.ac.uk/clustal).



a. Without RNase treatment



b. After RNase treatment

M - λ DNA/ *Eco*R I/ *Hind* III double digest marker, 1. *T. viride* - 1, 2. *T. harizianum* – 30, 3. *T. aureoviride*, 4. *T. viride* – 8

Plate 3 . Agarose gel electrophoresis of genomic DNA from *Trichoderma* isolates

Table 7. Quality and quantity of total DNA extracted from *Trichoderma* isolates

Sl. No.	*Trichoderma* spp.	Optical Density Ratio (OD_{260}/OD_{280})	Quantity of DNA (µg g^{-1} of mycelia)	Remarks on quality
1	*T. viride* – 1	1.81	204.08	Good
2	*T. harzianum* - 30	1. 79	191.55	Good
3	*T. aureoviride*	1.77	196.35	Good
4	*T. viride* – 8	1. 78	164.82	Good

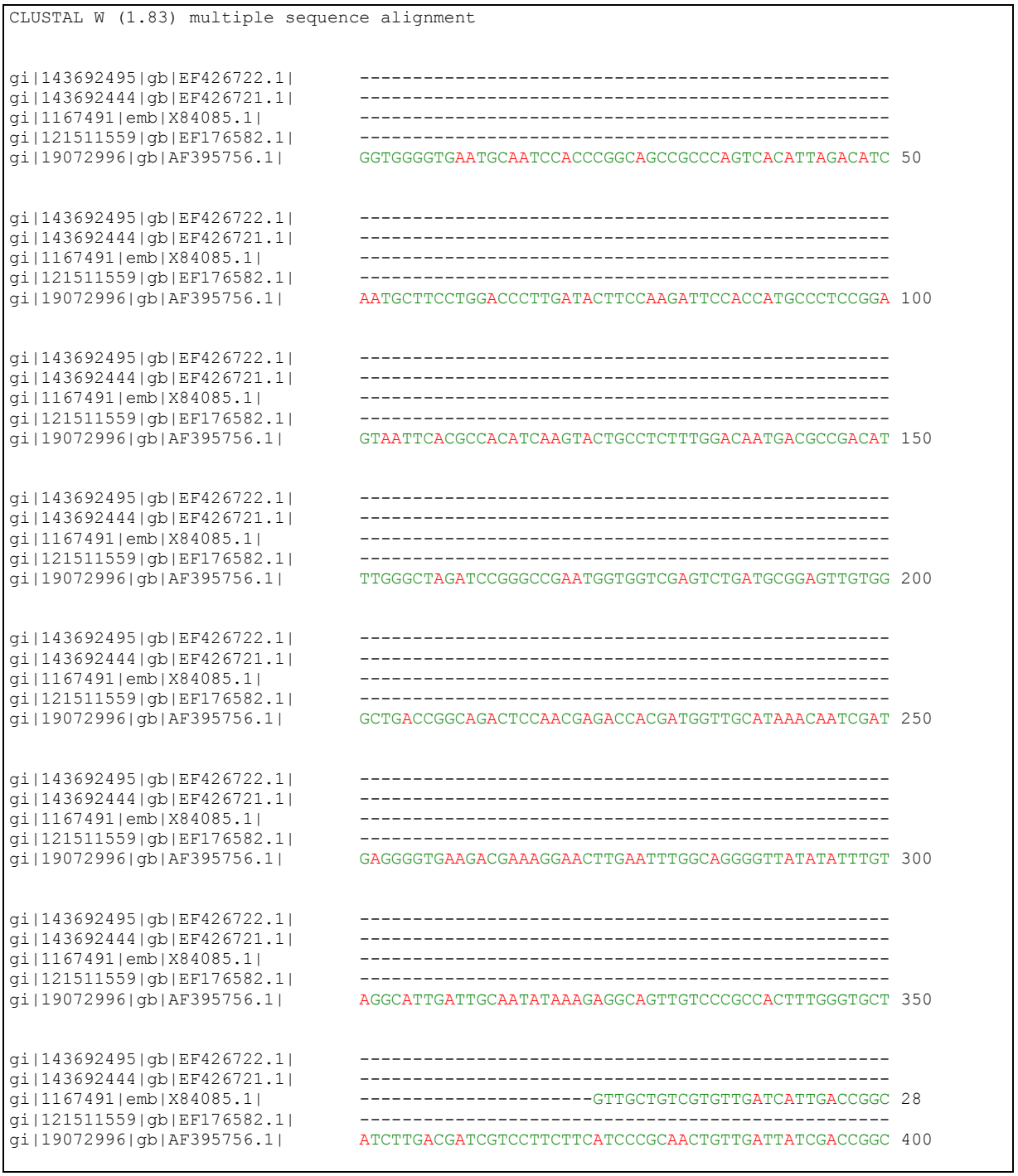
The conserved boxes selected through multiple sequence alignment at nucleotide level are provided in Plate 4. Based on these conserved regions, forward and reverse primers were designed. The details of the bases from conserved regions used for designing forward and reverse primers and details of the annealing temperature (Ta = Tm – 5) were given in Table 8. For different primer combinations with different annealing temperature, the expected amplicons sizes were provided in Table 9.

4.1.3.3. Amplification of β-1, 3- glucanase gene from *Trichoderma* isolates

All the four selected *Trichoderma* isolates were screened by PCR for the presence of β-1,3-glucanase gene using three sets of primers designed during the study and the result is presented in Table 10. In the screening of *Trichoderma* isolates using the first set of primer (Glucan F1 and Glucan R1), amplification of a single band of expected amplicon size about 550bp was obtained for two isolates *T. aureoviride* and *T. viride* - 8 (Plate 5a) and no amplification was obtained for isolates *T. viride* - 1 and *T. harzianum*-30. Amplicon size of the fragment being nearly 550 bp obtained for isolate *T. aureoviride* was used for cloning so as to confirm the gene sequence. This amplicon was named as GT-311.

All *Trichoderma* isolates were screened for β-1, 3- glucanase gene with second set of primer (Glucan F2 and Glucan R2). PCR amplification of glucanase gene with second set of primer showed a single band of expected amplicon size 600bp (Plate 5b). Amplification for glucanase gene was obtained for isolates *T. aureoviride* and *T. viride* - 8 as single band and no band was observed for isolate *T. viride* - 1 and *T. harzianum*-30. Amplicons of desired molecular weight from these isolates were sequenced. These amplicons were named as GT-322 and BPT-822.

Screening was also carried out for β-1, 3- glucanase gene with third set of primer (Glucan F3 and Glucan R3). Amplicon of expected size 550bp was obtained for the isolates of *T. aureoviride* as single band and for *T. viride* - 8 as two bands with less intensity (Plate 5c). Hence it was not selected for cloning.



```
CLUSTAL W (1.83) multiple sequence alignment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gi|143692444|gb|EF426721.1|      --------------------------------------------------
gi|1167491|emb|X84085.1|         --------------------------------------------------
gi|121511559|gb|EF176582.1|      --------------------------------------------------
gi|19072996|gb|AF395756.1|       GGTGGGGTGAATGCAATCCACCCGGCAGCCGCCCAGTCACATTAGACATC 50


gi|143692495|gb|EF426722.1|      --------------------------------------------------
gi|143692444|gb|EF426721.1|      --------------------------------------------------
gi|1167491|emb|X84085.1|         --------------------------------------------------
gi|121511559|gb|EF176582.1|      --------------------------------------------------
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gi|143692444|gb|EF426721.1|      --------------------------------------------------
gi|1167491|emb|X84085.1|         --------------------------------------------------
gi|121511559|gb|EF176582.1|      --------------------------------------------------
gi|19072996|gb|AF395756.1|       GTAATTCACGCCACATCAAGTACTGCCTCTTTGGACAATGACGCCGACAT 150


gi|143692495|gb|EF426722.1|      --------------------------------------------------
gi|143692444|gb|EF426721.1|      --------------------------------------------------
gi|1167491|emb|X84085.1|         --------------------------------------------------
gi|121511559|gb|EF176582.1|      --------------------------------------------------
gi|19072996|gb|AF395756.1|       TTGGGCTAGATCCGGGCCGAATGGTGGTCGAGTCTGATGCGGAGTTGTGG 200


gi|143692495|gb|EF426722.1|      --------------------------------------------------
gi|143692444|gb|EF426721.1|      --------------------------------------------------
gi|1167491|emb|X84085.1|         --------------------------------------------------
gi|121511559|gb|EF176582.1|      --------------------------------------------------
gi|19072996|gb|AF395756.1|       GCTGACCGGCAGACTCCAACGAGACCACGATGGTTGCATAAACAATCGAT 250


gi|143692495|gb|EF426722.1|      --------------------------------------------------
gi|143692444|gb|EF426721.1|      --------------------------------------------------
gi|1167491|emb|X84085.1|         --------------------------------------------------
gi|121511559|gb|EF176582.1|      --------------------------------------------------
gi|19072996|gb|AF395756.1|       GAGGGGTGAAGACGAAAGGAACTTGAATTTGGCAGGGGTTATATATTTGT 300


gi|143692495|gb|EF426722.1|      --------------------------------------------------
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gi|1167491|emb|X84085.1|         --------------------------------------------------
gi|121511559|gb|EF176582.1|      --------------------------------------------------
gi|19072996|gb|AF395756.1|       AGGCATTGATTGCAATATAAAGAGGCAGTTGTCCCGCCACTTTGGGTGCT 350


gi|143692495|gb|EF426722.1|      --------------------------------------------------
gi|143692444|gb|EF426721.1|      --------------------------------------------------
gi|1167491|emb|X84085.1|         ----------------------GTTGCTGTCGTGTTGATCATTGACCGGC 28
gi|121511559|gb|EF176582.1|      --------------------------------------------------
gi|19072996|gb|AF395756.1|       ATCTTGACGATCGTCCTTCTTCATCCCGCAACTGTTGATTATCGACCGGC 400
```

Plate 4. Multiple sequence alignment of β-1,3-glucanase gene of *Trichoderma* spp. The conserved sequences used for designing forward and reverse primers are shown in brackets

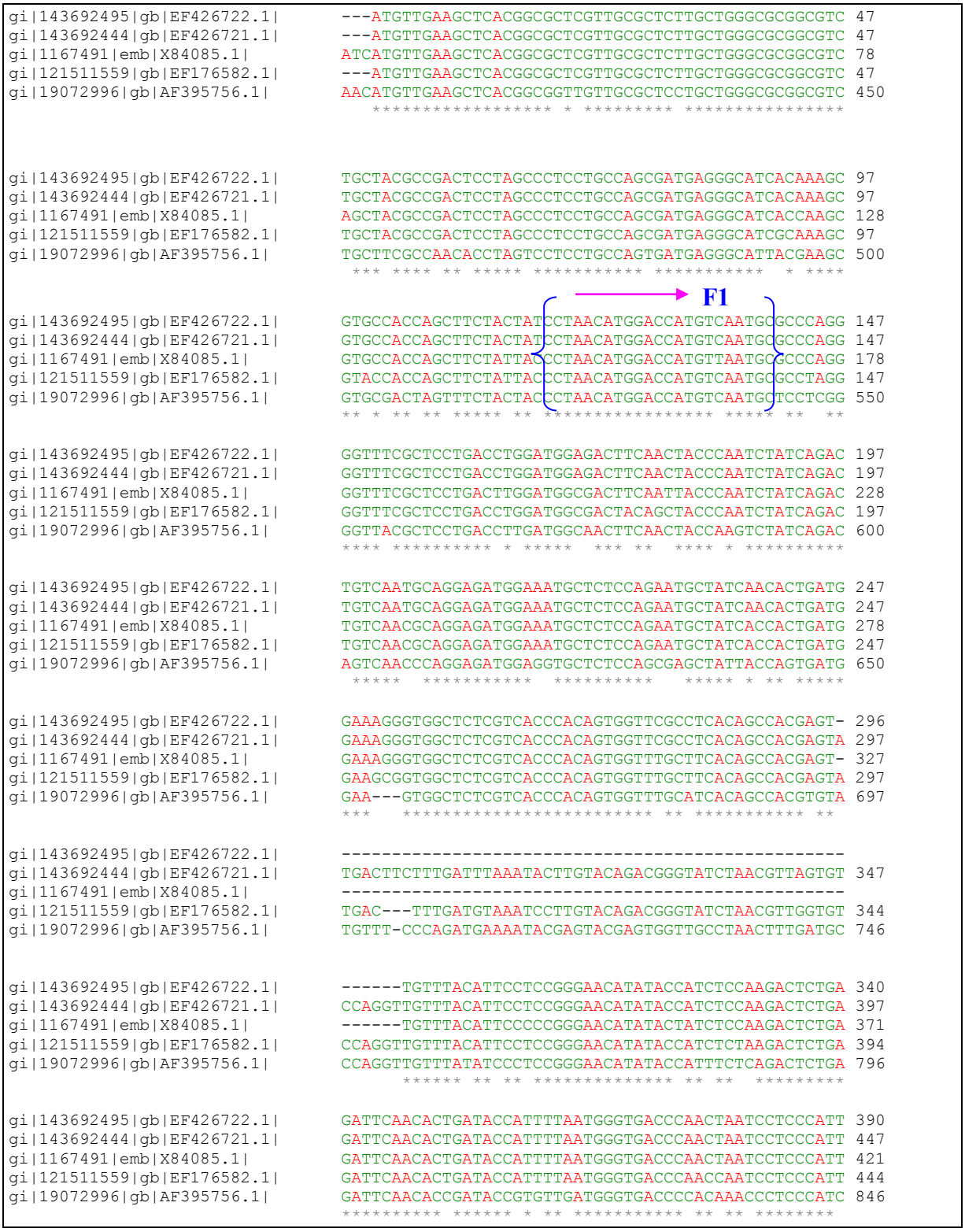


Plate 4. Multiple sequence alignment contd...

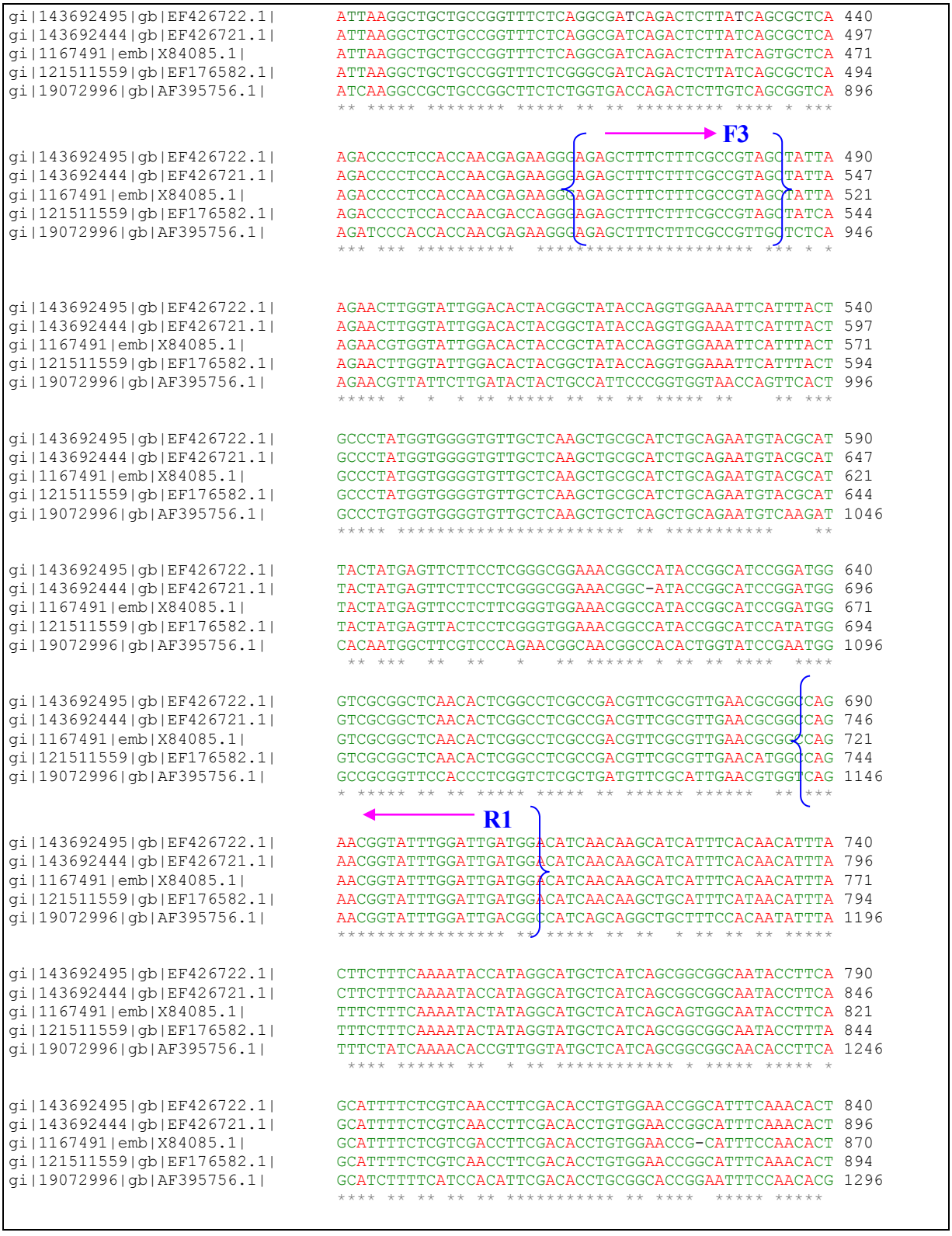


Plate 4. Multiple sequence alignment contd...

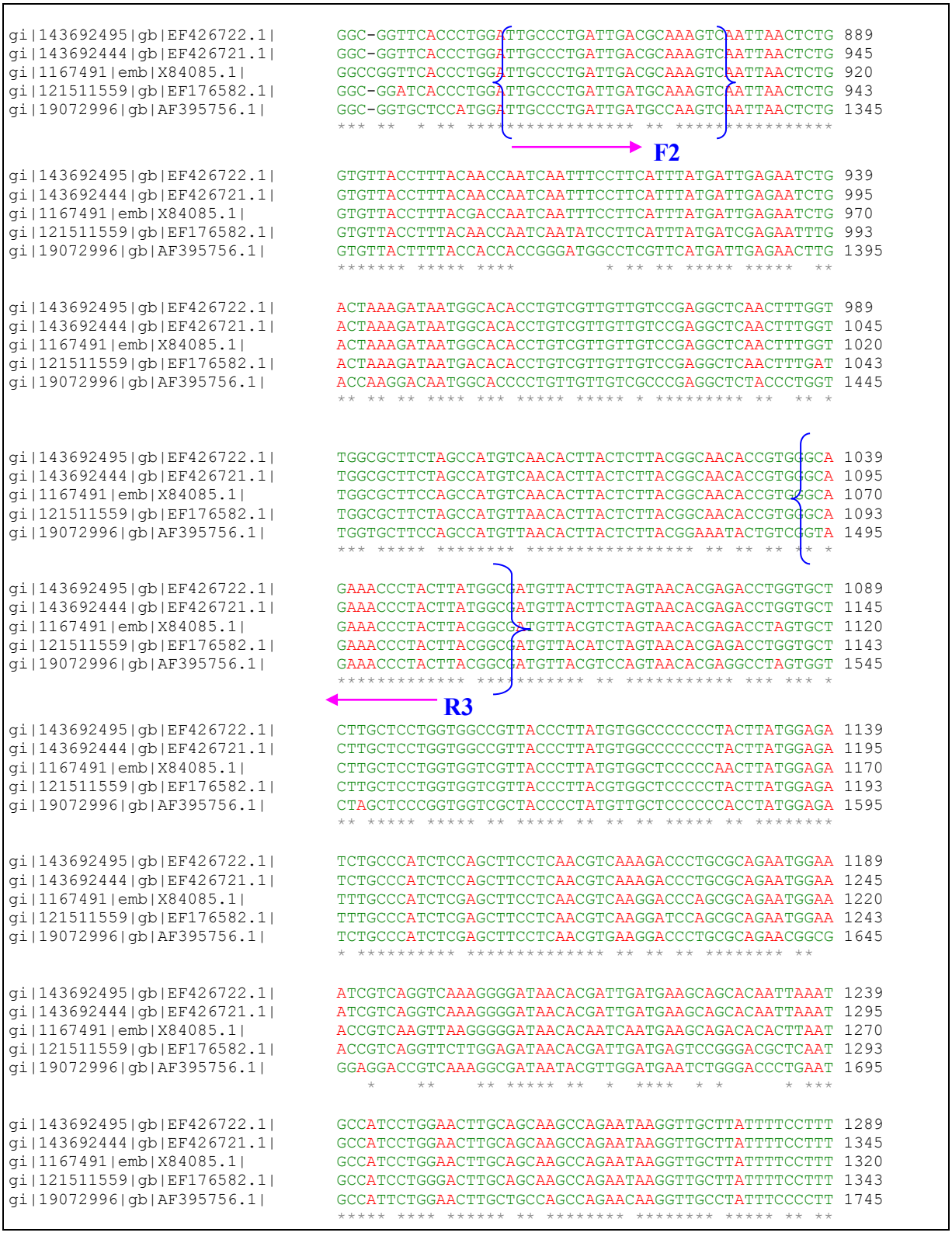


Plate 4. Multiple sequence alignment contd...

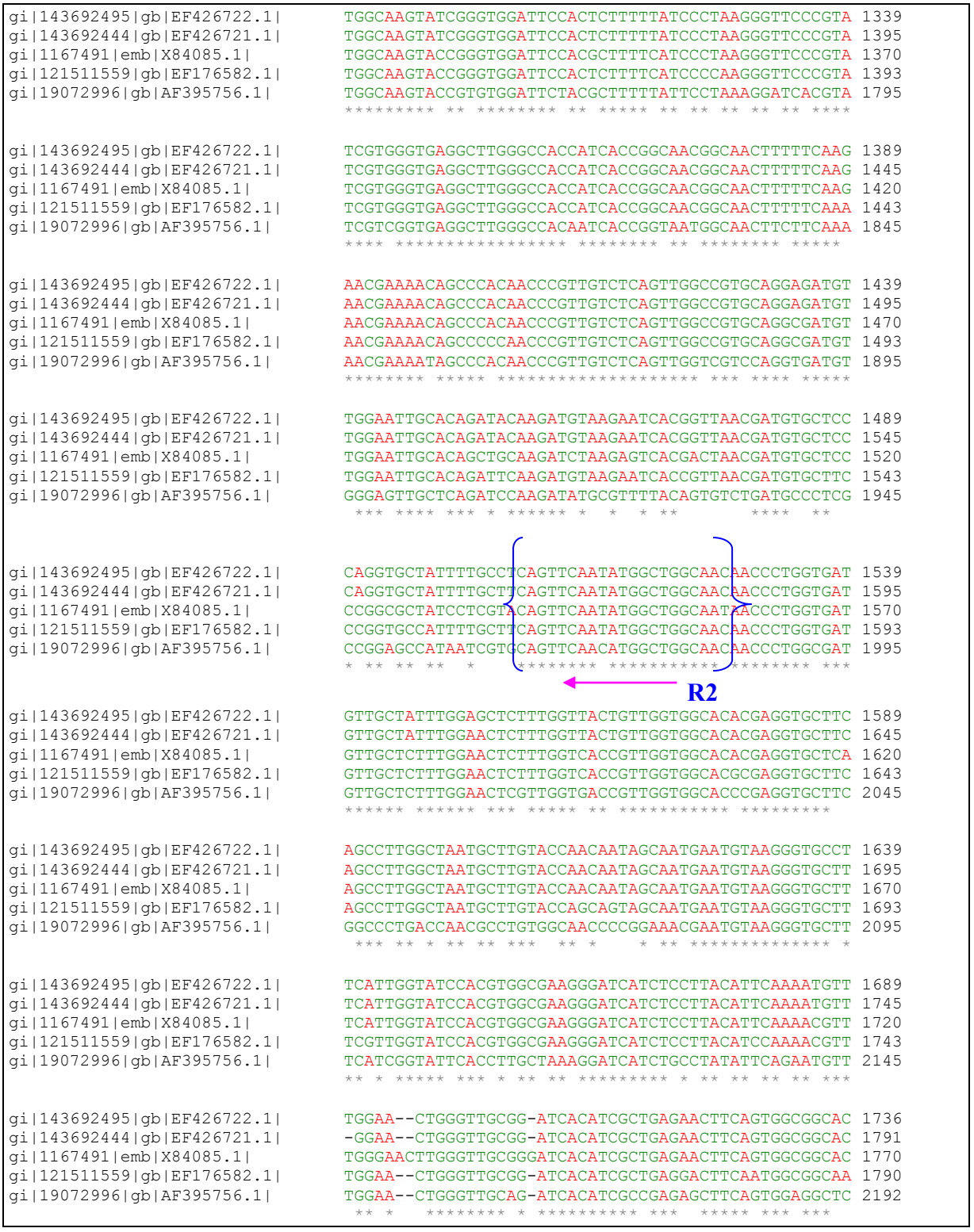


Plate 4. Multiple sequence alignment contd...

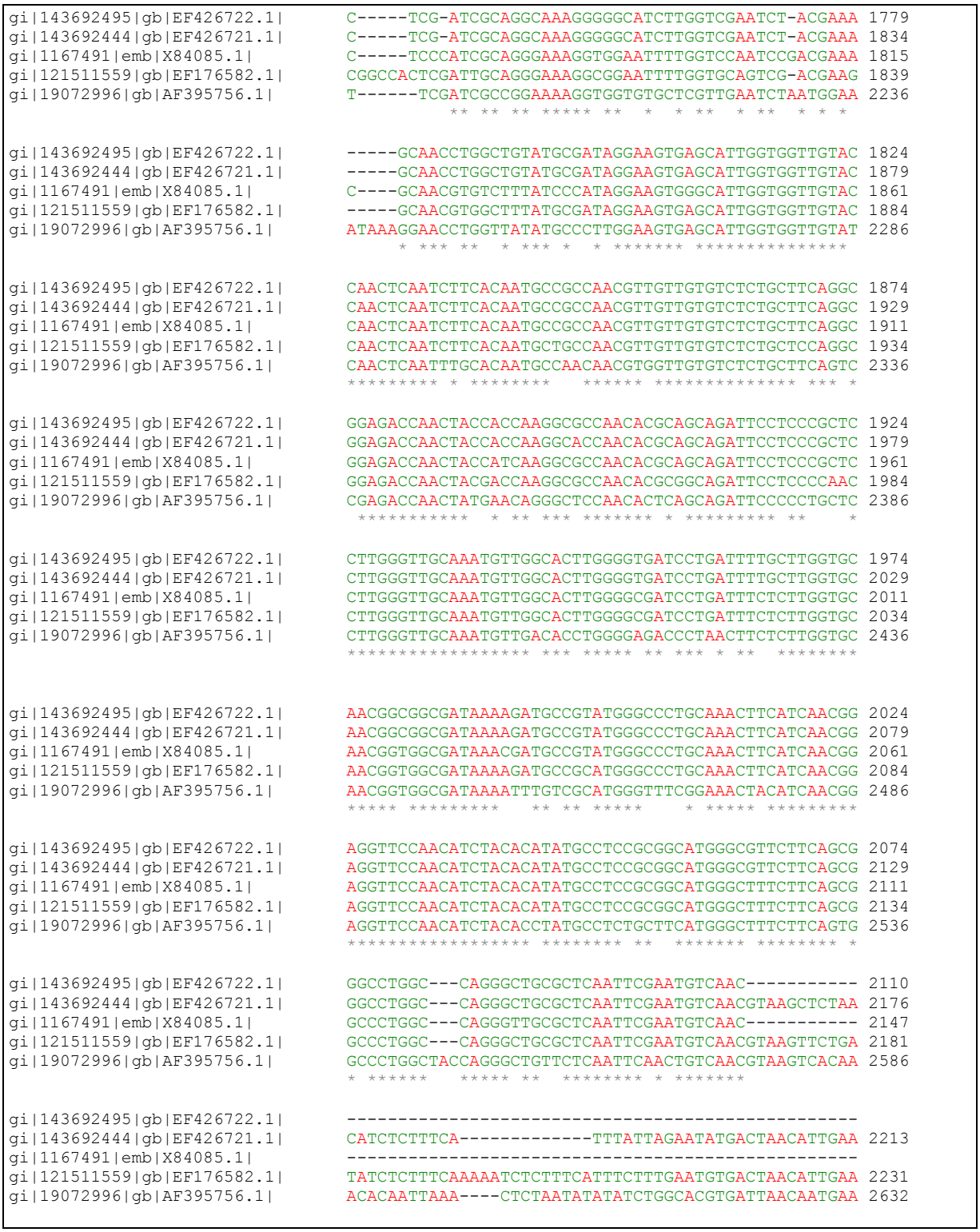


Plate 4. Multiple sequence alignment contd...

Plate 4. Multiple sequence alignment contd...

Table 8. Details of gene specific primers designed and used in the study

Sl. No.	Primer	Primer sequence	Length (bp)	Ta (^{0}C)
1	Glucan F_1	5' CCTAACATGGACCATGTYAATGC 3'	23	62
2	Glucan R_1	5' CCRTCAATCCAAATACCGTTCTG 3'	23	62
3	Glucan F_2	5' TTGCCCTGATTGAYGCMAAGTC 3'	22	60.5
4	Glucan R_2	5' RTTGCCAGCCATRTTGAACTG 3'	21	55.6
5	Glucan F_3	5' AGAGCTTTCTTTCGCCGTWG 3'	20	55
6	Glucan R_3	5' GCCRTAAGTAGGGTTTCTRC 3'	20	55

Glucan F1 - Forward primer 1, Glucan R1 - Reverse primer 1
Glucan F2 - Forward primer 2, Glucan R2 - Reverse primer 2
Glucan F3 - Forward primer 3, Glucan R3 - Reverse primer 3

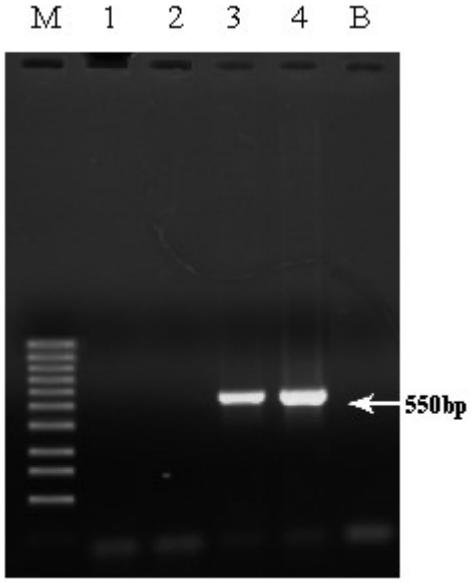
IUB codes used for degenerate codes : Y – C/T, R – A/G, M – A/C, W – A/T

Table 9. Details of annealing temperature and amplicon size expected for selected primers

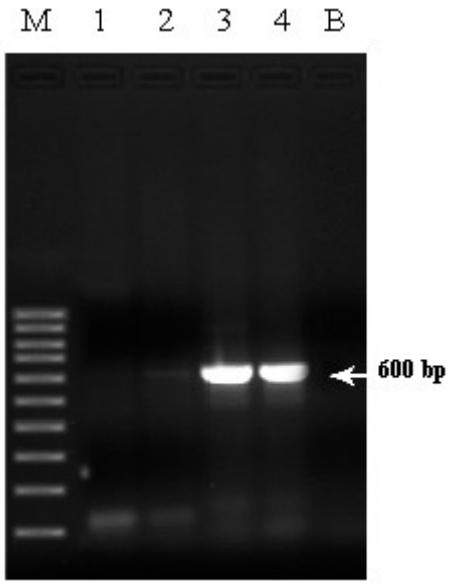
Sl. No.	Primer	Annealing Temperature (^{0}C)	Expected amplicon size (bp)
1	Glucan F1R1	62	548
2	Glucan F2R2	55	629
3	Glucan F3R3	55	553
4	Glucan F1R3	56	1360

Table 10. Details of amplicons obtained in β-1, 3- glucanase gene profile

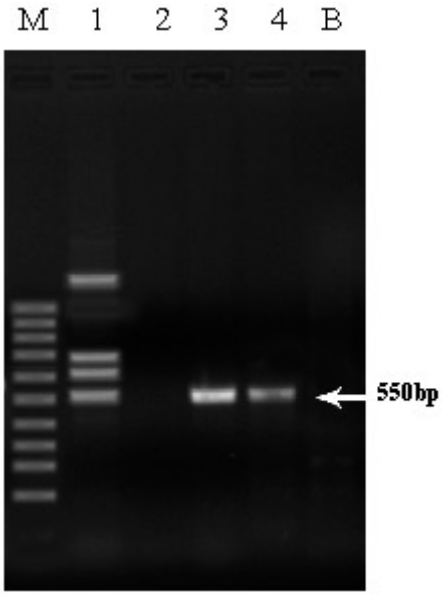
Sl. no.	Primer combination	Annealing temperature (^{0}C)	Presence of amplicon				No. of amplicons	Size of amplicons (bp)	Expected Size of amplicons (bp)
			T. viride - 1	*T. harzianum*	*T. aureoviride*	*T. viride* - 8			
1	Glucan F1R1	62	-	-	+	+	1, 1	~ 550, ~ 550	548
2	Glucan F2R2	52	-	-	+	+	1, 1	~ 600, ~ 600	629
3	Glucan F3R3	52	+	-	+	+	4, 1, 1	>1000, 700,550, 500, ~ 600, ~ 600	553
4	Glucan F1R2	56	+	+	+	-	1, 1, 1	~ 1300, ~ 1300	1360



a. With primer combination Glucan F1R2



b. With primer combination Glucan F2R2



c. With primer combination Glucan F3R3

M - 100 bp ladder, Lane 1. *T. viride* – 1, Lane 2. *T. harzianum* – 30, Lane 3. *T. aureoviride,* Lane 4. *T. viride* -8, Lane B. blank

Plate 5. PCR amplification with different primer combinations

Another primer combination of Glucan F1 and Glucan R2 were used for the screening of *T. viride* - 1*, T. harzianum*-30 and *T. viride* - 8 showed multiple bands in all isolates, when the amplification was carried out at annealing temperature 52^0C. Whereas single and discrete band was observed, when the annealing temperature was increased to 56^0C. Amplicon of expected size 1.3 kbp was obtained for isolates *T. viride* - 1 both in 1:10 dilution and without dilution, whereas in case of *T. harzianum*-30 and *T. aureoviride,* amplification was obtained only in 1:10 dilution as single band (Plate 6a, 6b). Amplicons of desired molecular weight from *T. viride* -1 and *T. harzianu*-30 were taken for cloning. These amplicons were named as T- 112 and CT – 3012.

4.1.3.4. Gel elution of PCR products

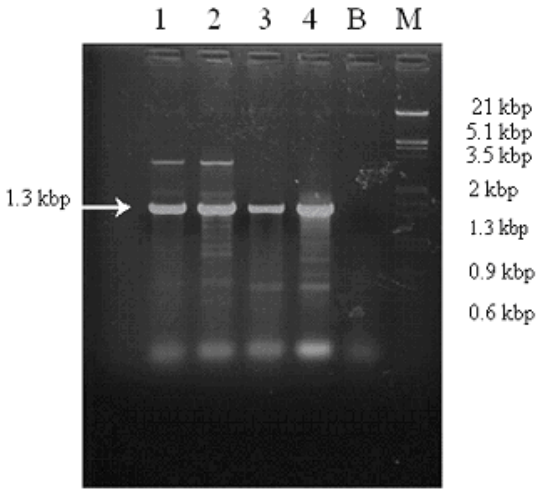
The amplicons GT-311, GT-322, BPT-822, T–112 and CT-3012 were eluted from the gel of PCR product. The eluted products when observed on agarose gel showed good concentration indicating good recovery from the gel slices (Plate 7Aa, 7Ab, 7Ac).

4.1.3.5. CLONING OF GENE ENCODING GLUCANASE

4.1.3.5.1. Preparation and screening of competent cells

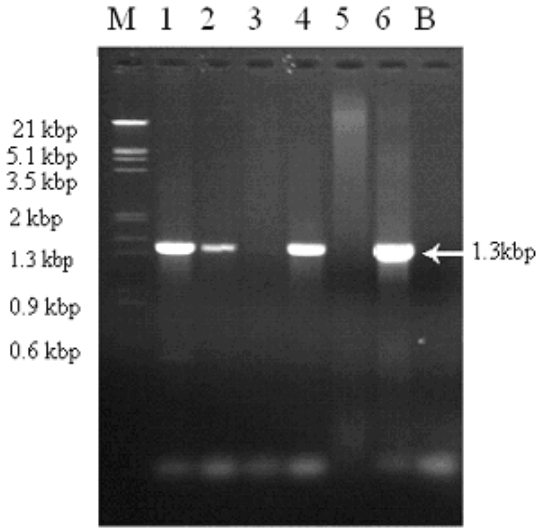
Competent cells were prepared from *E.coli* JM 109 strain. Large numbers of colonies were obtained when the competent cells were checked for competence by transformation using the pUC18 plasmid containing ampicillin resistance marker. The transformation efficiency of competent cells was calculated as $4.2x10^8$cfu μg^{-1} DNA. The cells were competent and showed a high degree of transformation efficiency. The colonies showed luxuriant growth, with no other contamination in LBA ampicillin plates (Plate 8a). Thus the competent cells prepared were found to be efficient for transformation and further cloning purposes.

The eluted bands corresponding to each isolate was ligated into pGEMT vector. After confirmation of competence, the ligated product was transferred into competent *E. coli* JM 109 cells using the heat shock method at 42^0C. When the transformed *E.coli* JM 109 cells



a. At annealing temperature 52°C

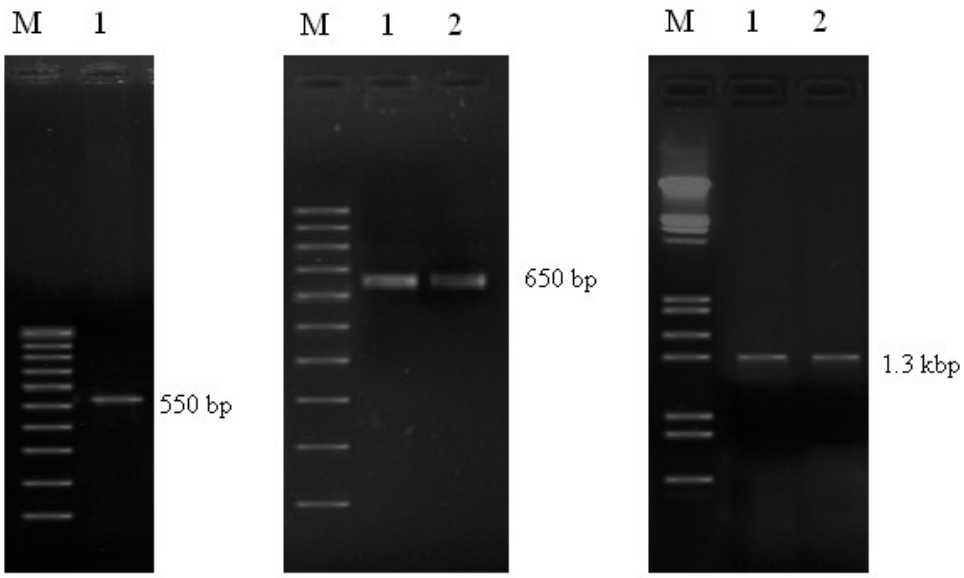
M - λ DNA/ *Eco* RI/ *Hind* III double digest marker, Lane 1. *T. viride* - 1, 2. *T. harizianum*-30, 3. *T. aureoviride,* 4. *T. viride* – 8, B. Blank



b. **At annealing temperature 56°C**

M - λ DNA/ *Eco* R I/ *Hind* III double digest marker, Lane 1. *T. viride* – 1 without dilution, 2. *T. viride* – 1 with 1:10 dilution, 3. *T. harizianum*-30 without dilution, 4. *T. harizianum*-30 with 1:10 dilution, 5. *T. aureoviride* without dilution, 6. *T. aureoviride* with 1:10 dilution, B. blank

Plate 6. PCR amplification with Glucan F1R2 primer combination



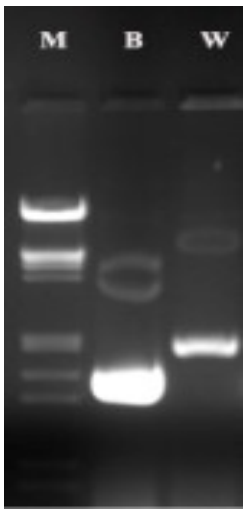
a. Glucan F1R1 **b.Glucan F2R2** **c.Glucan F1R2**

A. Eluted fragments from different primer combinations on agarose gel

M - 100 bp ladder,
1. *T. aureoviride* (GT-311)

M - 100 bp ladder,
1. *T. aureoviride* (GT-322),
2. *T. viride* – 8 (BPT-822)

M - Molecular weight marker,
1. *T. viride* – 1 (T-112),
2. *T. harizianum* (CT-3012)



B. Isolation of the plasmid containing the amplicon, CT-3012

M - λ DNA/ *Eco*R I/ *Hind* III double digest marker,
Lane W- Recombinant plasmid,
Lane B- Non recombinant plasmid

Plate 7. Eluted fragments and plasmid isolated from blue and white colony

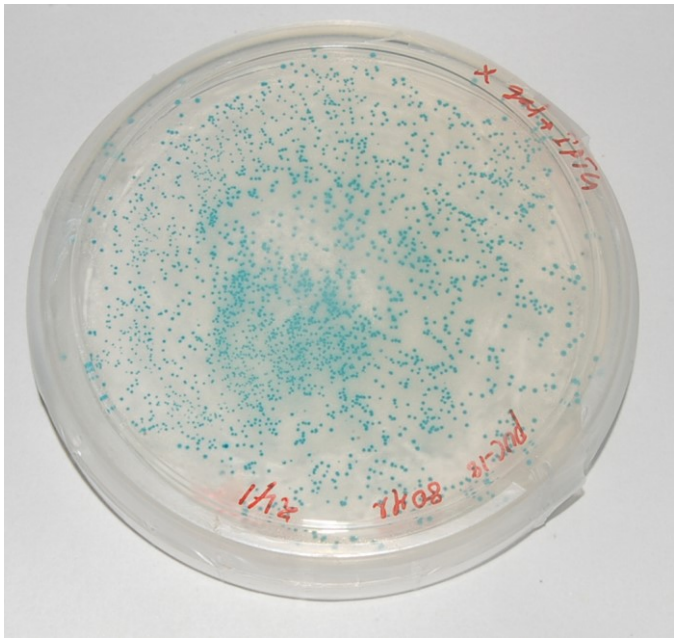
were grown in LBA ampicillin plates overlaid with X-gal and IPTG, a combination of blue and white colonies were obtained after overnight incubation confirming successful transformation (Plate 8b). The transformation efficiency varied from 66 per cent to 73.5 per cent indicating successful transformation (Table 11).

4.1.3.5.2. Screening of transformed colonies

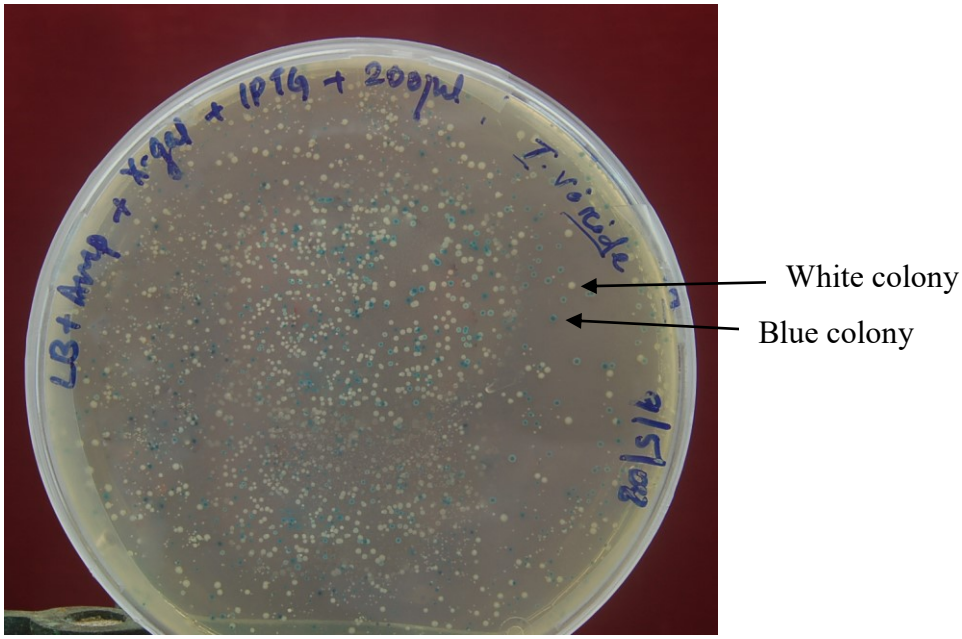
The LB ampicillin agar plates containing the transformed colonies were screened for recombinant plasmid. Three white colonies were picked from each plate and grown in LB ampicillin media separately. One blue colony was also inoculated in order to set the negative control. Plasmids were isolated and checked on 0.7 per cent agarose gel (Plate 7B). The plasmids isolated from white colonies had high molecular weight when compared with plasmid isolated from blue colonies. This confirmed presence of the insert in white plasmid.

4.1.3.5.3. Confirmation of insertion by PCR

The plasmid was additionally checked for the presence of fragment insert by PCR confirmation. The plasmid DNA was used as template and insert was amplified using gene specific primers. Amplification was obtained in the plasmids isolated from white colonies where as no amplification was detected in the plasmids isolated from blue colonies. The PCR products were checked on 1.0 per cent agarose gel (Plate 9Aa, 9Ba). The insert used for transformation was also loaded along with the amplified plasmid PCR products, to confirm the presence of insert in the plasmid. The plasmid was again confirmed by PCR using T7 forward and SP6 reverse universal primer. Higher amplicon sizes of about 800 bp, 900 bp and 1.5 kb of bands were obtained from the white plasmid of the clone of primer combinations Glucan F1R1, Glucan F2R2 and Glucan F1R2 respectively, which were little higher than the insert size and lower amplicon size than the white plasmid was obtained from blue plasmid by the primer (Plate 9Ab, 9Ba, 9Ca).



a. Competent cells grown in X-gal/IPTG plated ampicillin medium

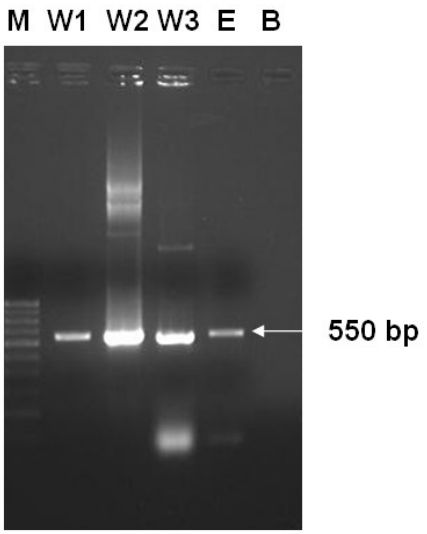


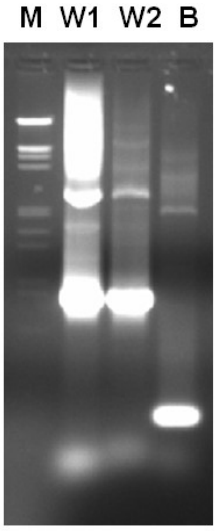
b. Blue and white colonies grown in X-gal/IPTG plated ampicillin medium

Plate 8. Competent cells of *E. coli* JM 109 and screening of transformants

Table 11. Transformation efficiency in *E. coli* JM 109 cells with different amplicons

Sl. No.	Details of amplicons	No. of white colonies	No. of blue colonies	Total no. of colonies	Transformation efficiency (%)
1	GT-311	69	30	99	70
2	GT-322	186	79	283	66
3	BPT-822	212	96	308	68.8
4	T-112	263	112	375	70.1
5	CT-3012	248	89	337	73.5

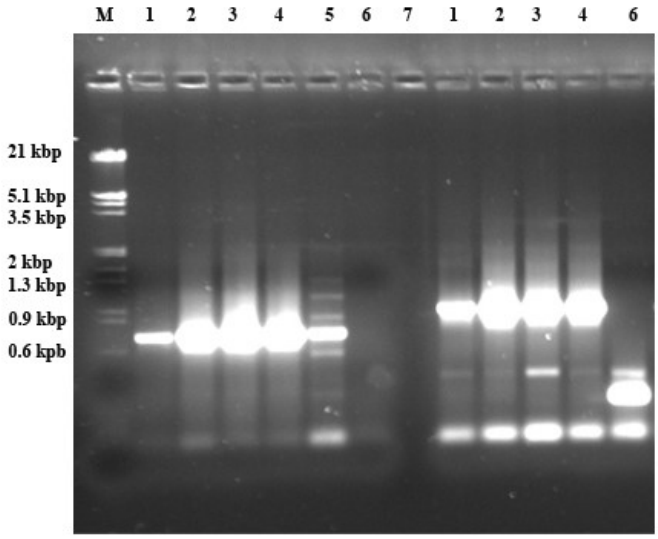


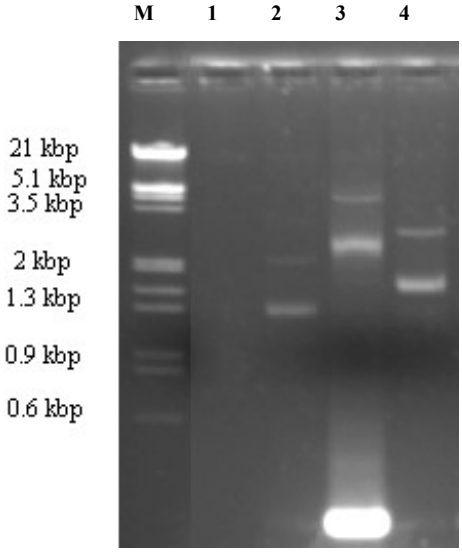


a. Sp. primer F1R1 **b. T7 & Sp6 primer**

A. GT-311 (*T. aureoviride*)

M - Molecular weight marker, W, W1, W2, W3 - Recombinant plasmid,
B - Non recombinant plasmid





a. Sp. primer F2R2 and T7 & Sp6 primer **a. T7 & SP6 primer**

B. GT-322 (*T. aureoviride*) and BPT- 822 (*T. viride* – 8)

M - λ DNA/ *Eco*R I/ *Hind* III double digest marker,
Lane 1,2 - Recombinant plasmid (GT-322), 3,4 - Recombinant plasmid (BPT-822), 5- PCR product of genomic DNA of *T. viride*-8 (BPT-8) with F2R2 primer, 6- non recombinant plasmid without dilution, 7 - Blank

C. T – 112(*T. viride*-1) and CT – 3012 (*T. harzianum*-30)

M - λ DNA/ *Eco*R I/ *Hind* III double digest marker, Lane 1- Blank, 2- Recombinant plasmid (T-112), 3- Non recombinant plasmid, 4 - Recombinant plasmid (CT-3012)

Plate 9. PCR confirmation of insertion of transformed clones

4.2. SEQUENCING OF THE CLONE AND AMPLICONS

The screened colonies in which the presence of insert was confirmed were used for automated sequencing using T7 universal primer. Of the four isolates, five recombinant clones were sent for sequencing to Genei, Bangalore, two sequences were obtained from the isolate *T. aureoviride* (GT-311 and GT-322) and only one sequence (BPT-822) from the isolate *T. viride* - 8.

4.3. THEORITICAL ANALYSIS OF SEQUENCE USING BIOINFORMATIC TOOLS

The nucleotide sequence of the clones obtained from the isolates *T. aureoviride* and *T. viride* – 8 were named as GT-311, GT-322 and BPT-822. The electropherograms of each sequence are presented in Annexure VI.

4.3.1. Nucleotide sequence analysis

4.3.1.1. Vector Screening

The nucleotide sequences were screened for the presence of vectors using VecScreen program of NCBI. The sequences cloned from isolate *T. aureoviride,* GT-311 and GT-322 showed significant similarity with vector regions. Both the sequences showed similarity to vectors starting from the region 1 to 47 bases. The sequence BPT822 from the isolate *T. viride* - 8 showed similarity to vectors starting from 1 to 52 bases and from 651 to 706 bases. Results of vector screen of cloned sequences are displayed in Plates 10B, 11B, 12B. The sequences after deletion of vector sequences and primer sequences were ready for further analysis. The sequences of amplicons obtained are given in Plates 10A, 11A, 12A and red colour region showing the vector sequence.

4.3.1.2. Homology search

Homology search of nucleotide sequences of clones GT-311, GT-322 and BPT-822 with other reported glucanase gene sequences were carried out. The sequences showed significant homology with different glucanase gene sequences of various species of *Trichoderma* or *Hypocrea* present in NCBI databank. Query coverage for GT-311 was 76 per cent, showing maximum identity of 93 per cent and 92 per cent to endoglucanse gene (bgn13.3) of *H. virens* and β-1,3-glucanase (glu) gene of *T. viride* respectively. For GT-322, 92 per cent homology was found to β-1,3- glucanase (glu) gene of *T. viride* and 90 per cent to endo–1,3(4)-β-glucanase of *T. harzianum*-30 and endoglucanase gene(bgn13.3) of *H. virens* with 79 per cent query coverage. The sequence BPT-822 had shown maximum homology of 96 per cent and 94 per cent to two accessions namely, EF176582.1 and X84085 respectively with minimum E–value. There were 24, 36 and 12 blast hits reported for sequences GT-311, GT-322 and BPT-822 respectively (Plates 10D, 10E, 11D, 11E, 12D, 12E).

Homology search through protein-protein BLAST (Blastp) using deduced amino acid sequence showed all three sequences had high similarity with endoglucanses of *H. virens*. GT-311 showed 87 blast hits and significant similarity to glucan endo-1,3-β-glucosidase (BGN13.1) of *T. harzianum*-30, β-1,3-glucanase and laminarinase of *T. viride* (Plates 10F, 10G). Similarity was also observed with protein β-1,3-glucanase and laminarinase of *T. viride* for GT-322 (Plate 11F, 11G). The sequence BPT8-22 showed similarity with β-1,3-glucanase precursor of *H. virens* and β-1,3-glucanase of *T. viride* (Plates 12F, 12G).

Diversity analysis of three glucanase sequences *viz.* GT-311, GT-322 and BPT-822 with glucanase genes of *Trichoderma* spp. reported in the public domain was carried out by constructing phylogram. GT-322 and BPT-822 showed close similarity with β-1,3-glucanase (glu) gene of *T. viride* having accession number EF176582.1 and GT-311 with endoglucanase (bgn13.1) gene of *H. virens* (accession

no. EF426721.1) and further connected to the gene β -1,3-glucanase precursor (bgn2) of *H. virens* (accession no. AF395756.1) in phylogenetic tree. The phylogram showing evolutionary relationship was given in fig. 3.

Fig. 3. Phylogram showing evolutionary relationship

4.3.1.3. Nucleic acid statistics

Nucleotide composition of the above sequences was determined using Biology Work Bench (http://seqtool.sds.edu/). Both the sequence GT-322 and BPT-822 were rich in A+T base pair composition (51.3%) as compared with C+G (48.7%), while GT-311 had equal proportion of AT and GC basepair. The details of nucleotide composition of sequences are given in Table 12. After deleting vector sequence and primer sequences, the nucleotide sequence length of cloned sequences GT-311, GT-322 and BPT-822 were 725 bp, 749 bp and 616 bp respectively (Table 12).

4.3.1.4. Detection of Open Reading Frame (ORF)

The sequences were translated in all six opening reading frames (http://www.ncbi.nlm.nih.gov.ORFfinder). There were six open reading frames in GT-311, with the longest one located on +2 strand starting from base 14 to 277, having a length of 264 bases and the other four were on +1 strand and rest one located on -2 strand (Plate 10H). When the longest significant open reading frame was subjected to BLASTp search, it detected homology with hypothetical protein of

Table 12. Nucleotide statistics of sequences from *Trichoderma* isolates

Sl. No.	Gene sequence	Sequence length (bp)	Nitrogen base percentage (%)					
			A	T	G	C	A and T	G and C
1	GT-311	725	25.5	25.0	25.5	24.0	50.5	49.5
2	GT-322	749	24.3	27.0	24.8	23.9	51.3	48.7
3	BPT-822	616	23.7	27.6	26.6	22.1	51.3	48.7

Rattus norvegicus (Plate 10I). GT- 322 had seven open reading frames, both +2 and +3 strand had two ORFs each and +3, -1 and +1 strands had one ORF each. The longest one was with a length of 423 bases coding 140 aminoacid residues, starting from base 68 to 490 located on +2 strands (Plate 11H). The result of the BLASTp showed 88 blast hits and homology to β-1,3-glucanase of *T. viride* and endoglucanse of *H. virens* (Plate 11I). BPT-822 possessed four open reading frames (Plate 12H) with the longest one located on +3 strand with a length of 441 bp, starting from base 72 to512, coding 146 aminoacid residues. The +1 strand possessed two ORFs and another one was located on +2 strand. The longest one was subjected to BLASTp and its result showed significant homology to β-1,3-glucanase of different *trichoderma* spp. and *Aspergillus fumigatus* (Plate 12I). The location and length of ORFs are specified in Table 13.

4.3.1.5. Restriction analysis

All the sequences were analyzed for the presence of restriction sites for ten enzymes (http://seqtool.sdsc.edu/). The restriction enzyme *Alu*I had four sites, followed by *Hae*III, three sites were present in GT311, whereas *Acl*I, *Dpn*I, *Mbo*I, *Nae*I and *Pvu*I had only one site (Plate 10J). There were no restriction sites for *Bam*HI and *Taq*I but only two restriction sites were seen in case of *Mse*I. In GT322, there were four restriction sites for *Dpn*I I and *Mbo*I. It had two restriction sites for *Taq*I and *Mse*I and one restriction site for *Alu*I, *Bam*HI, *Acl*I and *Hae*III (Plate 11J). Restriction enzymes *Nae*I and *Pvu*I lacked restriction sites both on GT-322 and BPT-822. The sequence BPT-822 was found to possess four restriction sites for *Taq*I, three restriction sites for *Alu*I, two restriction sites each for *Dpn*I and *Mbo*I, whereas there was only one restriction site for *Bam*HI, *Hae*III and *Mse*I (Plate 12J). Details of the restriction analysis of the sequences are given in Table 14 A, 14 B and 14 C.

Table 13. Open reading frames of glucanase genes cloned from different isolates

Sl .No.	Gene	ORF location	ORF length (bp)	Reading frame
1	GT-311	14- 277	264	+2
		7-183	177	+1
		1- 156	156	+2
		475 – 612	138	+1
		631 – 747	117	+1
		301 – 417	117	+1
2	GT-322	68 – 490	423	+2
		93 – 470	378	+3
		337 – 645	309	-3
		616 – 768	153	+1
		573 – 713	141	+3
		566 – 706	141	+2
		624 – 752	129	-1
3	BPT-822	72 – 512	441	+3
		97 – 405	309	+1
		427 – 627	201	+1
		506 – 627	123	+2

Table 14 A. Theoretical restriction analysis of the clone GT-311

Sl. No.	Restriction enzyme	Recognition sequence	No.of cut(s)	Position of restriction sites	Fragment sizes(bp)
1	*Alu* I	AG▾CT	4	312, 381, 396, 472	312, 67, 13, 74, 255
2	*Bam* HI	G▾GATC▴C	0	-	-
3	*Acl* I	AA▾CG▴TT	1	197	197, 530
4	*Taq* I	T▾CG▴A	0	-	-
5	*Dpn* I	GA▾TC	1	334	334, 393
6	*Hae* III	GG▾CC	3	575, 656, 725	575, 79, 67, 2
7	*Nae* I	GCC▾GGC	1	320	320, 407
8	*Mbo* I	▾GATC▴	1	332	332, 419
9	*Mse* I	T▾TA▴A	2	277, 497	277, 218, 237
10	*Pvu* II	CAG▾CTG	1	472	472, 256

Table 14 B. Theoretical restriction analysis of the clone GT-322

Sl. No	Restriction enzyme	Recognition sequence	No.of cut(s)	Position of restriction sites	Fragment sizes(bp)
1	*Alu* I	AG▾CT	1	278	278, 472
2	*Bam* HI	G▾GATC▴C	1	294	294, 456
3	*Acl* I	AA▾CG▴TT	1	710	710, 41
4	*Taq* I	T▾CG▴A	2	53, 274	53, 219, 477
5	*Dpn* I	GA▾TC	4	52, 296, 562, 573	52, 242, 264, 9, 178
6	*Hae* III	GG▾CC	1	632	632, 119
7	*Nae* I	GCC▾GGC	0	-	-
8	*Mbo* I	▾GATC▴	4	50, 294, 560, 571	50, 242, 264, 9, 180
9	*Mse* I	T▾TA▴A	2	5, 534	4, 527, 224
10	*Pvu* II	CAG▾CTG	0	-	-

Table 14 C. Theoretical restriction analysis of the clone BPT-822

Sl. No	Restriction enzyme	Recognition sequence	No.of cut(s)	Position of restriction sites	Fragment sizes(bp)
1	*Alu* I	AG▾CT	3	291, 415, 614	291, 122, 197, 5
2	*Bam* HI	G▾GATC▴C	1	307	307, 312
3	*Acl* I	AA▾CG▴TT	0	-	-
4	*Taq* I	T▾CG▴A	4	66, 287, 376, 599	66, 221, 89, 223, 20
5	*Dpn* I	GA▾TC	2	65, 309	65, 244, 310
6	*Hae* III	GG▾CC	1	420	420, 199
7	*Nae* I	GCC▾GGC	0	-	-
8	*Mbo* I	▾GATC▴	2	63, 307	63, 244, 312
9	*Mse* I	T▾TA▴A	1	18	18, 601
10	*Pvu* II	CAG▾CTG	0	-	-

4.3.1.6. Gene prediction

Gene prediction analysis of the sequence was carried out using the tool Genscan (www.genes.mi.edu/genscan/). GT-311 had an initial exon with a length of 130bp, starting from base 7 to 136 and a terminal exon of 191bp, starting from 334 to 524 bases (Plate 10K). There was no initial and terminal exon in GT-322, only two internal exons were present having a length 293bp (145 – 473) and 126bp (472 – 597), as shown on Plate 11K. BPT-822 possessed two internal exon having a length of 224bp (149 – 372) and 201bp (407 – 607), which presented on Plate 12K.

4.3.2 Amino acid sequence analysis

The deduced amino acid sequence was obtained from the nucleotide sequence using Molecular Tool Kit. The proportions of each amino acid in cloned sequences were calculated using 'AASTAT' tool (http://seqtool.sdsc.edu/). GT-311 had aminoacid serine with highest molar per cent of 11.3, followed by glycine (8.7%) as well as both leucine and alanine (8.3%). Glutamine and methionine content were least (1.3%) in GT-311 and GT-322 respectively. Both amino acids arginine and leucine content was highest (8.5%), followed by serine (7.2%) in GT-322. The BPT-822 sequence had leucine with the highest share of 19.70 per cent, whereas aspartic acid, tryptophan and tyrosine share was the least, 1.5 per cent each (Table 15).

The functional domains of GT-311 sequence were located as two transmembrane helices of 20 bp size in transmembrane region ranging from the amino acid sequence 37 to 57 and 140 to 160 using 'InterProScan' (www.ebi.ac.uk/InterProScan). In the sequence BPT-822, one signal peptide and two transmembrane helices were found through 'InterProScan'. The sequence region 1 to 52 amino acids coded for signal peptide and 36 to 56 and 71 to 91 amino acids coded for transmembrane helix. The graphical output of 'InterProScan' and 'TMHMM' are given in Plates 10L, 10M; 12L, 12M.

Table 15. Amino acid composition of different glucanase protein sequences

Amino acid			Molar percentage of amino acid (Mol%)		
			GT-311	**GT-322**	**BPT-822**
Non polar		Gly	8.7	5.9	3.4
		Ala	8.3	4.2	7.4
		Val	4.3	5.5	5.9
		Leu	8.3	8.5	19.7
		Ile	3.9	3.8	4.9
		Met	1.7	1.3	3.9
		Pro	7.0	6.4	7.4
		Phe	3.0	4.2	3.4
		Trp	2.6	5.9	1.5
Polar	Uncharged	Ser	11.3	7.2	8.9
		Thr	6.1	5.1	6.4
		Cys	3.9	5.9	2.0
		Tyr	4.3	3.8	1.5
		Asn	4.3	2.5	3.0
		Gln	5.7	5.5	3.4
	Basic	Lys	3.5	2.5	2.0
		Arg	6.5	8.5	7.4
		His	3.0	5.9	2.5
	Acidic	Asp	2.2	3.4	1.5
		Glu	1.3	3.4	3.4

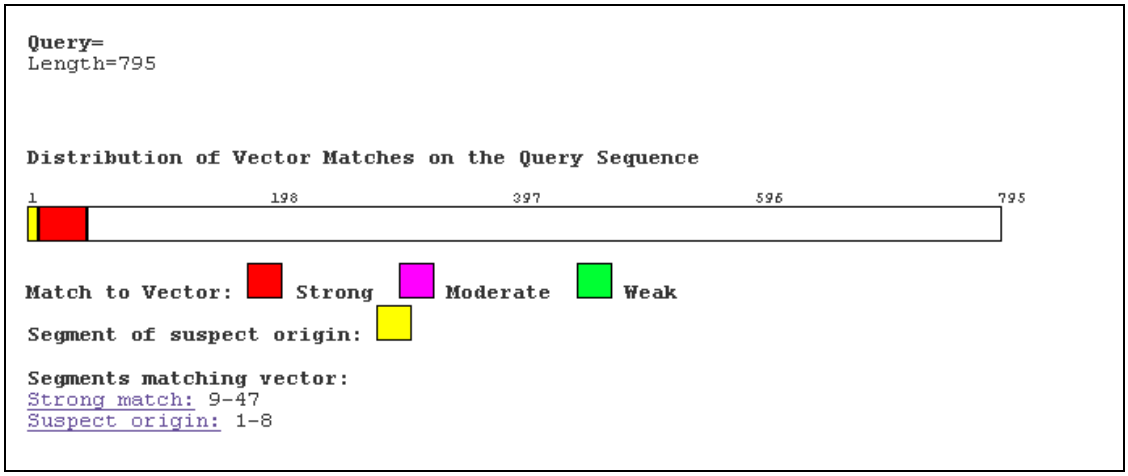
The ‘Motif Scan’ of aminoacid sequence of GT-311, GT-322 and BPT-822 revealed similarity with protein kinase and casein kinase phosphorylation site. BPT-822 had leucine rich region motif ranging from 6 to 87 amino acid sequence.

The secondary structure prediction of proteins was done using SOPMA programme provided by Expasy tools. The proportion of each structure type was found out. The sequences GT-311 and GT-322 were comparatively richer in random coils (47.83%, 47.03 %), where as BPT-822 was the richest in alpha helices (48.77%). The share of extended strands came to about 27.39 per cent in GT-311, 28.81 per cent in GT-322 and 12.81 per cent in BPT-822. The contributions of beta turns were 11.74, 7.20 and 4.43 per cent in GT-311, GT-322 and BPT-822 respectively. The proportion of random coil was 33.99 per cent in BPT-822 and the proportion of alpha helices were 13.04 and 16.95 per cent in GT-311 and GT-322 respectively. The results are displayed in Plates 10O, 10P; 11M, 11N; 12O, 12P).

Hydropathy plot of the sequences was constructed by means of Kyte and Doolittle Hydropathy Profile. All the three sequences were hydrphobic in nature being rich in hydrophobic amino acids. But GT-311 and BPT-822 had both hydrophobic and hydrophilic amino acids. Graphical outputs of the results were displayed in Plates 10N, 11L, 12N.

>GT-311
CGATTGGGCCGACGTCGCATGCTCCCGGCCGTCATGGCCGCGGGATTCCTAACATGGACCATGTTAAT
GCGCCCAGGGGTCTCGCTCCTGACCTGGATGGCGACTTCAACTACCCAATCTATCAGACTGTCAACGC
AGGAGATGGAAATGCTCTCCAGAATGCTATCAACACTGATGGAAAGGGTGGCTCTCGTCACCCACAGT
GGTTTGCTTCACAGCCAGGAGTATGACTTTGATGTAAATCCTTGTACAGACGGGTATCTAACGTTAGT
GTCCAGGTTGTTTACATTCCTCCGGGAACATATACCATCTCCAAGACTCTGAGATTCAACACTGATAC
CATTTTAATGGGTGACCCAACCAATCCTCCCATTATTAAGCTGCTGCCGGCTTCTCGGGCGATCAGAC
TCTTATCAGCGCTCAAGACCCTCCACCAACGAGAAGGAGAGCTGTCTTTCGCGTAGCTATTAGAACGT
GTATTGGACACTACGGCTATACTAGTGGAAATTCATTTACTGCCTATGGTGGGGTGTTGCTCAGCTGC
GCATCTGCAATGTACGCATTACTATGATTCTTCTCGGGGGAAACGGCATACGGCTCCGGATGGTCGGT
TCAAACTCGGTCCGCCAAGTTGGTTGACCGGCCAACGAATTGATTGACGAATCCTATGGCGGCGCCTG
CGGTCCCATTGGGAAGTCCAGCGTGAAGCAACTGAATTTCAAGGCCCAAATGGAATCTGCAAGTTCTG
GGAATTTACGCCAATTCCAAAAAAACCCAAAAATGTAAACTGGGGCC

A. Nucleotide sequence

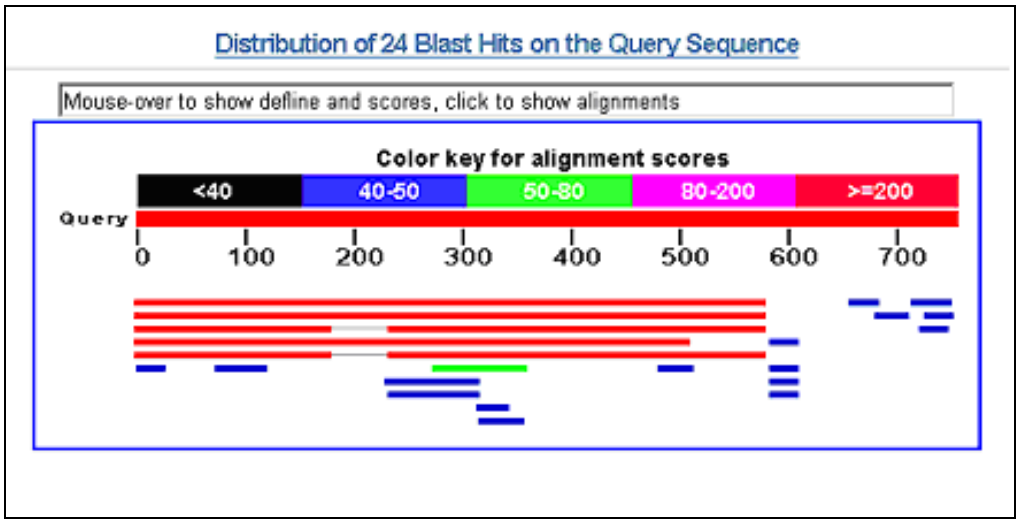


B. VecScreen Output

>GT_1 311
AQGSRS*PGWRLQLPNLSDCQRRRWKCSPECYQH*WKGWLSSPTVVCFTARSMTLM*ILV
QTGI*R*CPGCLHSSGNIYHLQDSEIQH*YHFNG*PNQSSHY*AAAGFSGDQTLISAQDP
PPTRRRAVFRVAIRTCIGHYGYTSGNSFTAYGGVLLSCASAMYALL*FFSGETAYGSGWS
VQTRSAKLVDRPTN*LTNPMAAPAVPLGSPA*SN*ISRPKWNLQVLGIYANSKKTQKCKL
GP

C. Deduced amino acid sequence

Plate10. Sequence analysis for the clone GT-311



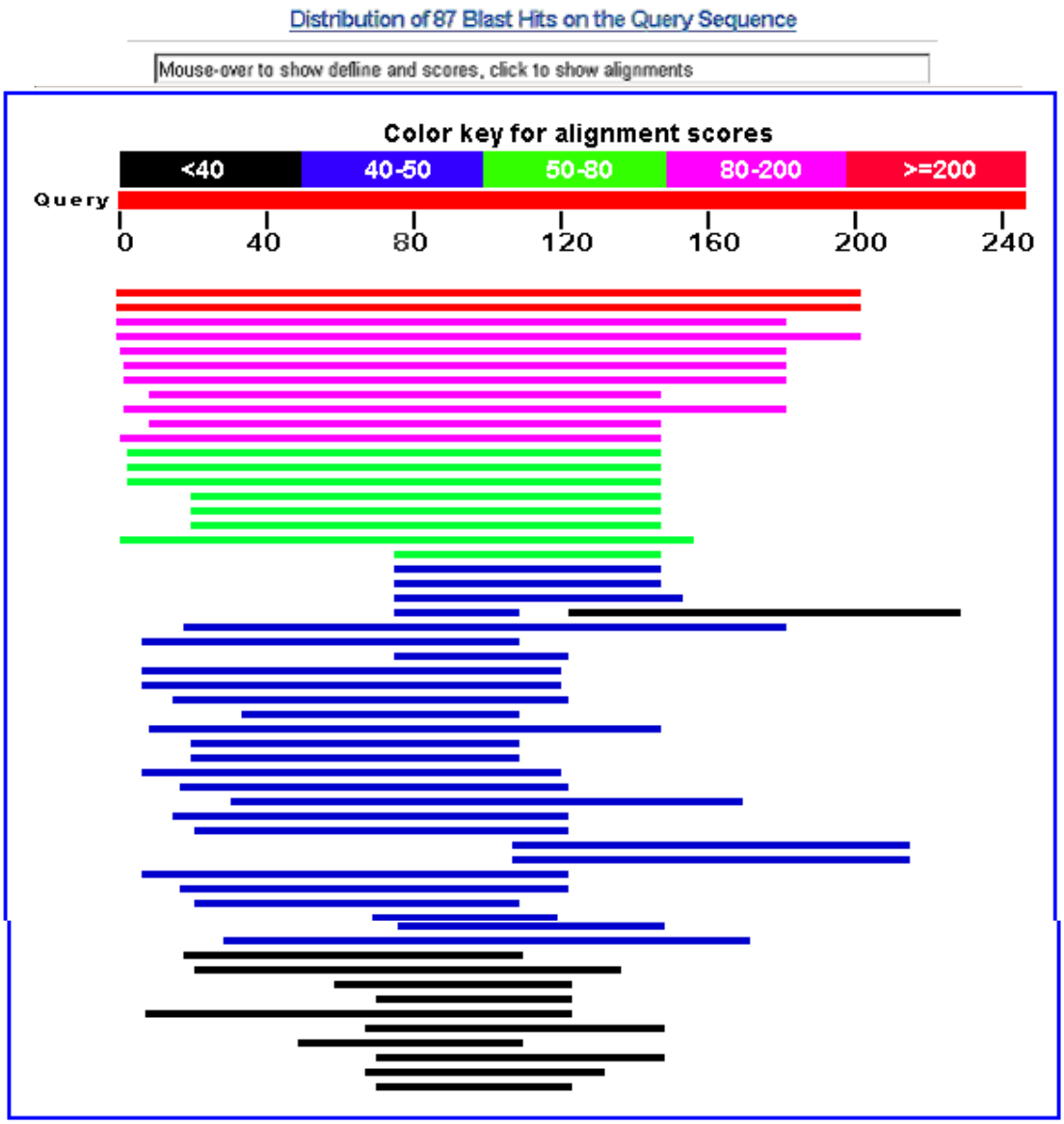
D. Blastn graphical output

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF426721.2	Hypocrea virens endoglucanase (bgn13.1) gene, complete sequence	832	832	76%	0.0	93%
EF176582.1	Trichoderma viride beta-1,3-glucanase (glu) gene, complete cds	814	814	76%	0.0	92%
EF426722.2	Hypocrea virens endoglucanase (bgn13.1) mRNA, complete cds	466	758	69%	6e-128	96%
AF395756.1	Hypocrea virens beta-1,3-glucanase precursor (bgn2) gene, complete	452	452	67%	1e-123	80%
X84085.1	T.harzianum mRNA for endo-1,3(4)-beta-glucanase	448	749	69%	2e-122	97%
XM_657364.1	Aspergillus nidulans FGSC A4 chromosome III	51.8	51.8	11%	0.004	73%
BX284752.1	Neurospora crassa DNA linkage group II BAC contig B23B10	48.2	48.2	11%	0.052	72%
XM_001761597.1	Physcomitrella patens subsp. patens predicted protein (PHYPADRAFT	44.6	44.6	6%	0.63	83%
CU633872.1	Podospora anserina genomic DNA	42.8	42.8	3%	2.2	92%
AC198144.2	Nomascus leucogenys BAC clone CH271-99L21 from chromosome unl	42.8	42.8	4%	2.2	90%
NM_001048558.1	Oryza sativa (japonica cultivar-group) Os01g0149200 (Os01g014920(	42.8	42.8	3%	2.2	92%
AP008207.1	Oryza sativa (japonica cultivar-group) genomic DNA, chromosome 1	42.8	42.8	3%	2.2	92%
AL157783.15	Human DNA sequence from clone RP11-134A8 on chromosome 10 Co	42.8	42.8	4%	2.2	86%
AP002540.2	Oryza sativa Japonica Group genomic DNA, chromosome 1, PAC clon	42.8	42.8	3%	2.2	92%
XM_951998.1	Neurospora crassa OR74A chromosome VI	42.8	42.8	11%	2.2	71%
AK069318.1	Oryza sativa Japonica Group cDNA clone:J023014M14, full insert sequ	42.8	42.8	3%	2.2	92%
XM_001866486.1	Culex pipiens quinquefasciatus conserved hypothetical protein, mRNA	41.0	41.0	4%	7.7	90%
AC198108.4	MACACA MULATTA BAC clone CH250-293L3 from chromosome 2, con	41.0	41.0	4%	7.7	88%
AC124250.11	Mus musculus chromosome 12, clone RP24-286P13, complete sequen	41.0	41.0	3%	7.7	92%
AC083799.17	Homo sapiens 3 BAC RP11-332M2 (Roswell Park Cancer Institute Hum	41.0	41.0	3%	7.7	92%
BX001032.12	Zebrafish DNA sequence from clone CH211-14K18 in linkage group 1	41.0	41.0	3%	7.7	93%
CP000155.1	Hahella chejuensis KCTC 2396, complete genome	41.0	41.0	5%	7.7	80%

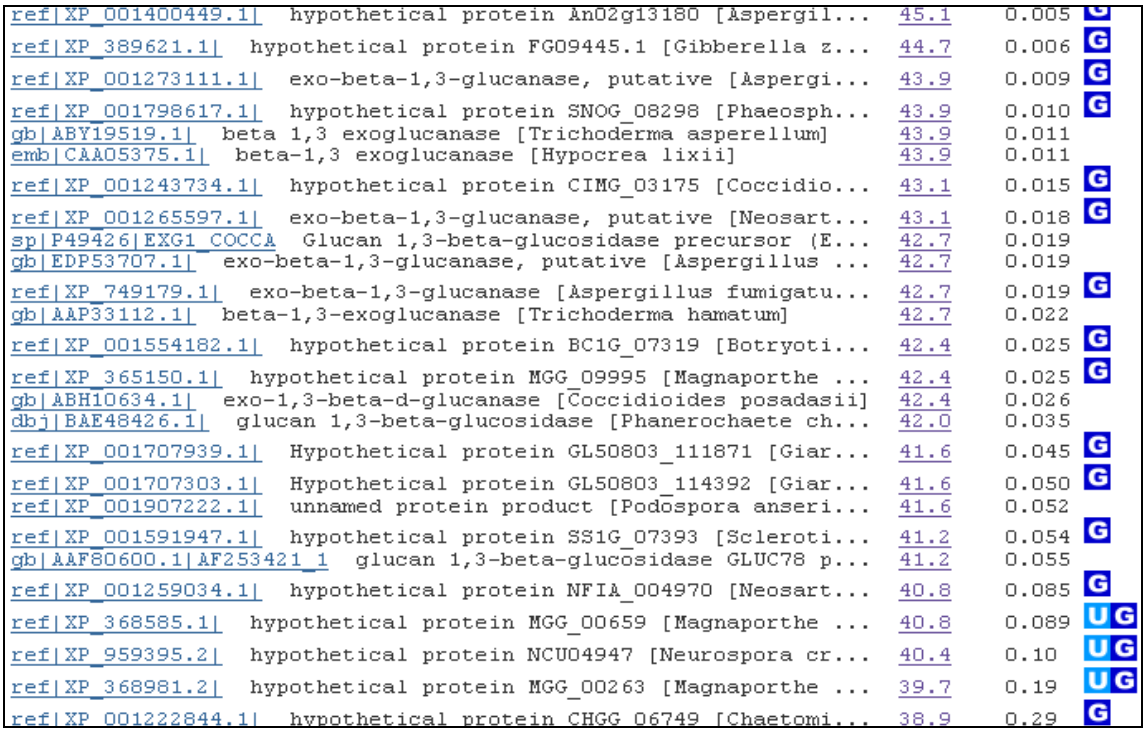
E. Blastn text output

Plate10. Sequence analysis for the clone GT-311 contd…



F. Blastp graphical output

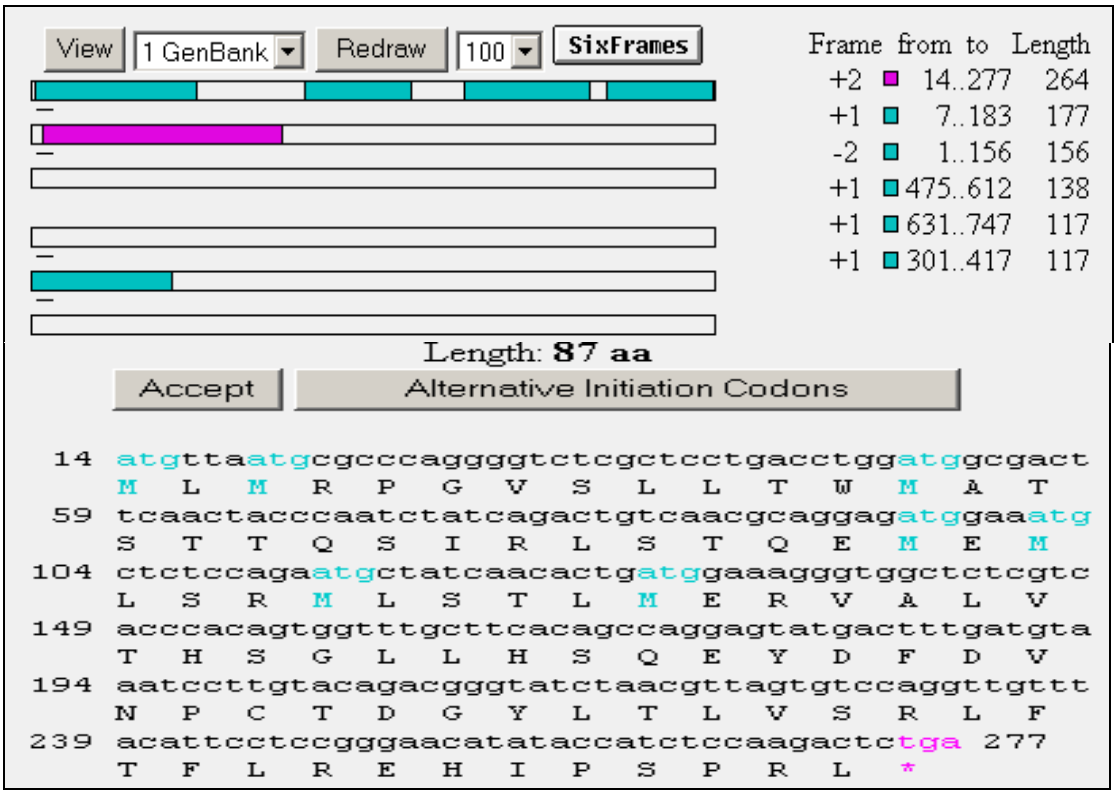
Plate10. Sequence analysis for the clone GT-311 contd…



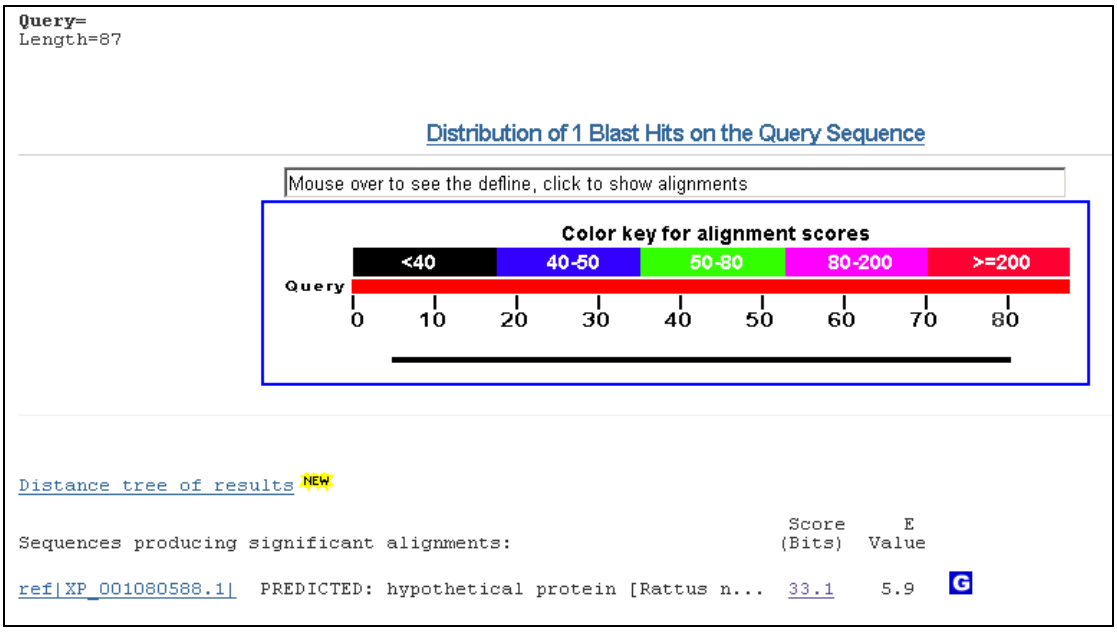
ref|XP_001400449.1| hypothetical protein An02g13180 [Aspergil...	45.1	0.005	G
ref|XP_389621.1| hypothetical protein FG09445.1 [Gibberella z...	44.7	0.006	G
ref|XP_001273111.1| exo-beta-1,3-glucanase, putative [Aspergi...	43.9	0.009	G
ref|XP_001798617.1| hypothetical protein SNOG_08298 [Phaeosph...	43.9	0.010	G
gb|ABY19519.1| beta 1,3 exoglucanase [Trichoderma asperellum]	43.9	0.011	
emb|CAA05375.1| beta-1,3 exoglucanase [Hypocrea lixii]	43.9	0.011	
ref|XP_001243734.1| hypothetical protein CIMG_03175 [Coccidio...	43.1	0.015	G
ref|XP_001265597.1| exo-beta-1,3-glucanase, putative [Neosart...	43.1	0.018	G
sp|P49426|EXG1_COCCA Glucan 1,3-beta-glucosidase precursor (E...	42.7	0.019	
gb|EDP53707.1| exo-beta-1,3-glucanase, putative [Aspergillus ...	42.7	0.019	
ref|XP_749179.1| exo-beta-1,3-glucanase [Aspergillus fumigatu...	42.7	0.019	G
gb|AAP33112.1| beta-1,3-exoglucanase [Trichoderma hamatum]	42.7	0.022	
ref|XP_001554182.1| hypothetical protein BC1G_07319 [Botryoti...	42.4	0.025	G
ref|XP_365150.1| hypothetical protein MGG_09995 [Magnaporthe ...	42.4	0.025	G
gb|ABH10634.1| exo-1,3-beta-d-glucanase [Coccidioides posadasii]	42.4	0.026	
dbj|BAE48426.1| glucan 1,3-beta-glucosidase [Phanerochaete ch...	42.0	0.035	
ref|XP_001707939.1| Hypothetical protein GL50803_111871 [Giar...	41.6	0.045	G
ref|XP_001707303.1| Hypothetical protein GL50803_114392 [Giar...	41.6	0.050	G
ref|XP_001907222.1| unnamed protein product [Podospora anseri...	41.6	0.052	
ref|XP_001591947.1| hypothetical protein SS1G_07393 [Scleroti...	41.2	0.054	G
gb|AAF80600.1|AF253421_1 glucan 1,3-beta-glucosidase GLUC78 p...	41.2	0.055	
ref|XP_001259034.1| hypothetical protein NFIA_004970 [Neosart...	40.8	0.085	G
ref|XP_368585.1| hypothetical protein MGG_00659 [Magnaporthe ...	40.8	0.089	UG
ref|XP_959395.2| hypothetical protein NCU04947 [Neurospora cr...	40.4	0.10	UG
ref|XP_368981.2| hypothetical protein MGG_00263 [Magnaporthe ...	39.7	0.19	UG
ref|XP_001222844.1| hypothetical protein CHGG_06749 [Chaetomi...	38.9	0.29	G

G. Blastp text output

Plate10. Sequence analysis for the clone GT-311 contd…

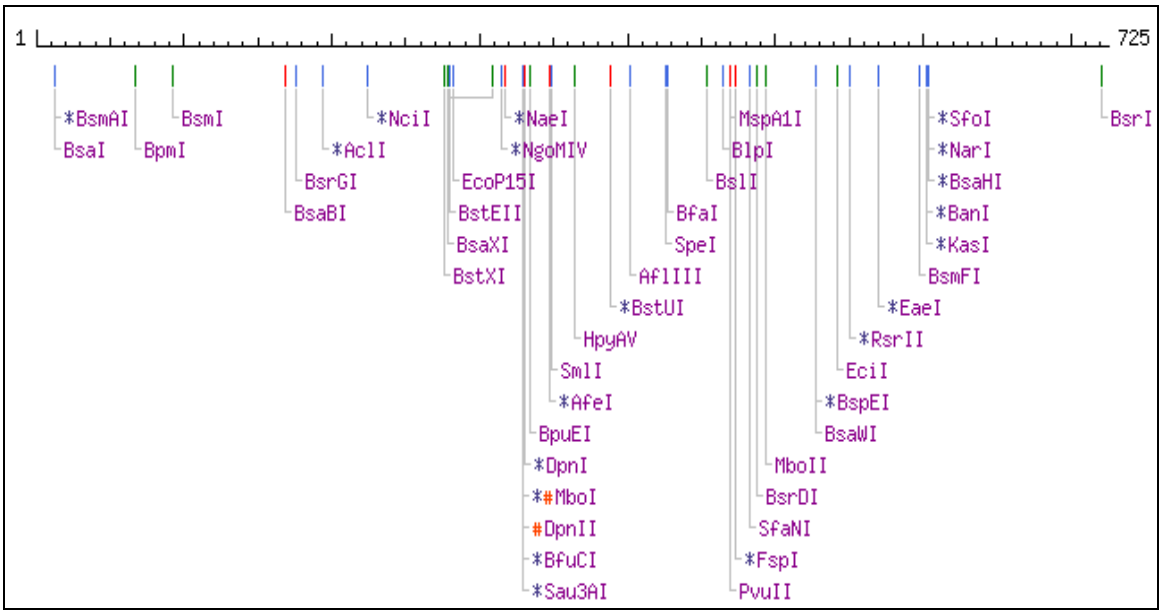


H. Open reading frame



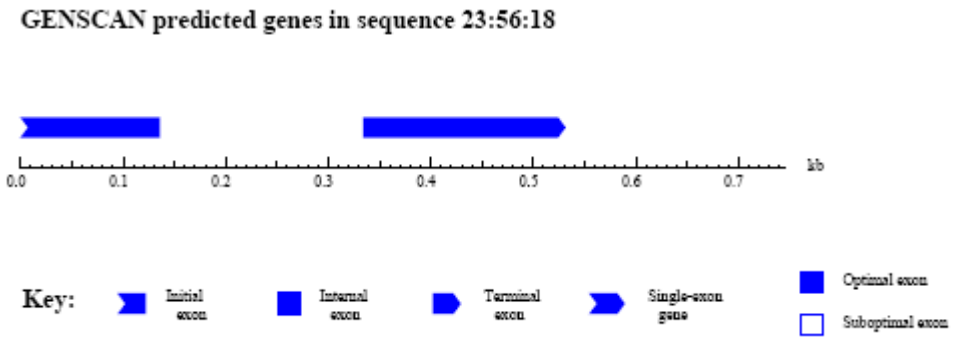
I. Blast result of Open Reading Frame

Plate10. Sequence analysis for the clone GT-311 contd…



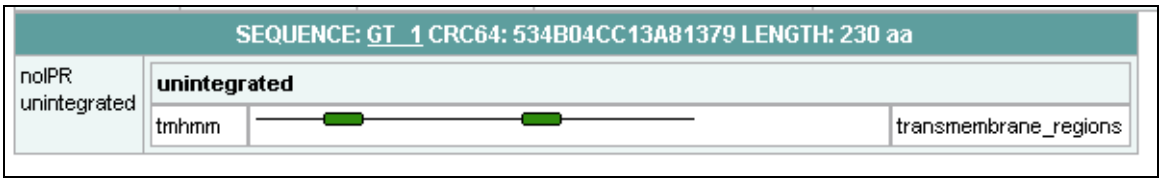
J. Restriction map

```
Predicted genes/exons:

Gn.Ex Type S .Begin ...End .Len Fr Ph I/Ac Do/T CodRg P.... Tscr..
----- ---- - ------ ------ ---- -- -- ---- ---- ----- ----- ------

 1.01 Init +      7    136  130  0  1    84    1   109 0.956   7.11
 1.02 Term +    334    524  191  2  2    33   37   143 0.247   6.31
 1.03 PlyA +    720    725    6                               -0.45
```

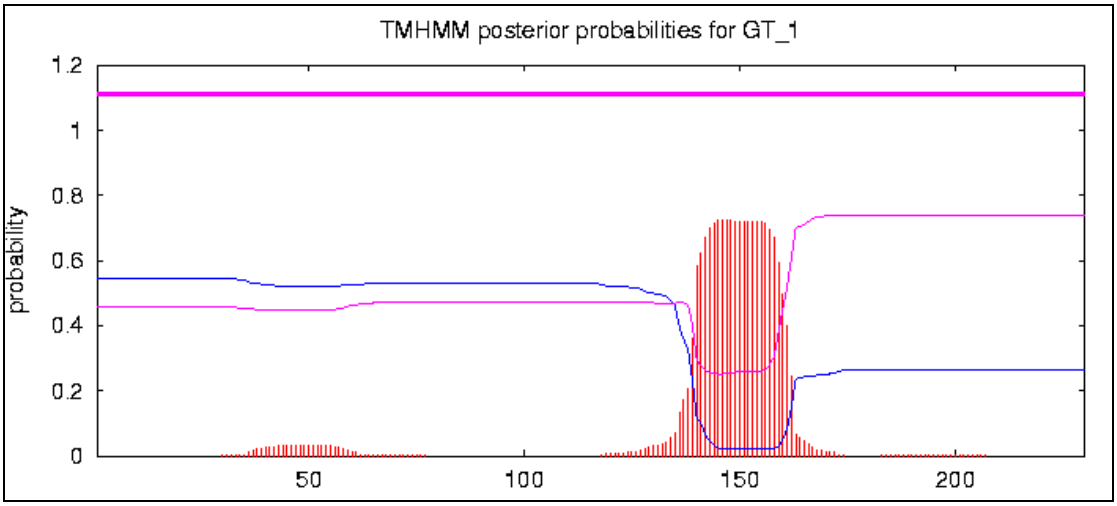


K. Genscan output

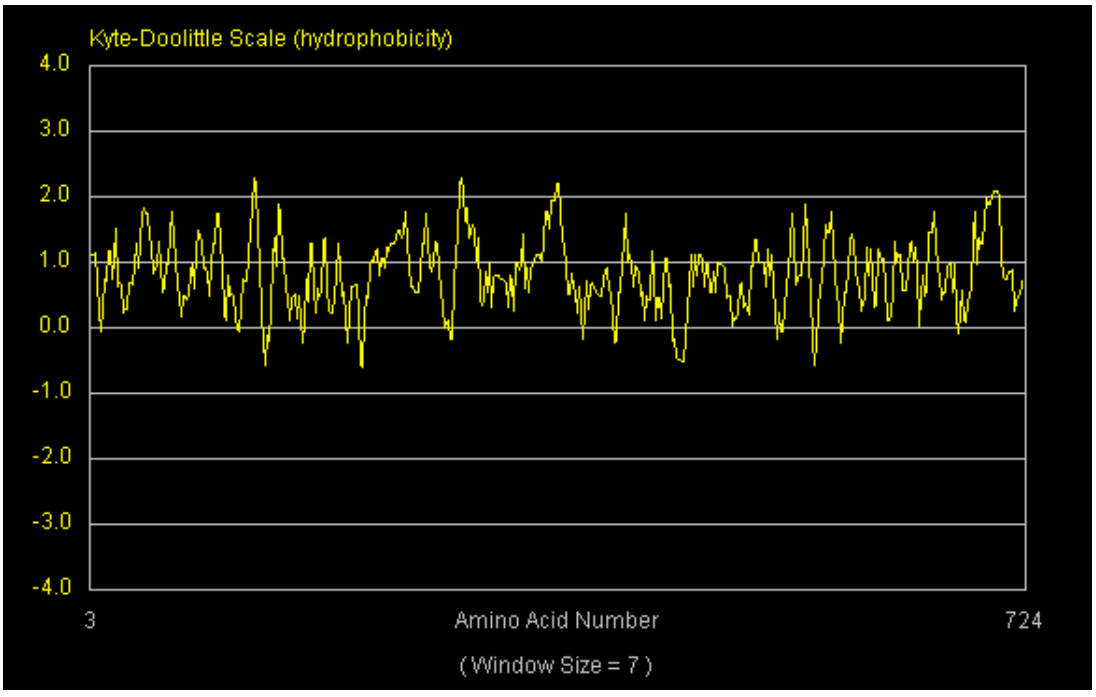
Plate10. Sequence analysis for the clone GT-311 contd…



L. InterProScan output



M. Transmembrane region

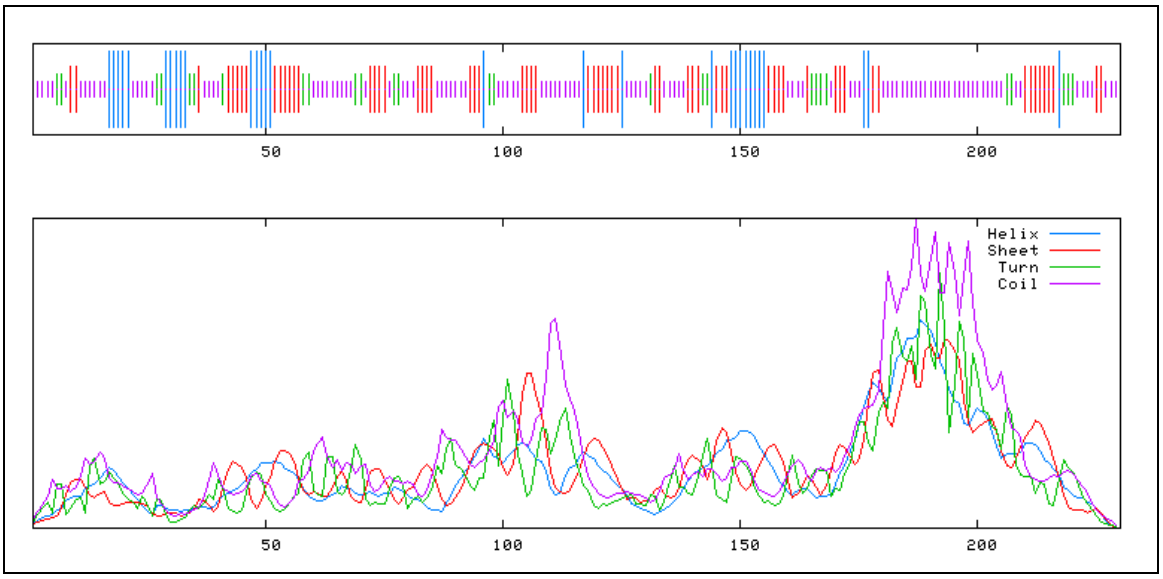


N. Kyte Doolittle Hydropathy Plot for deduced proteins

Plate10. Sequence analysis for the clone GT-311 contd…

```
        10         20         30         40         50         60         70
         |          |          |          |          |          |          |
AQGSRSPGWRLQLPNLSDCQRRRWKCSPECYQHWKGWLSSPTVVCFTARSMTLMILVQTGIRCPGCLHSS
ccccccttceeccccccchhhhhccccctthhhhhttecccceeeeehhhhheeeeeettcccccccccc
GNIYHLQDSEIQHYHFNGPNQSSHYAAAGFSGDQTLISAQDPPPTRRRAVFRVAIRTCIGHYGYTSGNSF
tceeeecttcccceeeecccccccceeehttcccccceeeeccccccccheeeeeeehccccteeccccce
TAYGGVLLSCASAMYALLFFSGETAYGSGWSVQTRSAKLVDRPTNLTNPMAAPAVPLGSPASNISRPKWN
eetttheeehhhhhhhheeeeccccettttceeecchheecccccccccccccccccccccccccttcc
LQVLGIYANSKKTQKCKLGP
eeeeeeehtttccccceeccc

Sequence length :   230

SOPMA :
   Alpha helix      (Hh) :    30 is  13.04%
   3(10) helix      (Gg) :     0 is   0.00%
   Pi helix         (Ii) :     0 is   0.00%
   Beta bridge      (Bb) :     0 is   0.00%
   Extended strand  (Ee) :    63 is  27.39%
   Beta turn        (Tt) :    27 is  11.74%
   Bend region      (Ss) :     0 is   0.00%
   Random coil      (Cc) :   110 is  47.83%
   Ambigous states  (?)  :     0 is   0.00%
   Other states          :     0 is   0.00%
```

O. Predicted secondary structure

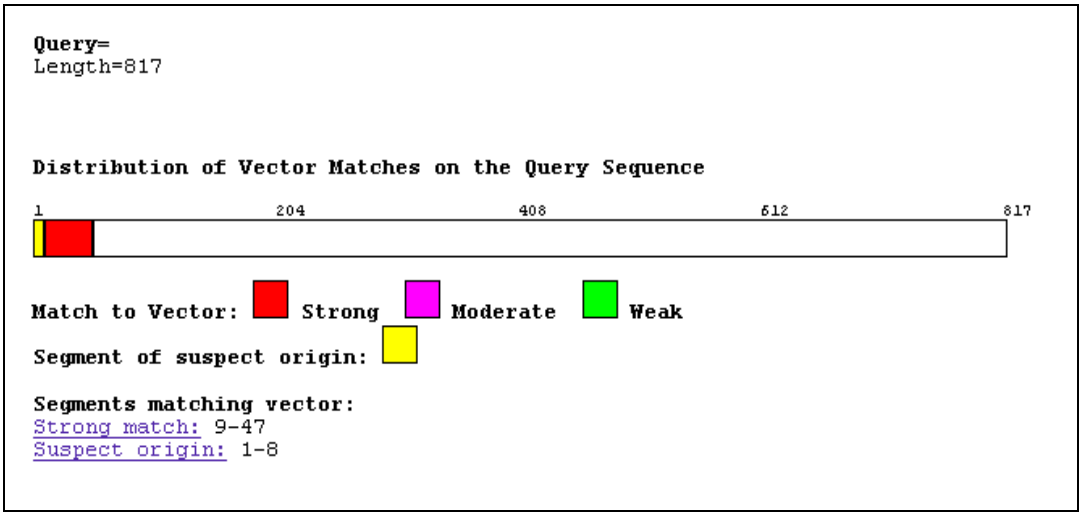


P. Graphical representation of secondary structure

Plate10. Sequence analysis for the clone GT-311 contd…

>GT-322
CGATTGGGCCGACGTCGCATGCTCCCGGCCGCCATGGCCGCGGGATTTTGCCCTGATTGACGCAAGTC
AATTAACTCTGGTGTTACCTTTACAACTAATCAATATCCTTCATTTATGATCGAGAATTTGACTAAAG
ATAATGGCACACCTGTCGTTGTTGTCCGAGGCTCAACTTTGGTTGGCGCTTCTAGCCATGTCAACACC
TACTCTTACGGCAACACCGTGGGCAGAAACCCTACTTACGGCGATGTTACGTCTAGTAACACGAGACC
TAGTGCTCTTGCTCCTGGTGGTCGTTACCCTTATGTGGCTCCCCCTACTTATGGAGATTTGCCCATCT
CGAGCTTCCTCAACGTCAAGGATCCAGCGCAGAATGGAAACCGTCAGGTTCTTGGAGATAACACGATT
GATGAGTCCGGGACGCTTAATGCTATCCTGGAACTTGCAGCAAGCCAGAATAAGTTGCTTATTTTCCT
TTTGGCAAGTACCGGGTGGATTCTACTCTTTTTATCCCTAAGGTTCCCGTATCGTGGGTGAGGCTTGG
GCATCATCACGGCAACGGCACTTTTCAGACGAAAACAGCCACAGCCGTGTCTCAGTGGCGTGCAGGCG
ATGTGGAATGCACAGATCAGATGTAGATCACGGTACGATGTGCTCGGGCATTGCTCGGTCAACATGGC
TGGCATATACATATGCGGCCGCTGCAGGTCAACATATGGGAAGTCCACGCGTGGATGCTACTGGAAAT
TCTAAGTACTAATACTTGGCAACAGCAACGTTTCTGTGATGTTTCCCCCATCCCACACAGCGGAATAG
A

A. Nucleotide sequence

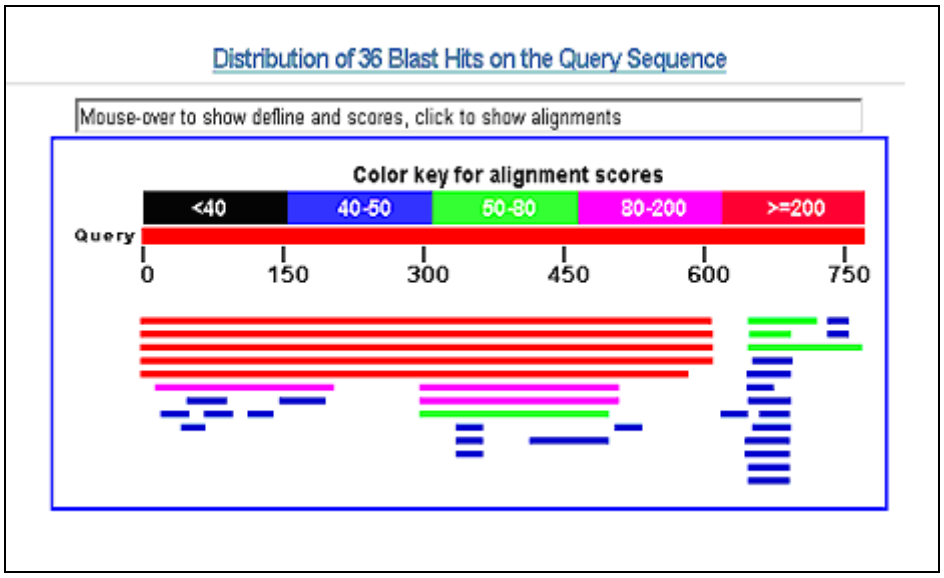


B. VecScreen Output

>GT_1 322
N*LWCYLYN*SISFIYDREFD*R*WHTCRCCPRLNFGWRF*PCQHLLLRQHRGQKPYLRR
CYV**HET*CSCSWWSLPLCGSPYLWRFAHLELPQRQGSSAEWKPSGSWR*HD**VRDA*
CYPGTCSKPE*VAYFPFGKYRVDSTLFIPKVPVSWVRLGHHHGNGTFQTKTATAVSQWRA
GDVECTDQM*ITVRCARALLGQHGWHIHMRPLQVNIWEVHAWMLLEILSTNTWQQQRFCD
VSPIPHSGIX

C. Deduced amino acid sequence

Plate 11. Sequence analysis for the clone GT-322



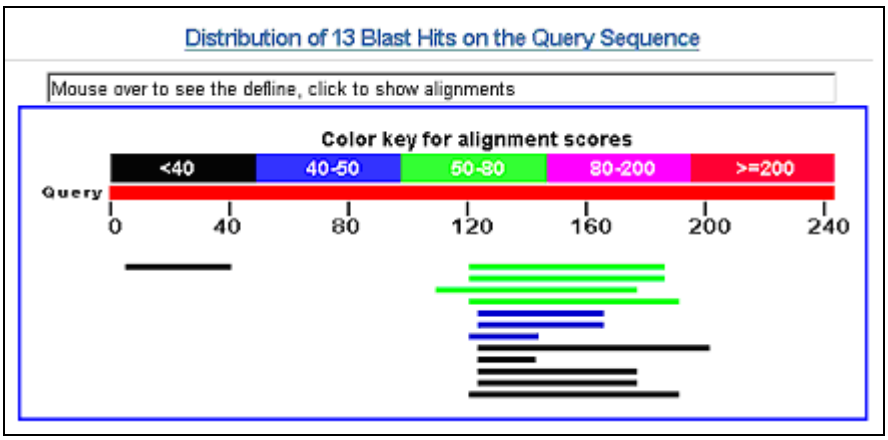
D. Blastn graphical output

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF176582.1	Trichoderma viride beta-1,3-glucanase (glu) gene, complete cds	883	883	79%	0.0	92%
X84085.1	T.harzianum mRNA for endo-1,3(4)-beta-glucanase	818	818	79%	0.0	90%
EF426722.2	Hypocrea virens endoglucanase (bgn13.1) mRNA, complete cds	803	803	79%	0.0	90%
EF426721.2	Hypocrea virens endoglucanase (bgn13.1) gene, complete sequence	803	803	79%	0.0	90%
AF395756.1	Hypocrea virens beta-1,3-glucanase precursor (bgn2) gene, complete	527	527	75%	2e-146	81%
BX284752.1	Neurospora crassa DNA linkage group II BAC contig B23B10	98.7	98.7	27%	3e-17	71%
XM_951998.1	Neurospora crassa OR74A chromosome VI	98.7	98.7	27%	3e-17	71%
AF395755.1	Hypocrea virens beta-1,3-glucanase precursor (bgn1) gene, complete	89.7	89.7	24%	2e-14	70%
AJ543749.1	Phanerochaete chrysosporium partial mRNA for putative polyketide s\	69.8	69.8	9%	2e-08	82%
DQ450434.1	Uncultured denitrifying bacterium clone S35 nitrite reductase-like (nir\	59.0	59.0	5%	3e-05	90%
XM_001219444.1	Chaetomium globosum CBS 148.51 hypothetical protein (CHGG_0022	55.4	55.4	26%	4e-04	66%
AY769624.1	Labeo rohita isolate B-9 transposon Tc1, complete sequence	51.8	51.8	15%	0.004	71%
DQ450428.1	Uncultured denitrifying bacterium clone S21 nitrite reductase-like (nir\	50.0	50.0	5%	0.015	90%
AJ879680.1	uncultured ectomycorrhiza (Tuber) partial ITS1, 5.8S rRNA gene and	50.0	50.0	6%	0.015	85%
AJ132700.1	Caenorhabditis elegans mRNA for centaurin gamma 1A	48.2	48.2	3%	0.054	96%
XM_001750843.1	Monosiga brevicollis MX1 predicted protein MONBRDRAFT_30400 mRN	46.4	46.4	3%	0.19	96%
AF072883.1	Siluania monomastiga 18S small subunit ribosomal RNA gene, partial	46.4	46.4	5%	0.19	83%
NW_001594046.1	Aspergillus niger CBS 513.88 contig An01c0470, complete genome >\	44.6	44.6	3%	0.65	93%
DQ450438.1	Uncultured denitrifying bacterium clone S24 nitrite reductase-like (nir\	44.6	44.6	5%	0.65	85%
AJ277968.1	Drosophila melanogaster TRPgamma gene for TRPgamma cation char	44.6	44.6	6%	0.65	84%
AJ277967.1	Drosophila melanogaster TRPgamma gene for TRPgamma cation char	44.6	44.6	6%	0.65	84%
XM_594793.3	PREDICTED: Bos taurus similar to olfactory receptor Olr223 (LOC516\	42.8	42.8	2%	2.3	100%
XM_542465.2	PREDICTED: Canis familiaris similar to olfactory receptor Olr223 (LOC	42.8	42.8	2%	2.3	100%
AJ420890.1	Entandrophragma cylindricum microsatellite DNA, clone pEcCIR156	42.8	42.8	5%	2.3	86%
AJ420887.1	Entandrophragma cylindricum microsatellite DNA, clone pEcCIR84	42.8	42.8	5%	2.3	86%
CR792454.8	Zebrafish DNA sequence from clone CH211-9F20 in linkage group 4 C	42.8	42.8	5%	2.3	85%
AB179717.1	Trichoderma viride lamAI mRNA for laminarinase, complete cds	42.8	42.8	10%	2.3	71%
AE004969.1	Neisseria gonorrhoeae FA 1090, complete genome	42.8	42.8	6%	2.3	80%
XM_001358731.1	Drosophila pseudoobscura GA20950-PA (Dpse\GA20950) mRNA, parti	41.0	41.0	4%	8.0	87%
CP000312.1	Clostridium perfringens SM101, complete genome	41.0	41.0	4%	8.0	90%
CP000356.1	Sphingopyxis alaskensis RB2256, complete genome	41.0	41.0	3%	8.0	92%
AC097354.12	Mus musculus strain 129/Sv clone ct7-297f7 map X, complete sequen	41.0	41.0	3%	8.0	90%
XM_741429.1	Aspergillus fumigatus Af293 exo-beta-1,3-glucanase (AFUA_4G03350)	41.0	41.0	3%	8.0	90%
AE010299.1	Methanosarcina acetivorans str. C2A, complete genome	41.0	41.0	3%	8.0	92%
AC096621.9	Mus musculus clone ct7-257p24, complete sequence	41.0	41.0	3%	8.0	90%
AL672082.15	Mouse DNA sequence from clone RP23-67B21 on chromosome X Cont	41.0	41.0	3%	8.0	90%

E. Blastn text output

Plate 11. Sequence analysis for the clone GT-322 contd…

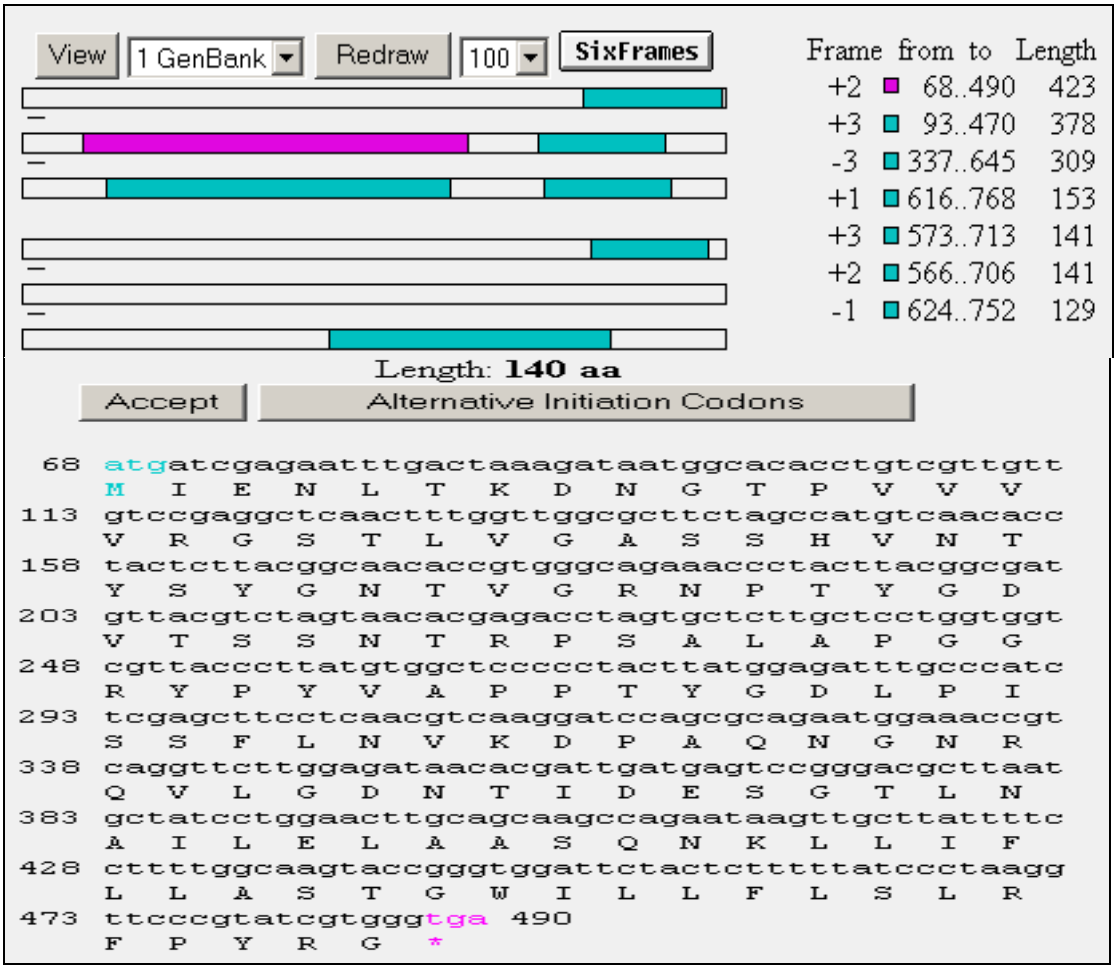


F. Blastp graphical output

Sequences producing significant alignments:	Score (Bits)	E Value	
gb|ABO93399.2| endoglucanase [Hypocrea virens]	55.5	3e-06	
gb|ABM55269.1| beta-1,3-glucanase [Trichoderma viride]	55.1	4e-06	
sp|P53626|E13B_TRIHA Glucan endo-1,3-beta-glucosidase BGN13.1...	54.7	5e-06	
gb|AAL84695.1|AF395756_1 beta-1,3-glucanase precursor [Hypocr...	53.1	2e-05	
emb|CAD70430.1| probable GLUCAN ENDO-1, 3-BETA-GLUCOSIDASE BG...	43.5	0.012	
ref|XP_957091.1| glucan endo-1,3-beta-glucosidase BGN13.1 pre...	43.5	0.013	G
ref|XP_001219445.1| hypothetical protein CHGG_00224 [Chaetomi...	41.2	0.055	G
ref|XP_001540220.1| predicted protein [Ajellomyces capsulatus...	38.9	0.30	G
ref|NP_869682.1| probable outer membrane protein [Rhodopirell...	38.1	0.48	G
ref|XP_391182.1| hypothetical protein FG11006.1 [Gibberella z...	37.4	0.98	G
gb|AAL84694.1|AF395755_1 beta-1,3-glucanase precursor [Hypocr...	35.8	2.4	
dbj|BAD67019.1| laminarinase [Trichoderma viride]	35.4	3.3	
ref|XP_001907222.1| unnamed protein product [Podospora anseri...	33.9	8.7	

G. Blastp text output

Plate 11. Sequence analysis for the clone GT-322 contd…

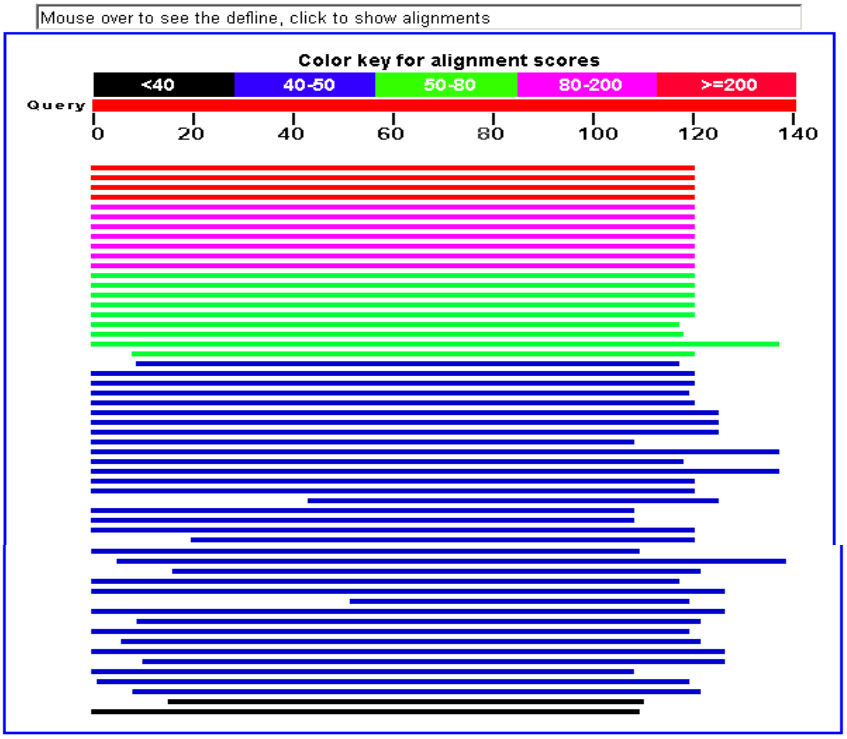


H. Open reading frame

Plate 11. Sequence analysis for the clone GT-322 contd…

Query=
Length=140

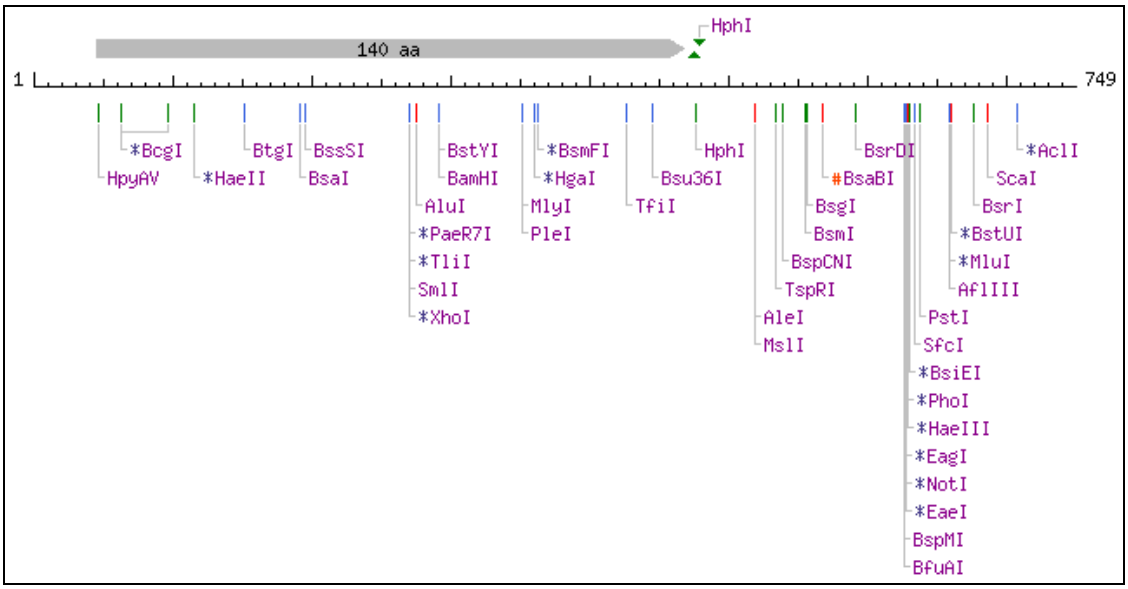
Distribution of 88 Blast Hits on the Query Sequence



Sequences producing significant alignments:	Score (Bits)	E Value	
gb|ABM55269.1| beta-1,3-glucanase [Trichoderma viride]	230	2e-59	
sp|P53626|E13B_TRIHA Glucan endo-1,3-beta-glucosidase BGN13.1...	229	3e-59	
gb|ABO93399.2| endoglucanase [Hypocrea virens]	228	1e-58	
gb|AAL84695.1|AF395756_1 beta-1,3-glucanase precursor [Hypocr...	227	2e-58	
gb|AAL84694.1|AF395755_1 beta-1,3-glucanase precursor [Hypocr...	147	2e-34	
ref|XP_001219445.1| hypothetical protein CHGG_00224 [Chaetomi...	138	1e-31	G
gb|ABR23667.1| endo-beta 1,3-glucanase [Hypocrea virens] >gb|...	130	2e-29	
dbj|BAD67019.1| laminarinase [Trichoderma viride]	130	2e-29	
ref|XP_391182.1| hypothetical protein FG11006.1 [Gibberella z...	123	3e-27	G
emb|CAD70430.1| probable GLUCAN ENDO-1, 3-BETA-GLUCOSIDASE BG...	109	6e-23	
ref|XP_957091.1| glucan endo-1,3-beta-glucosidase BGN13.1 pre...	109	6e-23	G
gb|EDP47369.1| exo-beta-1,3-glucanase, putative [Aspergillus ...	69.3	7e-11	
ref|XP_746522.1| exo-beta-1,3-glucanase [Aspergillus fumigatu...	69.3	7e-11	G
ref|XP_001262477.1| conserved hypothetical protein [Neosartor...	67.8	2e-10	G
ref|XP_001820924.1| [Aspergillus oryzae] >dbj|BAE58922.1| unn...	58.9	1e-07	G
sp|P49426|EXG1_COCCA Glucan 1,3-beta-glucosidase precursor (E...	56.2	7e-07	
ref|XP_001906371.1| unnamed protein product [Podospora anseri...	55.5	1e-06	
ref|XP_001226829.1| hypothetical protein CHGG_08902 [Chaetomi...	53.1	6e-06	G
ref|XP_001227389.1| hypothetical protein CHGG_09462 [Chaetomi...	51.2	2e-05	G
ref|XP_001589225.1| hypothetical protein SS1G_09858 [Scleroti...	50.1	5e-05	G
ref|XP_960189.1| hypothetical protein NCU09791 [Neurospora cr...	49.7	6e-05	G
gb|ABY19519.1| beta 1,3 exoglucanase [Trichoderma asperellum]	49.3	8e-05	
ref|XP_751203.1| exo-beta-1,3-glucanase [Aspergillus fumigatu...	49.3	8e-05	G
ref|XP_001905866.1| unnamed protein product [Podospora anseri...	48.5	1e-04	
emb|CAA05375.1| beta-1,3 exoglucanase [Hypocrea lixii]	48.5	1e-04	
gb|EDP52988.1| exo-beta-1,3-glucanase, putative [Aspergillus ...	47.8	2e-04	
ref|XP_001263693.1| exo-beta-1,3-glucanase, putative [Neosart...	47.8	2e-04	G

I. Blast result of Open Reading Frame

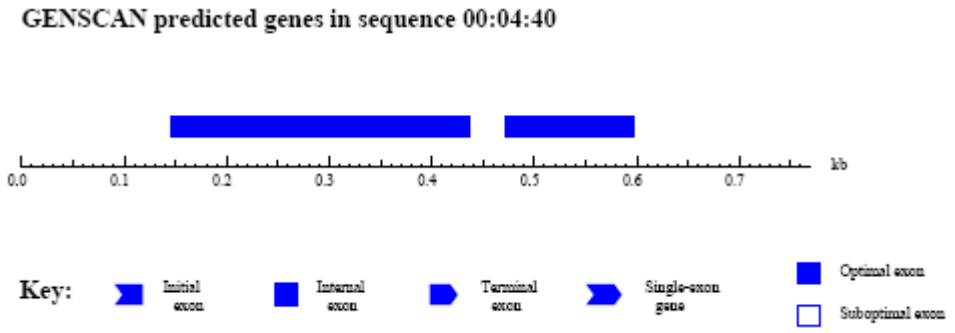
Plate 11. Sequence analysis for the clone GT-322 contd…



J. Restriction map

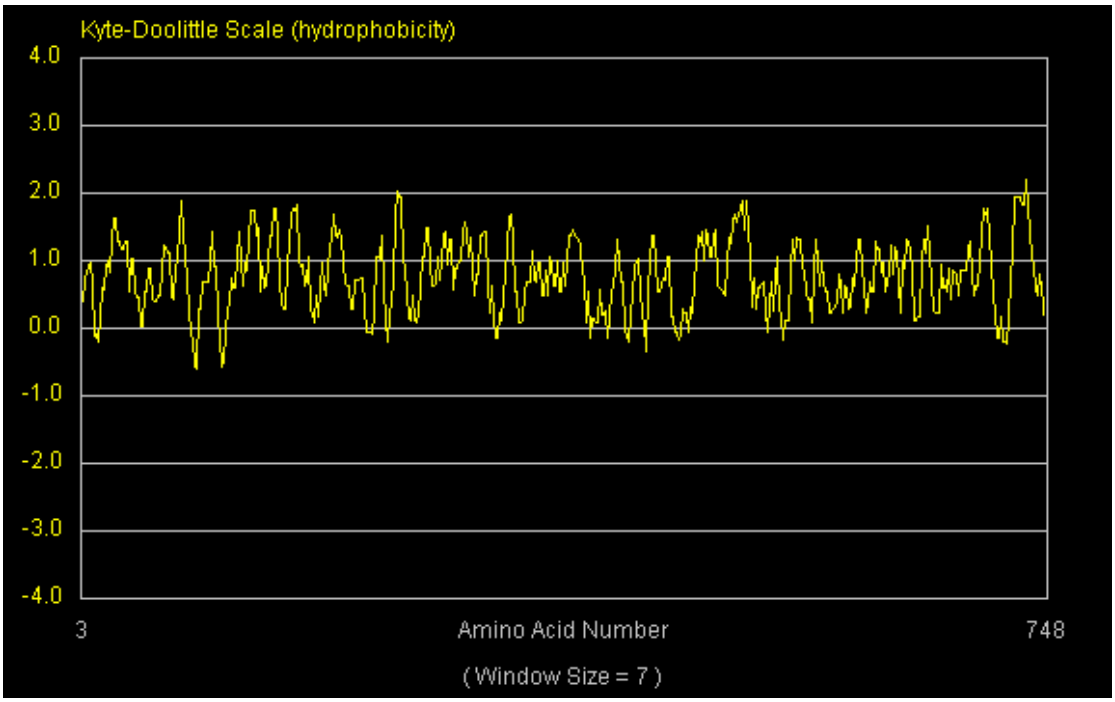
Predicted genes/exons:

Gn.Ex	Type	S	.Begin	...End	.Len	Fr	Ph	I/Ac	Do/T	CodRg	P....	Tscr..
1.01	Intr	+	145	437	293	1	2	51	25	351	0.637	26.88
1.02	Intr	+	472	597	126	2	0	56	80	70	0.650	8.65



K. Genscan output

Plate 11. Sequence analysis for the clone GT-322 contd…

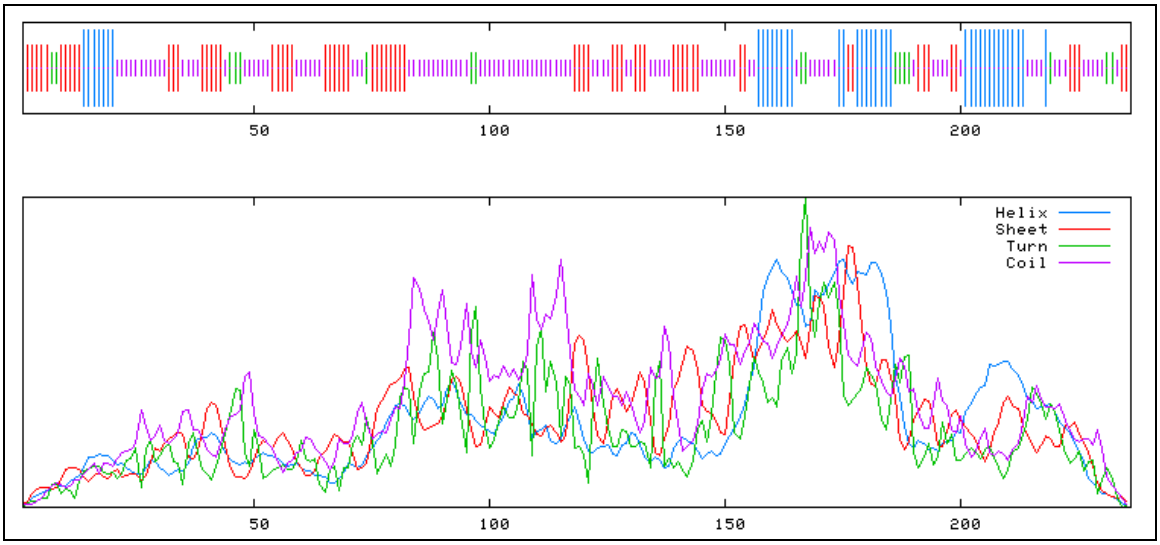


L. Kyte Doolittle Hydropathy Plot for deduced proteins

```
         10         20         30         40         50         60         70
          |          |          |          |          |          |          |
NLWCYLYNSISFIYDREFDRWHTCRCCPRLNFGWRFPCQHLLLRQHRGQKPYLRRCYVHETCSCSWWSLP
heeeeeetteeeeehhhhhhhccccccccccceeecccceeeeectttcccccceeeeecccccceeeee
LCGSPYLWRFAHLELPQRQGSSAEWKPSGSWRHDVRDACYPGTCSKPEVAYFPFGKYRVDSTLFIPKVPV
ecccteeeeeeeeccccccccccccttccccccccccccccccccceeeecccceeecceeecccccce
SWVRLGHHHGNGTFQTKTATAVSQWRAGDVECTDQMITVRCARALLGQHGWHIHMRPLQVNIWEVHAWML
eeeeecccccccceecchhhhhhhhcttcccccchheehhhhhhhhttttceeecccceechhhhhhhhh
LEILSTNTWQQQRFCDVSPIPHSGIX
hhhhcccchtcccceeeccccttcee

Sequence length :   236

SOPMA :
   Alpha helix      (Hh) :    40 is  16.95%
   3(10) helix      (Gg) :     0 is   0.00%
   Pi helix         (Ii) :     0 is   0.00%
   Beta bridge      (Bb) :     0 is   0.00%
   Extended strand  (Ee) :    68 is  28.81%
   Beta turn        (Tt) :    17 is   7.20%
   Bend region      (Ss) :     0 is   0.00%
   Random coil      (Cc) :   111 is  47.03%
   Ambigous states  (?)  :     0 is   0.00%
   Other states          :     0 is   0.00%
```

M. Predicted secondary structure

Plate 11. Sequence analysis for the clone GT-322 contd…

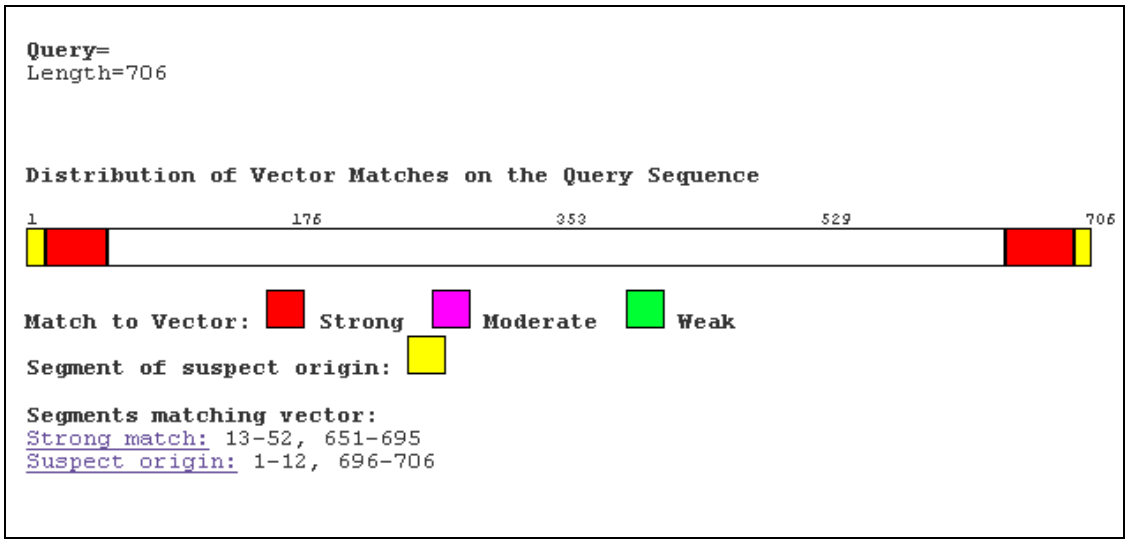


N. Graphical representation of secondary structure

Plate 11. Sequence analysis for the clone GT-322 contd…

>BPT-822
GCAACGATTGGGCCGACGTCGCATGCTCCCGGCCGTCCATGGCCGCGGGATTTTGCCCTGATTGTATC
CGCCAAGTCAATTAACTCTGGTGTTACCTTTACAACTAATCAATATCCTTCATTTATGATCGAGAATT
TGACTAAAGATAATGGCACACCTGTCGTTGTTGTCCGAGGCTCAACTTTGGTTGGCGCTTCTAGCCAT
GTCAACACCTACTCTTACGGCAACACCGTGGGCAGAAACCCTACTTACGGCGATGTTACGTCTAGTAA
CACGAGACCTAGTGCTCTTGCTCCTGGTGGTCGTTACCCTTATGTGGCTCCCCCTACTTATGGAGATT
TGCCCATCTCGAGCTTCCTCAATGTCAAGGATCCAGCGCAGAATGGAAACCGTCAGGTTCTTGGAGAT
AACACGATTGATGCAAGTACCGGGTGGATTCGACTCTTTTTATCCCTAAGGTTCCCGTATCGTGGTGA
GCTTGGCCACCATCACCGGCAACGGCAACTTTTTCAAGAACGAAAACAGCCACAGCCCGTTGTCTCAG
TTGCCGTGCAGCGATGTTGAATTGCACAGATTCAGATGTAAAATCCCGTTACGATGTGCTTCCCGTGC
ATTTGCTTCAGTTCACCTGCTGCAAAATCCATATGCGGCGCCTGCAGGTCGACCATTGGGAAAGCTCC
CACGCGTTGGATGCAAACCTGGAAAC

A. Nucleotide sequence

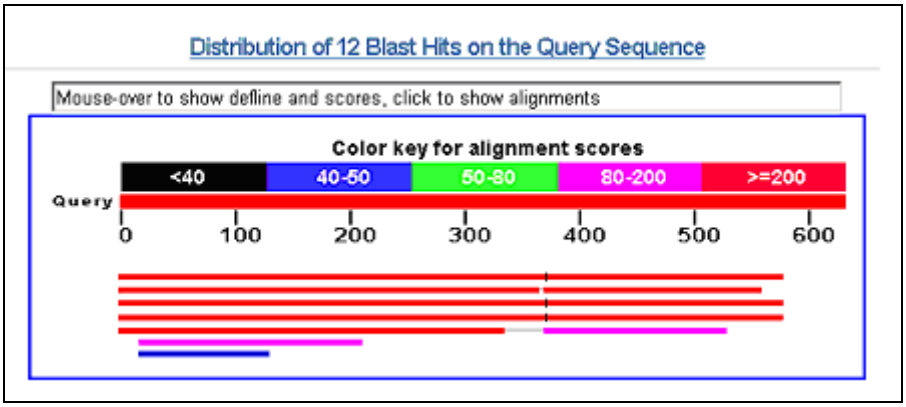


B. VecScreen Output

>BPT_1 822
YPPSQLTLVLPLQLINILHL*SRI*LKIMAHLSLLSEAQLWLALLAMSTPTLTATPWAET
LLTAMLRLVTRDLVLLLLVVVTLMWLPLLMEICPSRASSMSRIQRRMETVRFLEITRLMQ
VPGGFDSFYP*GSRIVVSLATITGNGNFFKNENSHSPLSQLPCSDVELHRFRCKIPLRCA
SRAFASVHLLQNPYAAPAGRPLGKLX

C. Deduced amino acid sequence

Plate 12. Sequence analysis for the clone BPT-822

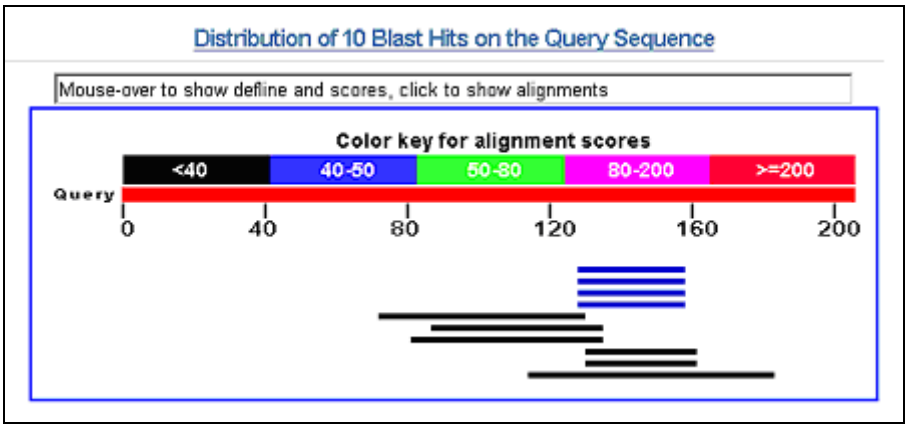


D. Blastn graphical output

Sequences producing significant alignments:
(Click headers to sort columns)

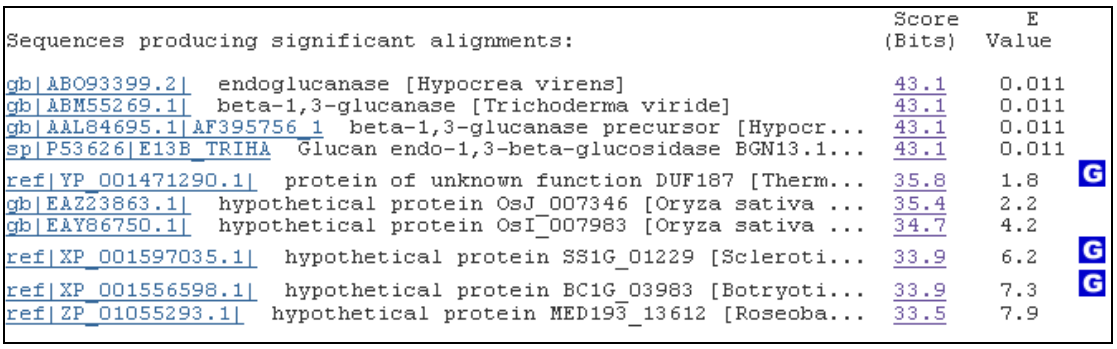
Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF176582.1	Trichoderma viride beta-1,3-glucanase (glu) gene, complete cds	601	853	91%	9e-169	96%
X84085.1	T.harzianum mRNA for endo-1,3(4)-beta-glucanase	560	781	88%	3e-156	94%
EF426722.2	Hypocrea virens endoglucanase (bgn13.1) mRNA, complete cds	542	785	91%	8e-151	92%
EF426721.2	Hypocrea virens endoglucanase (bgn13.1) gene, complete sequence	542	785	91%	8e-151	92%
AF395756.1	Hypocrea virens beta-1,3-glucanase precursor (bgn2) gene, complete	352	498	78%	8e-94	84%
AF395755.1	Hypocrea virens beta-1,3-glucanase precursor (bgn1) gene, complete	93.3	93.3	30%	1e-15	70%
AB179717.1	Trichoderma viride lamAI mRNA for laminarinase, complete cds	42.8	42.8	17%	1.8	68%

E. Blastn text output



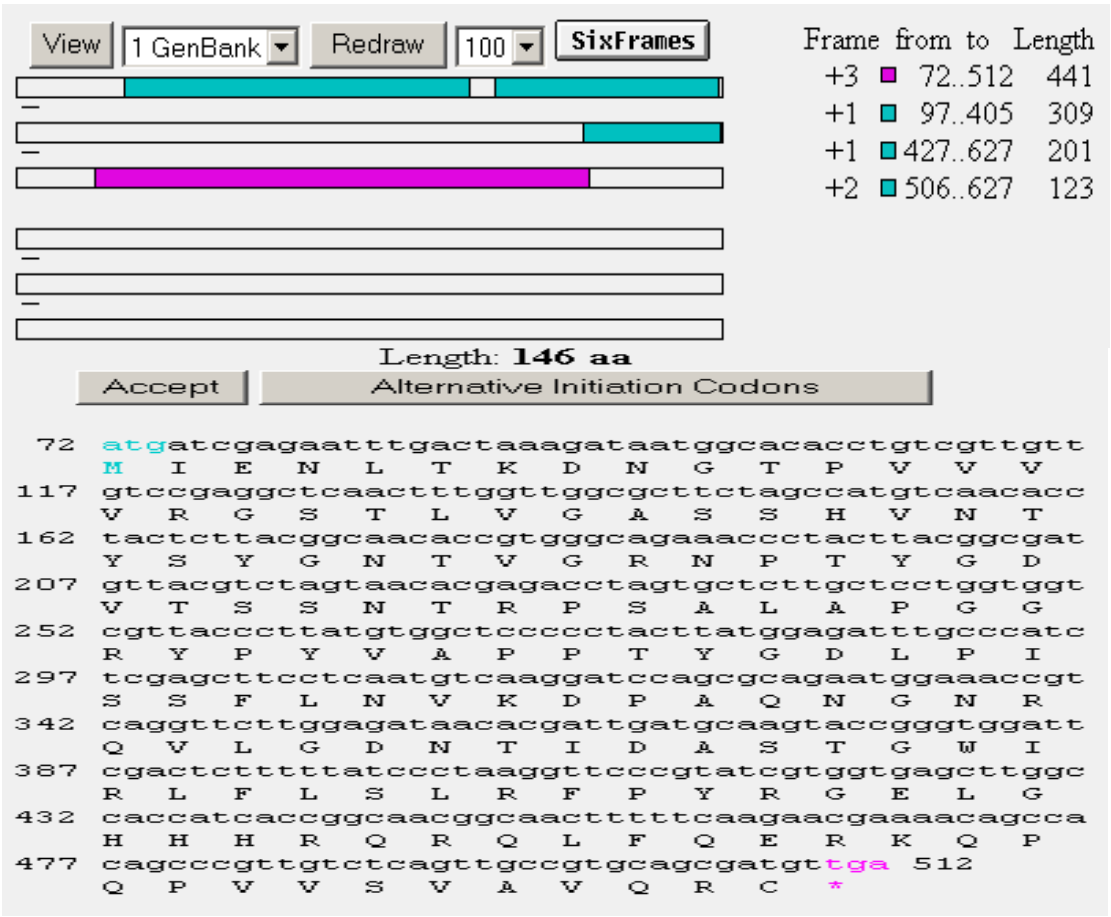
F. Blastp graphical output

Plate 12. Sequence analysis for the clone BPT-822 contd…



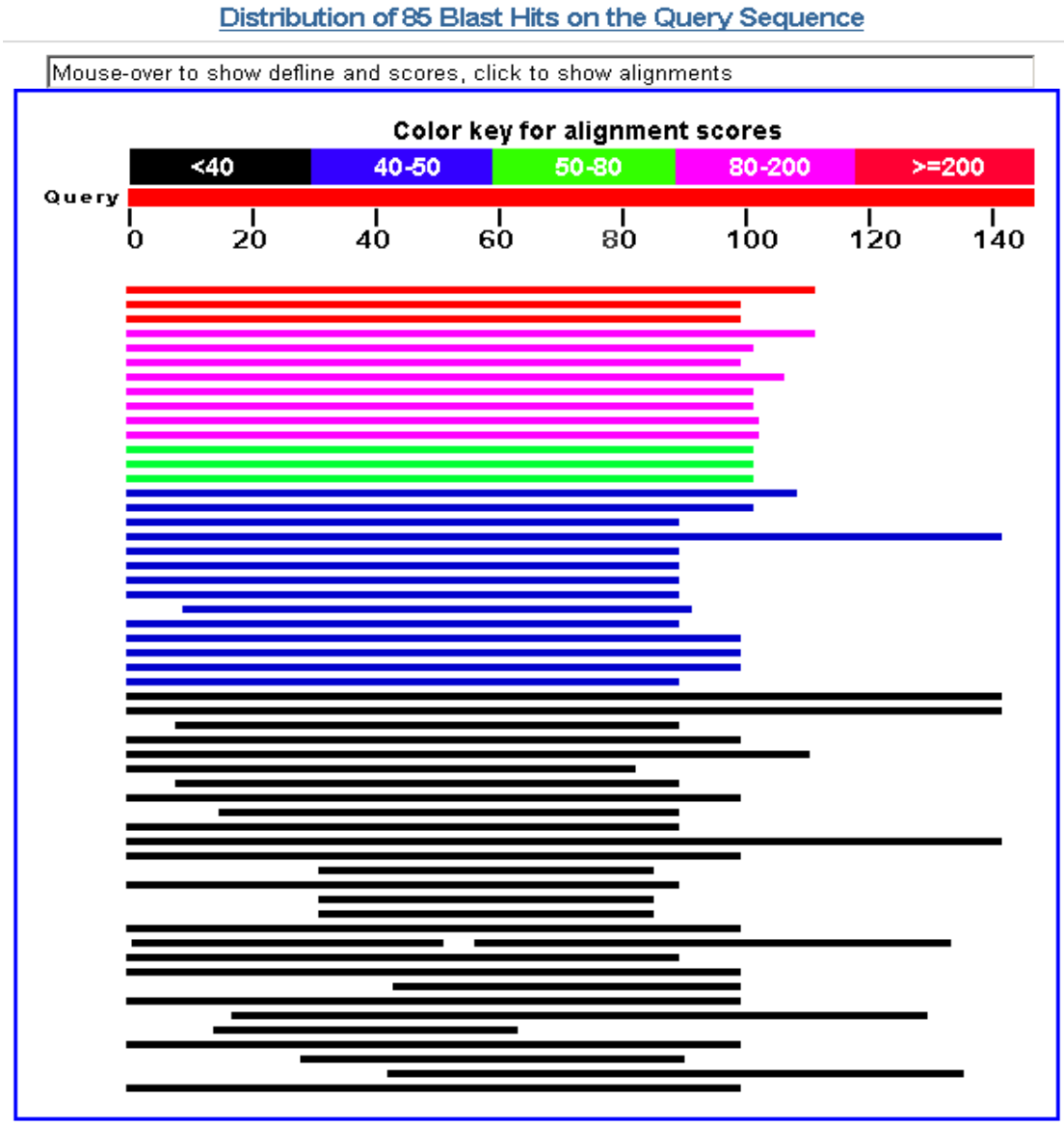
Sequences producing significant alignments:	Score (Bits)	E Value
gb|ABO93399.2| endoglucanase [Hypocrea virens]	43.1	0.011
gb|ABM55269.1| beta-1,3-glucanase [Trichoderma viride]	43.1	0.011
gb|AAL84695.1|AF395756_1 beta-1,3-glucanase precursor [Hypocr...	43.1	0.011
sp|P53626|E13B_TRIHA Glucan endo-1,3-beta-glucosidase BGN13.1...	43.1	0.011
ref|YP_001471290.1| protein of unknown function DUF187 [Therm...	35.8	1.8
gb|EAZ23863.1| hypothetical protein OsJ_007346 [Oryza sativa ...	35.4	2.2
gb|EAY86750.1| hypothetical protein OsI_007983 [Oryza sativa ...	34.7	4.2
ref|XP_001597035.1| hypothetical protein SS1G_01229 [Scleroti...	33.9	6.2
ref|XP_001556598.1| hypothetical protein BC1G_03983 [Botryoti...	33.9	7.3
ref|ZP_01055293.1| hypothetical protein MED193_13612 [Roseoba...	33.5	7.9

E. Blastp text output



F. Open reading frame

Plate 12. Sequence analysis for the clone BPT-822 contd…

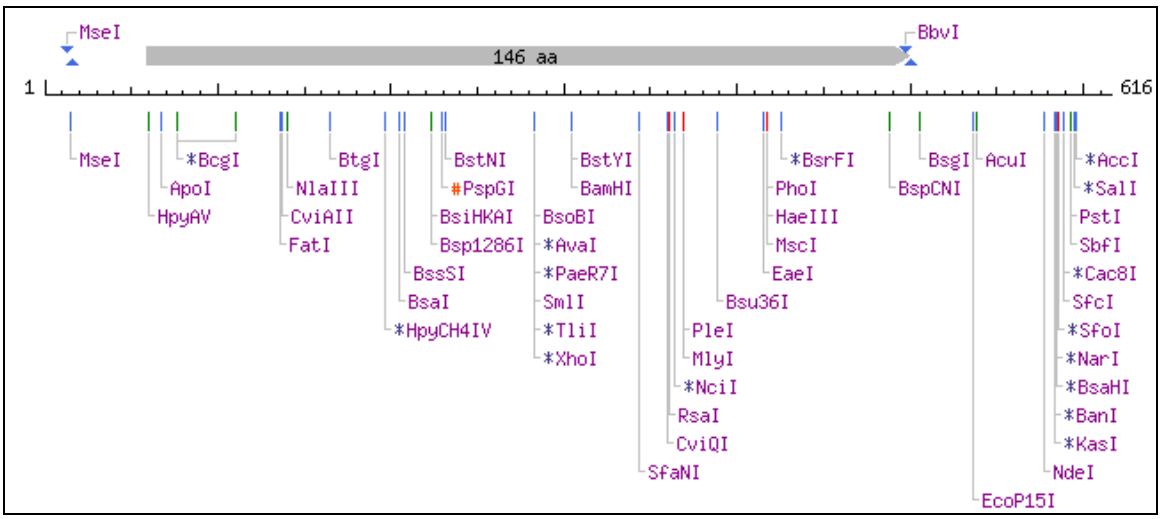


G. Graphical output of Blast of Open Reading Frame

Plate 12. Sequence analysis for the clone BPT-822 contd…

gb|EDP52988.1|	exo-beta-1,3-glucanase, putative [Aspergillus ...	41.2	0.022	
ref|XP_001263693.1|	exo-beta-1,3-glucanase, putative [Neosart...	41.2	0.022	G
ref|XP_754859.1|	exo-beta-1,3-glucanase [Aspergillus fumigatu...	41.2	0.022	G
ref|XP_001258465.1|	exo-beta-1,3-glucanase, putative [Neosart...	40.0	0.048	G
gb|EDP49765.1|	exo-beta-1,3-glucanase, putative [Aspergillus ...	39.7	0.063	
ref|XP_751077.1|	exo-beta-1,3-glucanase [Aspergillus fumigatu...	39.7	0.063	G
ref|XP_001940876.1|	glucan 1,3-beta-glucosidase [Pyrenophora ...	38.9	0.11	G
ref|XP_001556598.1|	hypothetical protein BC1G_03983 [Botryoti...	38.9	0.11	G
ref|XP_001227389.1|	hypothetical protein CHGG_09462 [Chaetomi...	38.9	0.11	G
ref|XP_001905866.1|	unnamed protein product [Podospora anseri...	38.5	0.14	G
ref|XP_001589225.1|	hypothetical protein SS1G_09858 [Scleroti...	38.5	0.14	G
ref|XP_001273111.1|	exo-beta-1,3-glucanase, putative [Aspergi...	38.1	0.18	G
gb|AAC09172.1|	exo-beta-1,3-glucanase [Ampelomyces quisqualis]	37.7	0.24	
ref|XP_001888863.1|	glycoside hydrolase family 55 protein [La...	37.4	0.31	G
ref|XP_001258312.1|	conserved hypothetical protein [Neosartor...	37.4	0.31	G
gb|EDP53707.1|	exo-beta-1,3-glucanase, putative [Aspergillus ...	36.6	0.53	
ref|XP_955880.2|	hypothetical protein NCU03493 [Neurospora cr...	36.6	0.53	U G
ref|XP_368585.1|	hypothetical protein MGG_00659 [Magnaporthe ...	36.6	0.53	U G

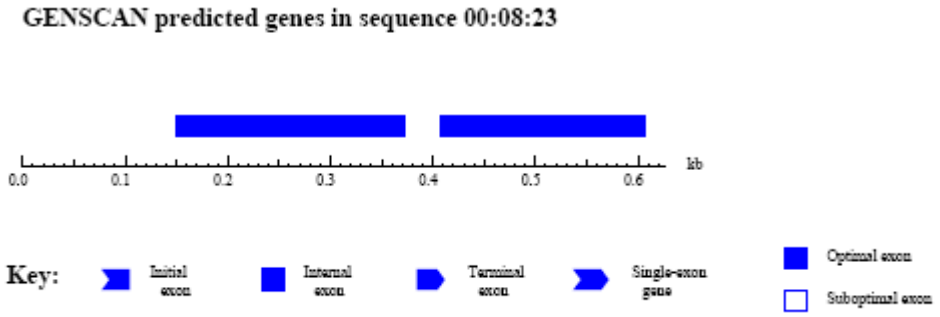
H. Text output of Blast of Open Reading Frame



J. Restriction map

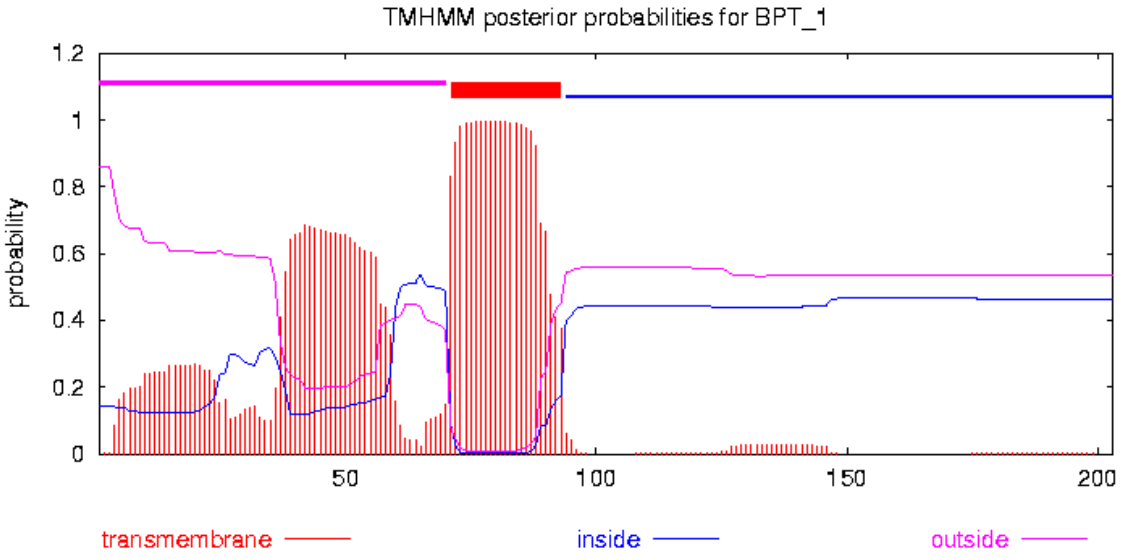
Plate 12. Sequence analysis for the clone BPT-822 contd…

Gn.Ex	Type	S	.Begin	...End	.Len	Fr	Ph	I/Ac	Do/T	CodRg	P....	Tscr..
1.01	Intr	+	149	372	224	2	2	51	25	297	0.675	22.47
1.02	Intr	+	407	607	201	0	0	47	24	194	0.951	13.16



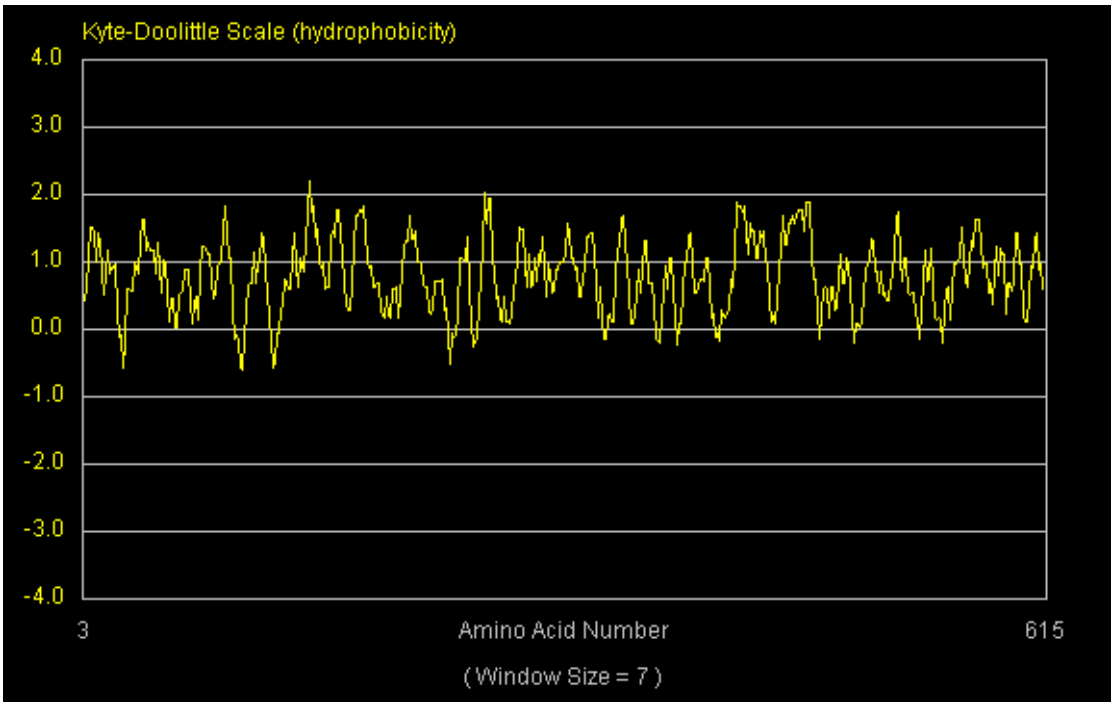
K. Genscan output

L. InterProScan output



M. Transmembrane region

Plate 12. Sequence analysis for the clone BPT-822 contd…

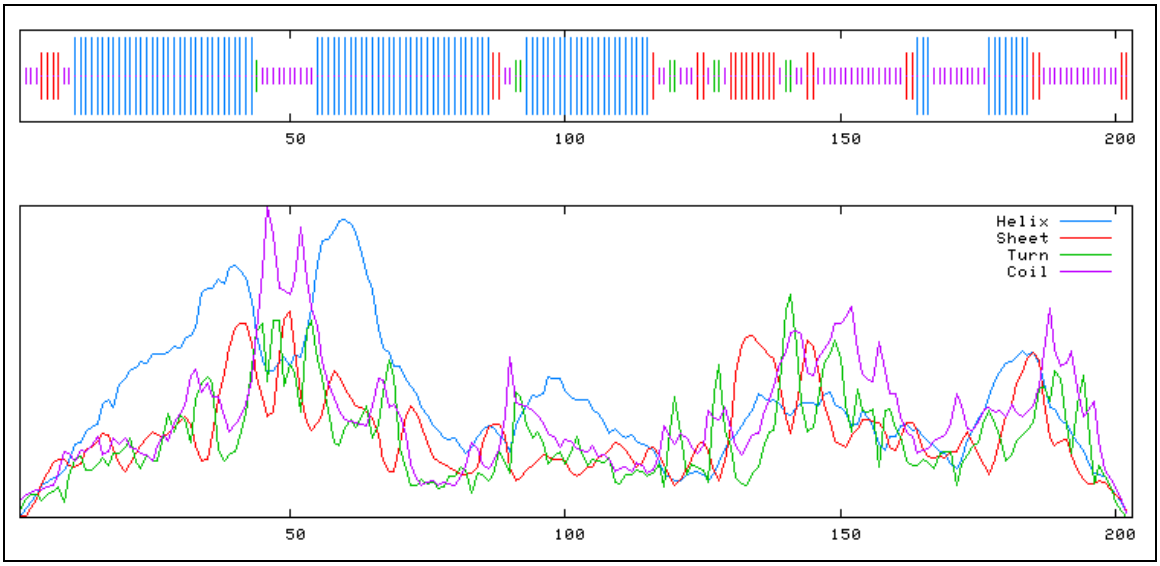


N. Kyte Doolittle Hydropathy Plot for deduced proteins

```
         10         20         30         40         50         60         70
          |          |          |          |          |          |          |
YPPSQLTLVLPLQLINILHLSRILKIMAHLSLLSEAQLWLALLAMSTPTLTATPWAETLLTAMLRLVTRD
ccccceeeeccchhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhtccccccccccchhhhhhhhhhhhhhh
LVLLLLVVVTLMWLPLLMEICPSRASSMSRIQRRMETVRFLEITRLMQVPGGFDSFYPGSRIVVSLATIT
hhhhhhhhhhhhhhhhheecctthhhhhhhhhhhhhhhhhhhhhhhhecctttccceecttceeeeeeeeeec
GNGNFFKNENSHSPLSQLPCSDVELHRFRCKIPLRCASRAFASVHLLQNPYAAPAGRPLGKLX
ttcceecccccccccccccccceehhhcccccccccchhhhhhhheeccccccccccccccee

Sequence length :   203

SOPMA :
   Alpha helix      (Hh) :    99 is  48.77%
   3_10 helix       (Gg) :     0 is   0.00%
   Pi helix         (Ii) :     0 is   0.00%
   Beta bridge      (Bb) :     0 is   0.00%
   Extended strand  (Ee) :    26 is  12.81%
   Beta turn        (Tt) :     9 is   4.43%
   Bend region      (Ss) :     0 is   0.00%
   Random coil      (Cc) :    69 is  33.99%
   Ambigous states  (?)  :     0 is   0.00%
   Other states          :     0 is   0.00%
```

O. Predicted secondary structure

Plate 12. Sequence analysis for the clone BPT-822 contd…



P. Graphical representation of secondary structure

Plate 12. Sequence analysis for the clone BPT-822 contd…

Discussion

5. DISCUSSION

The focus on the management of plant disease has been shifted from chemical pesticides to more ecofriendly biopesticides to reduce environmental hazards and to minimize the risk of development of pesticide resistant strains of plant pathogens. Research since the last few decades has clearly demonstrated that microorganisms possess a great potential for the management of many crop diseases. *Trichoderma* had been established as a potential biocontrol agent during past few decades, have created a new milestone in nonchemical plant disease management system and organic farming in Kerala. There is good evidence to support several mechanisms of disease control by *Trichoderma* species including mycoparasitism, cell lysis, antibiosis, induced resistance, competition for nutrient or space, inactivation of pathogen's enzyme etc. (Harman *et al*., 2004). *Trichoderma* spp. are known to produce many extracellular hydrolytic enzymes viz. β-1,3-glucanase, chitinase, cellulase, proteinase etc. by which they cause lysis or degradation of cell wall of many plant pathogenic fungi. In Kerala, *Phytophthora* is a major pathogen causing considerable damage to economically important crops. β-1,3-glucan is the main structural components of cell wall of oomycetes fungi particularly *Phytophthora* spp. and *Pythium* spp. β-1,3-glucanase has been proposed as one of the key enzyme in the lysis of cell wall of these fungi during the antagonistic action of *Trichoderma* (De la Cruz *et al*., 1995). A perusal of literature revealed many information regarding the characterization and cloning of chitinase and β-1,6-glucanase of *Trichoderma* spp. However, the reports on β-1,3-glucanase are rather meagre and scanty. Moreover no work has been reported from Kerala related to either chitinase or glucanase gene isolation and cloning in *Trichodrma* spp. So, in the view of above facts, the present investigation was carried out to study β-1,3-glucanase activity, total genomic DNA, molecular cloning and sequencing of different native antagonistic *Trichoderma* isolates. The results obtained during the present study are discussed in the light of earlier reports and possible interpretations are presented in this chapter.

5.1 BIOCHEMICAL CHARACTERIZATION

The production of extracellular β-glucanase was induced during the growth of *Trichoderma* isolates in TSB medium, when supplemented with 0.2 per cent *Phytophthora capsici* cell wall as the sole carbon source. The present findings revealed wide variation of glucanase enzyme activity with different native *Trichoderma* isolates. This variation was observed among the species and within the species, which might be due to the genetic variation among different strains. The glucanase enzyme activity of various *Trichoderma* isolates in the present study ranged from 1.3 U to 205.3U per 50 ml culture filtrate per 10 min. Of fifteen isolates, seven isolates showed high enzyme activity of more than 100 U and the maximum enzyme activity was observed in *T. viride*-1 (205.3U), *T. harzianum*-30 (157.8U), *T. aureoviride* (139.2U) and *T. viride*-8 (121.9U). De la Cruz *et al.* (1995), estimated β-1,3–glucanase activity produced by *T. harzianum,* grown in PDB supplemented with the mycelium of *Phytophthora citrophthora* as the sole carbon source and reported to be 700 mU/ mg of protein/ min. Noronha and Ulhoa (2000), also estimated β-1,3–glucanase activity of *T. harzianum* grown in the culture medium containing cell walls of *Pythium* spp. and reported to be 14.62±0.06 U/ mg of protein/ min. It is also interesting to note that *T. viride*-8, an endophytic *Trichoderma* isolated from black pepper has shown high glucanase enzyme activity, which can be further exploited for the gene isolation for the development of transgenic plants resistant to *Phytophthoa capsici.*

5.2 MOLECULAR CHARACTERIZATION

Molecular characterization of the gene encoding β-1,3-glucanase was carried out by polymerase chain reaction (PCR) with specific primer from the selected four *Trichoderma* isolates which were showing maximum enzyme activity.

5.2.1 Isolation of Genomic DNA

Good quality DNA without any contamination is a pre-requisite for PCR. For isolation of genomic DNA, *Trichoderma* isolates were grown in minimal medium for 5 days at room temperature as suggested by Geysens *et al*. (2005), which was found to be suitable for their growth. Large amounts of RNA in the DNA sample can chelate Mg^{2+} ions and reduce the yield of polymerase chain reaction (PCR). In order to overcome the problem of RNA contamination, the DNA samples were treated with RNaseA. As a result, good quality DNA with intact, discrete bands was obtained without any contamination, as visualized in 0.8 per cent agarose gel electrophoresis.

The quantity of DNA was estimated by Nano Drop® ND-1000 spectro photometer. The quantity of DNA isolated ranged from 164.82 to 204.08 µg g^{-1} of fresh mycelia. Since one PCR reaction requires only 25-50 ng template, the DNA recovered was found to be sufficient for further PCR reaction. The reaction of optical density value at 260 nm to that at 280 nm ranged from 1.77 to 1.81. The value of OD_{260} /OD_{280} between 1.8 and 2 indicates relatively pure DNA. Whereas according to Cenis (1992) method, the DNA yield from *T. harzianum* was reported to be 1040±22 µg g^{-1} of dry mycelia of OD_{260} /OD_{280} value 1.42±0.03.

Summing up the result already observed, it is revealed that, among the various *Trichoderma* isolates selected for the study, *T. viride* – 1 showed maximum glucanase activity and yielded high DNA concentration.

5.2.2 Primer Designing

When multiple sequence alignment of glucanases sequences from different fungi was done, the homology in conserved boxes was very less. Hence

only the sequences from different species of *Trichoderma/Hypocrea* were taken for further alignment and it showed a good number of conserved regions.

Oligonucleotides homologous to two regions in the gene of interest are used to amplify an intervening gene. While designing primers, complementary sequences and repeats of single nucleotide were avoided as much as possible. Primers were designed from conserved regions based on the degeneracy of amino acids to amplify the distance between forward and reverse primers and to get an amplicon of expected size. Degenerate oligonucleotides were designed considering codon bias and selection of aminoacids that require the least amount of degeneracy. From the glucanase gene specific primer, designed during this study, Glucan F1, Glucan R1 and Glucan R3 had degeneracy at single base, whereas Glucan F2, Glucan R2 and Glucan R3 had degeneracy at two bases. Even though degeneracies observed in primer sequences, but successfully amplified the β-1,3-glucanase gene from the genomic DNA of *Trichoderma* spp. Montero *et al*. (2007) also designed degenerate primer for the amplification of β-1,6-glucanase in *T. harzianum* and successfully able to amplify the gene from chromosomal DNA. Primer degeneracies of 10^5 to 10^6 or greater have been used successfully to isolate the correct gene from diverse species (Gould *et al*., 1989).

The primers, designed and synthesized have GC per cent about 50 per cent and Tm greater than 59°C. Highest annealing temperature was noticed for the primer combination Glucan F1R1 and lowest for Glucan F2R2 and Glucan F3R3.

5.2.3 Polymerase chain reaction

The PCR involves two origonucleotide primers, 17-30 nucleotides in length which flank the DNA sequence that is to be amplified. The primers hybridise to opposite strands of the DNA after it has been denatured and are oriented so that DNA synthesis by the polymerase, proceeds the region between two primers. By

repeated cycles of heat denaturation, primer hybridization and extension, there follows a rapid exponential accumulation of the specific target fragment of DNA (Mullis *et al*., 1986). PCR facilitates amplification of specific gene efficiently with gene specific primers.

While the PCR is simple in concept, practically there are a large number of variables which can influence the outcome of reaction (Pavlov *et al*., 2004). Therefore various PCR parameter like DNA concentration, Taq DNA polymerase concentration, annealing temperature and primer concentration were standardized for the specific amplification of β-1,3-glucanase. Good quality DNA template was found to be necessary for amplification of specific gene. Optimization of template DNA dilution was therefore to be carried out for each template lot. The pH of the reaction buffer should be optimum (8.3), which is necessary for the working of each reagent in reaction mixture. Taq DNA polymerase concentration was found to be effective at 0.6 units.

The primer combination Glucan F1R1 gave amplification for two species *T. aureoviride* and *T. viride*-8 and no amplification for *T. viride-1* and *T. harzianum*-30. For two species specific region corresponding to the primer combination has amplified whereas for other two isolates, no amplification could be obtained. Since most of the primers designed were derived from complete coding sequence of mRNA or cDNA, there exists the possibility of introns in the priming site. It is supposed that, mismatch of template and primer particularly had occurred at the 3' end of the primer or the occurrence of large introns immediately after priming region and hence amplification got disrupted. The same results were obtained with primer combination of Glucan F2R2.

Glucan F3R3 primer combination yielded single band in *T. aureoviride* and multiple bands in *T. viride*-1. It was also found that primer combination Glucan F1R2 yielded multiple bands in all isolated at 52°C annealing temperature, while increasing Ta to 56°C, there was only one discrete band in all isolates. It may be due

to the lower annealing temperature leads to non-specific binding. Since β-1,3-glucanase belongs to complex gene families (Beerhues *et al*., 1994) and different isoforms have a unique amino acid sequence that was encoded by a different gene. These genes shares conserved regions and might have amplified with this primer combination. There is also possibility of amplifying target DNA with varying repeat numbers, these yielding a population of PCR products differing slightly in size because of the variation in repeat number.

5.3 CLONING OF PCR PRODUCT

The cloning of PCR products is extremely valuable for sequencing, for mutation analysis, for *in vitro* mutagenesis as an intermediate in genetic engineering, in studies of gene expression and other applications. Amplification by PCR with Taq DNA polymerase lacking 3' – 5' exonulcease activity yields products that contain a single 3'-terminal nucleotide overhang, typically an A residue (Clark, 1988; Hu, 1993). Hence these PCR products can be frequently cloned into T-vectors, which contain a single T overhang. Amplicons obtained by PCR cloned into pGEM-T (Promega) Easy Vector System I.

5.3.1. CLONING OF GENE ENCODING GLUCANASE

Transformation may be described as the stable, heritable uptake of exogenous DNA into a host cell. In order to undergo transformation, the cell must be competent and bacteria such as *E. coli* may be artificially induced to become competent. The success of transformation is measured in terms of transformation efficiency. Many β-1,3-glucanases have been isolated, but only a few genes have been cloned, e.g. *bgn13.1* (De la Cruz *et al*.,1995) and *lam1.3* (Cohen-Kupiec *et al.*, 1999) from *T. harzianum*, *glu78* (Donzelli *et al.*, 2001) from *T. atroviride*, and *Tv-bgn1* (Kim *et al.,*2002) and *Tv-bgn2* (Djonovic *et al*., 2007) from *T. virens*.

The cells were made permeable to plasmid DNA by chilling the cells in presence of divalent cations such as $CaCl_2$. $CaCl_2$ affects the cell walls and might be responsible for binding of DNA to the cell surface (Hanahan, 1983). The uptake of DNA is also stimulated by the brief heat shock (Old and Primrose, 1994). *E. coli* cells were given a shock treatment by abruptly increasing the temperature from zero to 42°C for a short period of 90 seconds. The treated bacteria are more likely to take up foreign DNA. The temperature is an important factor to obtain the highest transformation efficiency. Competence of *E. coli* JM 109 cells was confirmed by transforming them with an uncut plasmid (pUC18) containing ampicillin resistance marker. High efficiency of transformation of $4.2x10^8$ cfu μg^{-1} DNA was obtained with the competent cells prepared. This was in accordance with Liu and Rashdbaigi (1990) as they reported the optimum efficiencies of transformants were 10^7 to 10^9 cfu μg^{-1}. The competent cells harbouring the plasmid alone could grow in ampicillin containing medium. If any other ampicillin resistant bacteria thrive in the transformation plate, they will give white colonies that could be misinterpreted as transformed cells. Strict aseptic condition was maintained through out the preparation of competent cells, since contamination in competent cells can produce white colonies after blue-white screening.

The cloning vector used was pGEM-T easy vector having a size of 3kb, which is the convenient system for the cloning of PCR products. The vector was custom made by cutting with *Eco*RV and adding 3'-terminal thymidine to both ends. These single 3'-T overhangs at the insertion site improve the efficiency of ligation of a PCR product into the plasmid by preventing recircularization of the vector and providing a compatible overhang for the product generated by *Taq* polymerase. This thermostable enzyme often adds a single deoxyadenosine, in a template independent fashion, to the 3' ends of the amplified fragments (Clark, 1988). The high copy number pGEM-T Easy Vector contain T7 and SP6 RNA polymerase promoter flanking a multiple cloning site within the *lac*Z region. This facilitates blue-white screening or selection of recombinants by insertional inactivation of β-galactosidase. The amplified DNA fragments of GT-311, GT-322, BPT-822, T–112 and CT–3012

were eluted from the gel and ligated into pGEM-T Easy Vector. It was found that incubation of ligation reaction at room temperature for one hour followed by overnight incubation at 4°C yields maximum number of transformants.

Ligated product containing the specific amplicon was used to transform *E. coli* JM 109 cells, which could be later picked up from the media containing 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal) and isopropyl thiogalactoside (IPTG) based on blue white screening. *E. coli* cells after transformation when plated on LBA plate overlaid with X-Gal and IPTG; produced blue and white colonies after overnight incubation. The pGEM-T Easy Vector contained polycloning sites inside the β-galactosidase encoding gene (*lac*Z). Insertion of a foreign sequence will disrupt the reading frame of β-galactosidase encoding gene. During transformation, the host encoded and vector encoded protein regions of β-galactosidase undergoes α-complementation to form enzymatically active proteins. *E. coli* cells that are not transformed with the recombinant plasmid can further utilize the chromogenic substrate X-Gal and appear as blue colonies (Ullman *et al*., 1967). Colonies which have not taken up the plasmid can further utilize the substrate and appear as blue colonies on X-gal chromogenic substrate (Horwitz *et al.,* 1964). *E. coli* cells carrying recombinant plasmid were not capable of α-complementation and developed into white colonies.

It is observed from the above result that, all transformants showed high transformation efficiency more than 65 per cent. Among all transformants, CT–3012 showed maximum and GT–322 showed lowest transformation efficiency.

5.3.2. CONFIRMATION OF INSERTION

Plasmids isolated from blue and white colonies gave bands with different molecular weights. All the plasmids isolated from white colonies had higher molecular weight than the pGEM-T vector alone. Plasmid from blue colony was

self-ligated vector alone, since no transformation has occurred. A band corresponding to 3.1 kb corresponding to the actual size of plasmid was obtained.

PCR with gene specific primers amplified fragments of expected size obtained in white colonies only and there were no amplification in blue colonies. This confirmed the presence of insert in the vector. But in clones T-112 and CT-3012, multiple bands were observed in recombinant clones with the expected size of band, when PCR was carried out with gene specific primer Glucan F1R2. It was further confirmed with T7 forward and SP6 reverse universal primer. Higher amplicon sizes of about 800 bp, 900 bp and 1.5 kb of bands were obtained from the white plasmid of the clone of primer combination Glucan F1R1, Glucan F2R2 and Glucan F1R2 respectively, which were little higher than the insert and lower amplicon size than the white plasmid was obtained from blue plasmid by the primer.

5.3.3. SEQUENCING AND THEORETICAL SEQUENCE ANALYSIS

5.3.3.1. Sequencing of the cloned fragment

The cloned fragment when sequenced by automated sequencing with T7 forward primer provided the 5'-3' sequence data in the downstream direction since it is a forward primer. The sequences obtained after cloning, were subjected to vector screening to delete the sequences of vector, if any present. Vector screening of the sequence GT-311 and GT-322 showed significant similarity starting from the region 1 to 47 bases. The vector screen of sequence BPT-822 showed strong match to vectors starting from region starting from 1 to 52 bases and from 651 to 706 bases. Hence, the regions showing similarity to the vector and the primer sequences which were externally supplied during PCR, were deleted and only the insert sequence was retrieved. The actual insert sequences data obtained were further analysed. It is worthwhile to mention that, this is the first attempt on biochemical characterization and molecular cloning of *T. aureoviride.*

5.3.6.2. Theoretical analysis of sequence using bioinformatics tools

The analysis of sequence using bioinformatics tools was theoretically relevant to know the details of the sequence obtained, which can be helpful for isolating the full length gene. Basic Local Alignment Search Tool (BLAST) is the tool most frequently used for calculating sequence similarity. BLAST comes in variations for use with different query sequences against different databases. Homology search through BLAST is a heuristic method to find the highest scoring locally optimal alignments between a query sequence and a database sequence (Altschul *et al*., 1990, 1997). The BLAST analysis can determine the sequence homology to predict the identity and function of the query sequence.

When the cloned sequences were subjected to Blastn, they were identified as partial glucanase genes. The sequences showed significant homology with different glucanase gene sequences of various species of *Trichoderma* or *Hypocrea* present in NCBI databank. Query coverage for GT-311 was 76 per cent, showing maximum identity of 93 per cent and 92 per cent to endoglucanse gene (bgn13.3) of *H. virens* and β-1,3- glucanase (glu) gene of *T. viride* respectively. For GT-322, 92 per cent homology was found to β-1,3- glucanase (glu) gene of *T. viride* and 90 per cent to endo–1,3(4)-β-glucanase of *T. harzianum* and endoglucanase gene(bgn13.3) of *H. virens* with 79 per cent query coverage. The sequence BPT-822 showing maximum homology of 96 per cent and 94 per cent to two accessions namely, EF176582.1 and X84085 respectively with minimum E–value. There were 24, 36 and 12 Blast hits reported for sequences GT-311, GT-322 and BPT-822 respectively. This report was in accordance with the result obtained by Gao *et al.* (2008). They found that cloned beta-1,3-glucanase gene (glu) from *Trichoderma viride* LTR-2 cDNA sequence showed 93 per cent homology to β -1,3-glucanase genes from *T. harzianum* (bgn3.1) and *Hypocrea virens* (bgn13.1).

Homology search through protein-protein BLAST (Blastp) using deduced amino acid sequence showed all three sequences had significant similarity with endoglucanses of *H. virens,* glucan endo-1,3-β-glucosidase (BGN13.1) of *T. harzianum*, β-1,3-glucanase and laminarinase of *T. viride,* β-1,3-glucanase precursor of *H. virens.* GT-311, GT-322 and BPT-822 showed 87, 13 and 10 blast hits respectively.

The phylogram of GT-311, GT-322 and BPT-822 with glucanase genes of different species of *Trichoderma/Hypocrea* in NCBI databank showing evolutionary relationship was constructed. The result of phylogram revealed that GT-322 and BPT-822 showed close similarity with beta-1, 3-glucanase (glu) gene of *T. viride* having accession EF176582.1 and GT-311 with endoglucanase (bgn13.1) gene of *H. virens* (accession EF426721.1) and further connected to the gene beta-1,3-glucanase precursor (bgn2) of *H. virens* (AF395756.1) in phylogenetic tree.

When the sequences were subjected to nucleotide statistics tool, both the sequences GT-322 and BPT-822 were rich in A+T base pair composition (51.3%) as compared with C+G (48.7%), while GT-311 had C+G (49.5%) and A+T (50.5%).

Open reading frame (ORF) is the part of protein coding gene and the longest ORF of the DNA sequence contains the protein-coding region. It starts with an initiation codon and ends with a termination codon (Old and Primrose, 1994). When the ORFs of the cloned sequence were examined, GT-311 encoded the longest ORF on +2 reading frame, with a length of 264bp of nucleotide and 87bp of aminoacids. When subjected to BLASTp search, it detected homology with hypothetical protein of *Rattus norvegicus.* The longest ORF was located on the +2 reading frame for GT-322 sequence, with a length of 423 bases coding 140 aminoacid residues, starting from base 68 to 490. The result of the BLASTp showed 88 blast hits and homology to β-1, 3- glucanase of *T. viride* and endoglucanse of *H. virens.* The ORF analysis of BPT-822, revealed that the longest ORF (441bp) was encoded on +3 reading frame. The longest one was subjected to BLASTp and its result showed significant

homology to β-1,3-glucanase of different *Trichoderma* spp. and *Aspergillus fumigatus*. A gene fragment of 453bp was obtained from *bgn16.3* by ORF (Montero *et al.*, 2007). Even though ORF is detected from the sequence obtained by ORF finder, it can only be confirmed after the cloning of full length gene.

The theoretical restriction analysis for ten enzymes showed that *Alu*I had four sites, followed by *Hae*III three sites in GT-311, whereas *Acl*I, *Dpn*I, *Mbo*I, *Nae*I and *Pvu*I had only one site and only two restriction sites for MseI. In GT-322, there were four restriction sites for *Dpn*I I and *Mbo*I. It had two restriction sites for *Taq*I and *Mse*I and one restriction site for *Alu*I, *Bam*HI, *Acl*I and *Hae*III. The sequence BPT-822 was found to possess four restriction sites for *Taq*I, three restriction sites for *Alu*I, two restriction sites each for *Dpn*I and *Mbo*I, whereas there were only one site for *Bam*HI, *Hae*III and *Mse*I. Restriction enzymes *Nae*I and *Pvu*I lacked restriction sites both on GT-322 and BPT-822 and there was no restriction site for *Bam*HI and *Taq*I in GT-311.

'Genscan' tool was used to analyze the exons present in the cloned sequence. In GT-311, initial exon of 130bp and terminal exon of 191bp separated by 198bp of intron. Both GT-322 and BPT-822 possessed only two internal exons each of 293bp, 126bp and 224bp, 201bp respectively. Poly A tail region of 6bp present in GT-311. Even though the exonic regions were found from the cloned sequences, it will be practically more relevant after the cloning of full length gene.

Aminoacid analysis revealed composition of different aminoacids in glucanase gene sequences cloned. The molar percentage of serine was found to be highest (11.3%, 26 residues), followed by glycine (8.3%, 20 residues) in GT311 sequence. Both amino acids arginine and leucine content was highest (8.5%, 20 residue), followed by serine (7.2%, 17 residue) in GT-322. The BPT-822 sequence had leucine with the highest share of 19.70 per cent with 40 residues.

Functional aspects of domains of GT-311 sequence discovered through 'InterProscan' revealed two transmembrane helix of 20bp region ranging from the amino acid sequence 37 to 57 and 140 to 160. Transmembrane domain is a three dimensional protein structure, which is thermodynamically stable. This can be predicted on the basis of hydrophobicicity. Using hydrophobicity analysis, transmembrane helices enable prediction of membrane topology of a protein. In the sequence BPT-822, one signal peptide and two transmembrane helices were found through 'InterProScan'. The sequence region 1 to 52 amino acids coded for signal peptide and 36 to 56 and 71 to 91 amino acids coded for transmembrane helix. The cleavage site of signal peptide located between A-52 and T-53 was predicted. This was in accordance with the findings of Nobe *et al.* (2004). He got only one signal peptide cleavage site at A-17 and S-18 by SignalP from the 33 residue of N-terminal sequence of LAMI from *T. viride.* Montero *et al.* (2007) also found a typical signal peptide region comprising 18 amino acid residues in *bgn16.3* sequence from *T. harzianum.*The sequence GT-322 has no functional domain.

The 'Motif Scan' of aminoacid sequence of GT-311, GT-322 and BPT-822 revealed similarity with protein kinase and casein kinase phosphorylation site. The protein sequence BPT-822 from *T. viride*–8 had a Leucine rich motif region of 82 residue length through 'Motif Scan' search. While it was reported that β-1, 3- glucanase contain a cysteine rich motif from *T. harzianum* (De la Cruz *et al.*, 1995).

The secondary structure prediction of the cloned sequences showed the proportion of different structures namely alpha helix, beta sheet and random coils. The sequences GT-311 and GT-322 were comparatively richer in random coils (47.83%, 47.03 %), where as BPT-822 was the richest in alpha helices (48.77%). The proportion of random coil was 33.99 per cent in BPT-822 and the proportion of alpha helices were 13.04 and 16.95 per cent in GT-311 and GT-322 respectively. The beta turns were contributed to 11.74, 7.20 and 4.43 per cent in GT-311, GT-322 and BPT-822 respectively.

When the sequences are subjected to hydropathy plot by means of Kyte Doolittle Hydropathy Profile, all the three sequences were showing hydrphobic in nature, rich in hydrophobic amino acids. But GT-311 and BPT-822 had both hydrophobic and hydrophilic amino acids. All the sequences were analysed for the transmembrane helices.

Summing up the discussion presented so far, it may be concluded that, the present findings have enriched our knowledge on various molecular aspects of *Trichoderma* isolates of Kerala, particularly the glucanase enzyme activity, glucanase gene isolation, cloning and its sequencing which provided the pioneer steps not only for the genetic manipulation of valuable crops for developing disease resistance but also for the enhancement of biocontrol activity of microorganisms.

Summary

6. SUMMARY

An investigation on "Molecular cloning and characterization of the gene encoding β-1, 3- glucanase in *Trichoderma* spp." was conducted at the Centre for Plant Biotechnology and Molecular Biology and Department of Plant Pathology, College of Horticulture, Vellanikkara, Thrissur from 2006 to 2008. The objectives of the study were biochemical and molecular characterization of the enzyme β-1,3-glucanase in *Trichoderma* spp. The salient findings of the study are summerized in this chapter.

1. Twelve antagonistic *Trichoderma* isolates with three reference cultures of *T. viride*, *T. virens*, *T. harzianum*, having potential antagonistic activity against the major soil borne pathogens of Kerala viz. *Pythium* spp., *Phytophthora capsici, P. meadii, Ralstonia solanacearum* were used for the present study.

2. Biochemical characterization of fifteen *Trichoderma* isolates was done by determinining the glucanase activity. Among the 15 *Trichoderma* isolates, four isolates such as *T. viride*-1, *T. harzianum*-30*, T. aureoviride* and *T. viride*-8 showed high β-1,3- glucanase activity ranging from 205.3U to 121.9U per 50ml culture filtrate at 10 min incubation, were selected for genomic DNA isolation.

3. Genomic DNA was isolated from the four selected *Trichoderma* isolates. The quantity of DNA in the sample varied from164.82 μg g^{-1} to 204.08 μg g^{-1} of mycelia. The OD_{260}/OD_{280} ranged between 1.77 and 1.81 indicating the good quality of DNA without any contamination.

4. Three pairs of primers for β-1,3-glucanase genes were designed, based on the homology within the conserved regions of glucanase genes present in

different species of *Trichoderma* / *Hypocrea.* All Primers had melting temperature more than 56^0C and sequence length of 20 to 23 bp.

5. The PCR amplification with primer combination Glucan F1R1 and Glucan F2R2 yielded discrete bands both in *T. aureoviride* and *T. viride* – 8. The expected amplicon sizes of 550bp and 600bp were obtained in both cases respectively. To confirm the gene sequence, the amplicons were eluted and cloned.

6. Multiple bands were obtained for primer combination Glucan F1R2 at annealing temperature 52^0C in all four isolates. When the annealing temperature increased to 56^0C, single and discrete band of expected size (1.3 kbp) was observed. To confirm the gene sequence, the amplicons of *T. viride* - 1 and *T. harzianum*-30 were eluted and cloned.

7. The amplicons were effectively eluted, ligated in pGEM-T vector and competent *E.coli* JM 109 cells were transformed with the ligated product.

8. Plasmid was isolated from blue and white colonies and the electrophoretic profile confirmed the presence of insert in it. Further confirmation tests were done using PCR amplification with the same gene specific primer and T7 forward and SP6 reverse universal primer.

9. The cloned insert of β-1,3-glucanase genes were sequenced using T7 universal primer. After removing the vector sequence and primer sequence, the nucleotide sequence of 725 bp, 749 bp and 616 bp were obtained for GT-311, GT-322 and BPT-822 respectively.

10. Theoretical analysis of the sequence using blastn and blastp progammes showed 90-96 per cent identity with glucanase genes and protein present in different species of *Trichoderma.*

11. Phylogram of three cloned sequences with glucanase genes of different species of *Trichoderma/Hypocrea* from NCBI databank showed GT-322 and BPT-822 had lower phylogenetic distance and closely related to beta-1, 3-glucanase (glu) gene of *T. viride* having accession number EF176582.1. Where as GT3-11 is more divergent from other two sequences and closely related to endoglucanase (bgn13.1) gene of *H. virens* (accession EF426721.1).

12. Restriction analysis revealed that the GT-311, GT-322 and BPT-822 fragment have restriction sites for frequent cutter *Alu*I , *Dpn* I, *Mbo* I and *Taq* I respectively. All the three sequences were rich in A+T base pair composition as compared with C+G.

13. The cloned fragments had largest ORF of size 264, 423 and 441 bases that coded 87, 140 and 146 amino acids in GT-311, GT-322 and BPT-822 respectively.

14. GT-311 had an initial exon with a length of 130bp and a terminal exon of 191bp. GT-322 and BPT-822 possessed two internal exons each of 293, 126 and 224, 201 base pairs respectively.

15. The amino acid composition of the cloned sequences was analysed using AASTAT tool available in Biology Workbench. Leucine content was high both in GT-322 and BPT-822 where as GT-322 was rich in serine content.

16. No conserved domain was detected for any of the three sequences. The 'Motif Scan' of aminoacid sequence of GT-311, GT-322 and BPT-822 revealed similarity with protein kinase and casein kinase phosphorylation site. BPT-822 had leucine rich region motif ranging from 6 to 87 amino acid sequence.

17. The 'InterProScan' result showed that GT-311 had two transmembrane helices where as BPT-822 had one signal peptide of 52bp long and two transmembrane helices.

18. Kyte and Doolittle hydropathy plot analysis revealed the presence of transmembrane regions in the deduced amino acid sequences of GT-311 and BPT-822 clones. All the three sequences were hydrphobic in nature being rich in hydrophobic amino acids.

19. The secondary structure prediction of the sequences were done using SOPMA programme. The sequences GT-311 and GT-322 were comparatively richer in random coils (47.83%, 47.03 %), where as BPT-822 was the richest in alpha helices (48.77%).

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*Originals not seen

Annexures

ANNEXURE I

Composition of different media used in the study

1. *Trichoderma* Selective Medium (TSM)

$MgSO_4$	-	0.20 g
K_2HPO_4	-	0.90 g
NH_4NO_3	-	1.00 g
KCl	-	0.15 g
Glucose	-	3.00 g
Rose Bengal	-	0.15 g
Agar	-	15 g
Water	-	1 l

***2. Trichoderma* Selective Broth (TSB)**

$MgSO_4$	-	0.2 g
K_2HPO_4	-	0.9 g
NH_4NO_3	-	1 g
KCl	-	0.15 g
Cell wall extract (*Phytophthora capsici*)	-	2.0 g
Water	-	1 l

3. Potato Dextrose Agar medium (PDA)

Potato	-	200 g
Dextrose	-	20 g
Agar	-	20 g
Water	-	1 l

4. Carrot agar medium

Carrot	-	200 g
Agar	-	20 g
Water	-	1 l

5. Carrot broth

Carrot	-	200 g
Water	-	1 l

ANNEXURE II

I. Reagents used for glucanase assay

1. Sodium acetate buffer (50mM and pH – 5.0)

Sodium acetate	–	0.68 g
Distilled water	-	100ml

2. Sodium hydroxide (4.5%)

NaOH	–	4.5 g
Distilled water	–	100ml

3. Dinitrosalicylic acid (DNS)

DNS	–	8.8 g
Sodium patasium tartarate	–	2.55 g
NaOH (4.5%)	–	30 ml
Distilled water	-	80 ml

4. Laminarin (4%)

Laminarin	–	40 mg
Distilled water	–	1 ml

ANNEXURE III

I. Medium used for DNA isolation

1. Minimal medium

Dextrose monohydrate	-	20 g
$(NH_4)_2SO_4$	-	5 g
$KH_2 PO_4$	-	15 g
$CaCl_2$	-	0.3 g
$MgSO_4$	-	0.3 g
$FeSO_4.7H_2O$	-	5 mg
$ZnSO_4.7H_2O$	-	1.4 mg
$CoCl_2$	-	3.7 mg
$MnSO_4.7H_2O$	-	1.6 mg
Distilled Water	-	1 l

I1. Reagent used for DNA isolation

1. 10mM Tris Cl

1M Tris Cl (pH -8.0)	-	1ml
Distilled water	-	100 ml

2. Extraction buffer

200mM Tris HCl	–	3.152 g
250mM NaCl	-	1.461 g
25mM EDTA	-	0.930 g
SDS (0.5%)	-	0.5 g
Distilled water	-	100 ml

3. TE buffer
(Tris Cl- 50mM; EDTA - 20mM)

Tris Cl - .05 M (pH -8.0)	-	0.394 g
.02M EDTA (pH -8.0)	-	0.372 g
Distilled water	-	100 ml

4. RNase stock

RNase	-	10 mg
Distilled water	-	1 ml

Stock was prepared by dissolving 10 mg RNase in 1 ml water and was stored under refrigerated conditions at -20^0C.

5. Phenol : Chloroform : Isoamylalcohol (25:24:1, v/v/v)

To 25 parts of Tris saturated Phenol, 24 parts of chloroform and 1 part of isoamylalcohol were added and mixed properly. The mixture was stored in refrigerator before use.

6. 5M Ammonium acetate (pH 7.4)

Ammonium acetate	-	57.75 g
Distilled water	-	100 ml

7. 3M sodium acetate

Sodium acetate	-	20.412 g
Distilled water	-	50 ml

8. Chilled isopropanol

9. 70% ethylacohol

To 70 parts of absolute ethanol, 30 parts of double distilled water was added.

ANNEXURE IV

Buffers and dyes used in gel electrophoresis

1. 6x Loading/ tracking dye

Bromophenol blue	-	0.25%
Xylene cyanol	-	0.25%
Glycerol	-	30%

The dye was prepared and kept in fridge at 4^0C

2. Ethidium bromide (intercalating dye)

The dye was prepared as a stock solution of 10 mg/ ml in water and was stored at room temperature in a dark bottle.

3. 50x TAE buffer (pH 8.0)

Tris base	-	242.0 g
Glacial acetic acid	-	57.1 ml
0.5M EDTA (pH 8.0)	-	100 ml
Distilled water	-	1000 ml

The solution was prepared and stored at room temperature.

ANNEXURE V

I. Media used for competent cell preparation

1. Luria Bertani (LB) broth

Tryptone	-	10 g
Yeast Extract	-	5 g
NaCl	-	5 g
pH adjusted to	-	7± 0.2
Distilled water	-	1 l

2. Luria Bertani Agar (LBA) medium

Tryptone	-	10 g
Yeast Extract	-	5 g
NaCl	-	5 g
Agar	-	20g
pH adjusted to	-	7± 0.2
Distilled water	-	1 l

II. Reagent used for competent cell preparation

1. Solution A

   Ice- cold 100mM $CaCl_2$

III. Reagents used for plasmid isolation

1. Solution I (Resuspension buffer)

Glucose	-	50mM
Tris	-	25mM
EDTA	-	10mM
pH	-	8.0

2. Solution II (Lysis buffer)

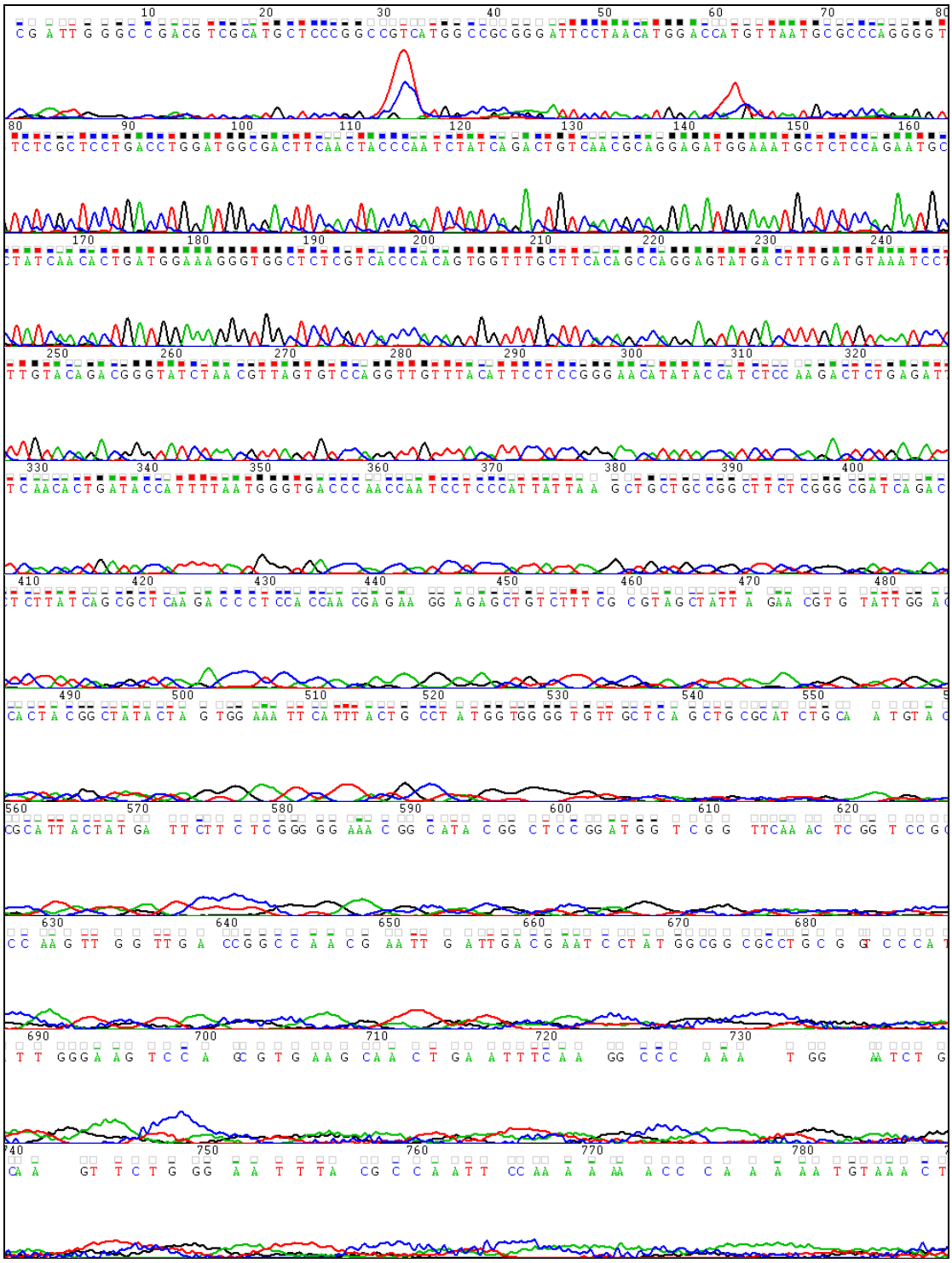
NaOH	-	0.2 M
SDS	-	1 %

3. Solution III

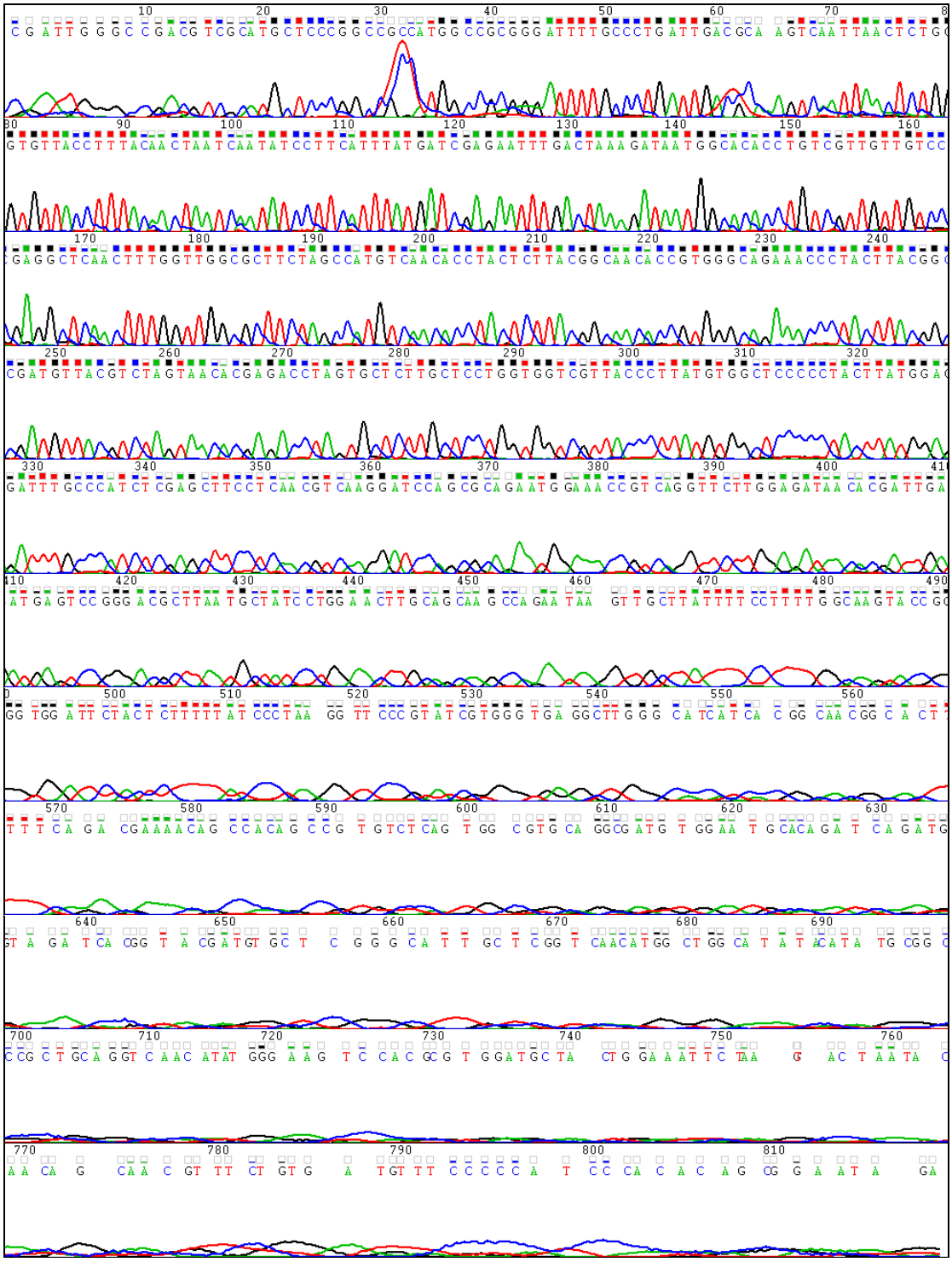
CH_3COOK	-	5M
pH	-	5.5

ANNEXURE VI

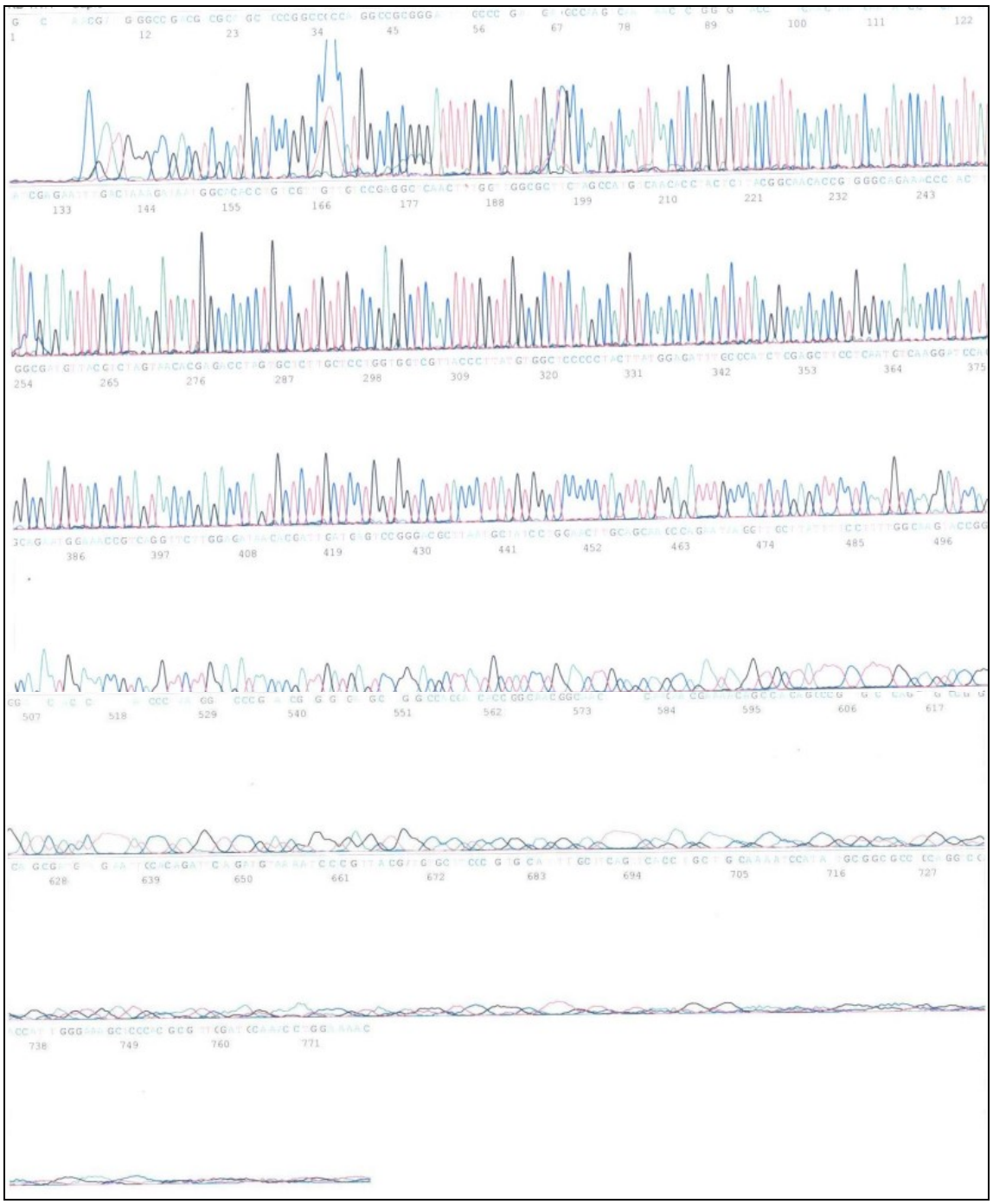
Electropherogram obtained for the cloned fragment GT-311



Electropherogram obtained for the cloned fragment GT-322



Electropherogram obtained for the cloned fragment BPT-822



MOLECULAR CLONING AND CHARACTERIZATION OF THE GENE ENCODING β-1, 3- GLUCANASE IN *Trichoderma* spp.

By

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(2006-11-109)

ABSTRACT OF THE THESIS

Submitted in partial fulfillment of the

requirement for the degree of

Master of Science in Agriculture

(PLANT BIOTECHNOLOGY)

Faculty of Agriculture

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2008

ABSTRACT

β–1,3–glucanase enzyme is widely distributed among bacteria, fungi and higher plants. Fungal β–1,3–glucanase produced by *Trichoderma* spp. is the key enzyme in the lysis of cell wall during their mycoparasitic action against several phytopathogenic fungi. This creates the potentiality of *Trichoderma* to be used as biocontrol agent.

Biochemical characterization of the enzyme β–1,3–glucanase from different *Trichoderma spp.* has been reported. The gene encoding β–1,3–glucanase has been cloned and sequenced in several plants, bacteria and fungi. The study entitled "Molecular cloning and characterization of the gene encoding β-1,3-glucanase in *Trichoderma* spp." was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB) and Department of Plant Pathology, College of Horticulture, Vellanikkara during the period from 2006 to 2008. In the present study an attempt was made to determine the glucanase activity and to characterize β–1,3–glucanase gene of *Trichoderma* spp.

Fifteen *Trichoderma* isolates, antagonistic to *Phytophthora capsici, P. meadii, Pythium aphanidermatum* and *Ralstonia solanacearum* were used for the present study. The enzyme activities of different isolates were found to be ranged from 1.3 U to 205.3 U per 50 ml culture filtrate for 10 min incubation. Among the fifteen isolates, the highest enzyme activity was found in *T. viride*–1 (205.3U) followed by *T. harzianum*-30 (157.8U), *T. aureoviride* (139.2U), *T. viride*-8 (121.9U) and these four were selected for gene isolation.

The informations on glucanase gene sequences of different species of *Trichoderma* available in the public domain NCBI were collected and subjected to multiple sequence alignment to detect conserved boxes of the gene among species.

Based on the data, three pairs of gene specific primer were designed for amplification of β–1,3–glucanase gene fragment of about 500 - 1400bp in *Trichoderma* spp.

Genomic DNA was isolated from the four selected *Trichoderma* isolates. Amplification was obtained from two isolates by the primer combination of Glucan F1R1 and Glucan F2R2, whereas by Glucan F1R2, the amplification was obtained in all isolates. The amplicons obtained by Glucan F1R1 from *T. aureoviride*, by Glucan F2R2 from *T. aureoviride* and *T. viride*-8, by Glucan F1R2 from *T. viride*-1 and *T. harzianum*-30, were used for cloning.

The amplicons were eluted, cloned in pGEM-T Easy Vector and transformed into *E. coli* JM 109 competent cells. High level of recombination was observed on blue-white screening. Recombination of the insert was confirmed by PCR of the plasmid, isolated from white colonies. The cloned fragments were sequenced to obtain the nucleotide sequence information.

The sequences obtained after vector screening were named as GT-311, GT-322, BPT-822 and were subjected to Blast search. All the three sequences revealed significant levels of homology with glucanase genes of different *Trichoderma* spp. The sequences were also subjected to various theoretical sequence analysis using bioinformatics tools, which include ORF finder, GENSCAN, SOPMA, NEB cutter, Hydropathy plot, Interproscan, Motifscan, NASTATS and AASTATS tools of Biology Workbench.

Sequences diversity of β–1,3–glucanase gene among different species of *Trichoderma/Hypocrea* was determined. The evolutionary distance of GT-322 from *T. aureoviride* and BPT-822 *T. viride*-8 was much lower and closely related to β-1,3-glucanase (glu) gene of *T. viride* having accession number EF176582.1. The

two sequences cloned from *T. aureoviride* showed divergence, indicating the divergence of β–1,3–glucanase within the species.

The sequence information obtained from the *Trichoderma* isolates during the present study can be further exploited for full length gene isolation to develop transgenic black pepper and ginger plants with resistance to *Phytopthora capsici* and *Pythium aphanidermatum* and can also be exploited to develop transgenic microorganism with high biocontrol potential.