ISOLATION AND CHARACTERISATION OF β-1, 3-GLUCANASE GENE FROM *Piper* spp.

By

MABLE ROSE GEORGE

THESIS

submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture

(PLANT BIOTECHNOLOGY)

Faculty of Agriculture
Kerala Agricultural University, Thrissur

Centre for Plant Biotechnology and Molecular Biology
COLLEGE OF HORTICULTURE
VELLANIKKARA, THRISSUR - 680 656
KERALA, INDIA

2005

DECLARATION

I hereby declare that the thesis entitled **"Isolation and characterisation of β -1, 3-glucanase gene from *Piper* 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, fellowship or other similar title, of any other University or Society.

Mabl

Vellanikkara

Mable Rose George

CERTIFICATE

Certified that this thesis entitled **"Isolation and characterisation of β -1, 3-glucanase gene from *piper* spp."** is a record of research work done independently by **Miss. Mable Rose George** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship or fellowship to her.

Vellanikkara

Dr. P. A. Nazeem
Chairman, Advisory committee
Associate Professor& Head
Centre for Plant Biotechnology and
Molecular Biology (CPBMB)
College of Horticulture
Kerala Agricultural University,
Thrissur

CERTIFICATE

We, the undersigned members of the advisory committee of **Miss. Mable Rose George,** a candidate for the degree of **Master of Science in Agriculture**, with major field in **Plant Biotechnology**, agree that the thesis entitled **"Isolation and Characterisation of β-1, 3-glucanase gene from *Piper* spp"** may be submitted by Miss. Mable Rose George in partial fulfilment of the requirement for the degree.

Dr. P.A. Nazeem
(Major Advisor, Advisory Committee)
Associate Professor & Head
Centre for Plant Biotechnology and Molecular Biology
College of Horticulture
Kerala Agricultural University
Vellanikkara

Dr. D. Girija
(Member, Advisory Committee)
Assistant Professor,
Centre for Plant Biotechnology and
Molecular Biology,
College of Horticulture,
Vellanikkara

Dr. N.K. Vijyakumar
(Member, Advisory Committee)
Professor (Genetics),
College of Forestry,
Vellanikkara

Dr. P.A. Valsala.
(Member, Advisory Committee)
Associate Professor,
Centre for Plant Biotechnology
and Molecular Biology,
College of Horticulture,
Vellanikkara

Dr. V. Udayasuriyan
External examiner
Professor
Centre for Plant Molecular Biology
Tamil Nadu Agricultural University
Coimbatore

ACKNOWLEDGEMENT

I place deep sense of gratitude and devotion to Jesus Christ for utilizing my efforts to carry out this research work.

I express my unreserved gratitude and incredible indebtedness towards Dr. P. A. Nazeem, Associate Professor and Head, CPBMB, College of Horticulture and the chairman of my advisory committee for the constant support, encouragement, unfailing patience, motherly affection, keen interest, sustained and valuable guidance to me during the course of my research work and preparation of the thesis. I consider myself being fortunate in having the privilege of being guided by her.

I am greatly obliged to Dr.D Girija, Assistant Professor, CPBMB, College of Horticulture for meticulous guidance and constructive suggestions. It would have been impossible to complete this research work without her constant encouragement and unfailing help extended towards me.

I respectfully thank to Dr. N.K. Vijyakumar, Professor (Genetics), College of Forestry for his concern, valuable suggestions and critical evaluation of thesis.

I am grateful to Dr. P.A. Valsala, Associate Professor, CPBMB, College of Horticulture for careful evaluation of manuscript.

I thankfully acknowledge Dr. R. Keshavachandran for giving amble freedom in Bioinformatics centre for computer access.

My sincere thanks to all the staff members of CPBMB, especially Dr. A. Augustin, P.C. Rajendran and Smt. Lissamma for their valuable suggestions and whole hearted support during the period of study.

I deeply express my whole hearted thanks to Ms. P.S. Beena, Senior research fellow, CPBMB for her valuable suggestions and help throughout the research.

My sincere thanks to the labourers of CPBMB for their co-operation and help throughout the study.

I avail this opportunity to thank my loving friends Smitha Jose, Resmy Henry, Smini Varghese, Smita Nair, Blessyl, Smisha, Jismy, Niraj, Nima, Jaliya, Rethidevi, Sreerekha, Seena for making Msc programme meaningful.

I extend myriad of gratitude to Resmy Henry and her family for their affection and guardiance during my study period.

My special thanks to research workers of CPBMB Jithesh, Anish, Preetha, Binoy, Rejith, Sunil, Fathima and Feroz for their wholehearted co-operation.

I thankful acknowledge Shaju chettan, Pradeep chettan, Shileja chechi, Smimi, Sujith and Shayam for their assistance and support for successful completion of thesis work.

My profound thanks to Santhosh chettan, computer club for the assistance rendered during the prepration of the thesis.

Above all, I am highly indebted to my father Kurian George, mother Rosamma Kurian, grandmother and brother Melbin George for their inspiration and moral support.

Mable Rose George

Dedicated to my loving parents and brother

TABLE OF CONTENTS

Chapter	Title	Page. No.
1	INTRODUCTION	1-2
2	REVIEW OF LITERATURE	3-19
3	MATERIALS AND METHODS	20-37
4	RESULTS	38-57
5	DISCUSSION	56-67
	SUMMARY	68-70
	REFERENCES	i-xiv
	APPENDICES	
	ABSTRACT	

List of Tables

Sl. No.	Title	Page No.
1.	Sequence information of ß-1, 3-glucanase of important plants deposited in NCBI databank	11
2.	Details of ß-1, 3-glucanase sequence information used for primer designing.	39
3.	Details of primers derived from the conserved sequences.	39
4.	Expected annealing temperature and amplicon size for the selected primer combinations.	41
5.	Quality and quantity of genomic DNA from *Piper* spp detected using spectrophotometer	41
6.	Yield of amplicon with different primer combinations in *Piper* spp	42
7.	Recombination efficiency in the *E. coli* cells transformed with different amplicons.	45
8.	Homology of *Pnglu* ORF with the other β-1, 3 -glucanase sequences in the public domain.	51
9.	Homology of *Pcglu* ORF with the other β-1, 3 - glucanase sequences in the public domain.	52
10.	Nucleotide statistics of *Pnglu* and *Pcglu* obtained using NASTATS.	54
11.	Restriction sites of *Pnglu*, from *Piper nigrum* with common restriction enzymes.	54
12.	Restriction sites of *Pcglu* with common restriction enzymes.	55
13.	Amino acid composition of deduced sequences from *Pnglu* and *Pcglu*.	56

List of Figures

Sl.No.	Title	Between Page No.
1	pGEM-T easy vector used for cloning PCR product.	31-32
2A.	Multiple sequence alignment of the amino acids of β-1, 3-glucanase from different crops.	38-39
2B	Multiple sequence alignment of the nucleotide sequence for β-1, 3- glucanase from wheat, rice and rubber.	38-39
2C	Multiple sequence alignment of the nucleotide sequence for β-1, 3- glucanase from tobacco, *Prunus* and *Arabdiopsis*.	38-39
3A	Graphical output of sequence in *P. nigrum* obtained with *GluF1R2* primer combination.	48-49
3B	Graphical output of sequence in *P. nigrum* obtained with *GluF2R2* primer combination.	48-49
3C	Graphical output of sequence in *P. colubrinum* obtained with *GluF1R2* primer combination.	48-49
4A	Homology search in cloned fragment in *P. nigrum* obtained using primer combination *GluF1R2* through blastn.	48-49
4B	Results of homology search in cloned fragment obtained in *P. nigrum* using primer combination *GluF2R2.*	48-49
4C	Homology search results of *Pnglu* using blastn tool.	48-49
4D	Homology search results of *Pnglu* using blastx tool.	49-50
4E	Results of homology search in *Pnglu* through blastp tool.	49-50
5A	Homology search results for the cloned fragment *Pcglu* using blastn tool.	49-50

List of Figures contd.

Sl.No.	Title	Between Page No.
5B	Results of homology search in the cloned fragment *Pcglu* through blastx tool	50-51
5C	Results of homology search in the cloned fragment *Pcglu* through blastp tool	50-51
6	Multiple sequence alignment of *Pnglu* with β-1, 3-glucanase sequences in the databank	50-51
7	Phylogram of *Pnglu* showing evolutionary relationship with β-1, 3-glucanase genes deposited in the databank	50-51
8	Multiple sequence alignment of *Pcglu* with β-1, 3-glucanase sequences in the databank	50-51
9	Phylogram of *Pcglu* showing evolutionary relationship with β-1, 3-glucanase genes deposited in the databank.	50-51
10	Open reading frames of *Pnglu* with its largest ORF	50-51
11	Open reading frames of *Pcglu* with its largest ORF	51-52
12A	Exons predicted from *Pnglu* using GENSCAN	55-56
12B	Exons predicted from *Pcglu* using GENSCAN	55-56
13A	The secondary structure of protein *Pnglu* predicted by CHOFAS tool	57-58
13B	The secondary structure of protein *Pcglu* predicted by CHOFAS tool.	57-58

List of Figures contd.

Sl.No.	Title	Between Page No.
14A	Results of conserved domain in the deduced aminoacids from *Pnglu.*	57-58
14B	Results of conserved domain in the deduced aminoacids from *Pcglu*	57-58
15A	Determination of functional domains in *Pnglu* using InterProScan.	57-58
15A	Determination of functional domains in *Pcglu* using InterProScan.	57-58
16	Domain structure of Glycosyl hydrolases family	57-58
17	Kyte-Doolittle hydropathy plot of *Pnglu and Pcglu* protein.	57-58
18A	Restriction map of *Pnglu* with common restriction	65-66
18B	Restriction map of *Pcglu* with common restriction	65-66
19	Depiction of secondary structure of the deduced aminoacids from *Pcglu* and *Pnglu.*	67-68

List of Plates

Sl. No.	Title	Between pages
1	Source plants used for DNA isolation	23-24
2	DNA isolation,amplification and elution in *P. nigrum* and *P. colubrinum* with primer combinations *GluF1R2* and *GluF2R2*	43-44
3	Amplification and elution of DNA in *P. nigrum* and *P. colubrinum* with primer combinations *GluF2R1* and *GluF3R3*	43-44
4	Screening of competent cells and transformation with PCR product	43-44
5	Plasmid DNA isolation from the transformed colonies	44-45
6	Confirmation of recombination in plasmid DNA	46-47

List of Appendices

Sl. No.	Title
1	Composition of reagents and media used for molecular biology works.
2	Sequence information of β-1, 3- glucanase used for primer designing.

ABBREVIATIONS

%	Per centage
A	Adenine
AASTATS	Aminoacid Stastistics
BCM	Baylor College of Medicine
BLAST	Basic Local Alignment Search Tool
bp	Base pair
C	Cytosine
CATH	Class, Architecture, Topology and Homologous Superfamily
CDD	Conserved Domain Database
cDNA	Complementary DNA
CPBMB	Centre for Plant Biotechnology and Molecular Biology
DNA	Deoxy ribonucleic acid
dNTPs	Deoxyribo Nucleoside Triphosphate
EDTA	Ethylene diamine tetraacetic acid
G	Guanine
hr	Hour(s)
IPTG	Iso propyl thiogalactosidase
KD	Kilodalton
LB	Luria Broth
LBA	Luria Bretani Agar
Min.	Minute(s)
mRNA	Messenger RNA
NASTATS	Nucleotide Statistics
NCBI	National Centre for Biotechnological Information
ng	Nanogram
^{o}C	degree celius
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PR	Pathogenesis related
RACE	Rapid amplified cDNA ends

RNA	Ribonucleic Acid
rpm	rotations per minute
RT PCR	Reverse Transcriptase Polymerase Chain Reaction
SDS	Sodium dodecyl Sulphate
T	Thymine
TAE	Tris Acetate EDTA Buffer
TE	Tris EDTA
UV	Ultra Violet
v/v	volume per volume
X-gal	5bromo-4-chloro-3-indoyl-β-D-galactosidase
ηg	Nanogram
µl	Microlitre

INTRODUCTION

1. INTRODUCTION

Black pepper (*Piper nigrum* L), renowned as 'King of spices' is one of the most important export oriented spice crop of India. Kerala accounts for more than 90 per cent of the area under black pepper in India. Unfortunately, productivity of the crop in the state is no way near the world average. The foot rot disease of black pepper incited by the soil borne fungus *Phytophthora capsici* is the major constraint that drastically reduces the production in the country. It is estimated that on a global scale, crop loss due to *Phytophthora* foot rot disease accounts for about 4.5-7.5 million US dollars (Rajan *et al.*, 2002) and a loss of 30 per cent was reported from Kerala (Samaraj and Jose, 1966).

Piper colubrinum Link is an exotic wild species of *Piper*, which shows high degree of resistance to many diseases of black pepper, especially *Phytophthora* foot rot. It is even resistant to root knot nematode infestation. It has good potential as a donor plant in breeding programmes for the improvement of the cultivated species, *Piper nigrum*. It is commonly used as rootstock for black pepper.

Infection of plants by pathogens results in the accumulation of a novel class of proteins referred to as pathogenesis related (PR) proteins (Van Loon, 1985). Recent reports reveal that several classes of PR proteins correspond to hydrolytic enzymes like chitinases and β-1, 3-glucanases. Parab (2000) had revealed the positive role of β -1, 3- glucanase in the defense mechanism of black pepper against foot rot caused by *Phytophthora capsici*.

Presently there are 14 PR protein families and in the PR two group, three basic and four acidic proteins were found to have glucanase activity. The basic isoforms were localized primarily in the central vacuole of the cells, while the acidic forms were extracellular (Bulcke *et al.*, 1989). They are capable of

breaking β glucan chain of cell wall of *Phytophthora capsici*, thus providing induced resistance to the crop.

The incorporation of resistance genes into important crop plants is the major disease control method utilized worldwide in agriculture. This strategy epitomizes effective biological disease control. The gene for β-1, 3-glucanase differs significantly at levels of gene expression, localization and substrate specificity. The gene for β-1, 3-glucanase was isolated from various crops including potato (Beerhues and Kombrink, 1994), tobacco (Linthorst *et al.*, 1995), *Musa* (Chen *et al.*, 1997) and rice (Romero *et al.*, 1998) and is being utilized in crop improvement programme.

Transgenic potato plants expressing soybean β-1, 3-glucanase gene exhibited an increased level of resistance to *Phytophthora infestans* (Borkowska *et al.*, 1998) Isolation and characterization of this native gene in the resistant and susceptible species would be of immense use in modifying the defense mechanism in the cultivated species. Crop improvement programme can thus be targeted for the expression of this defense gene, in the cultivated species.

To understand the regulatory mechanism and for better expression of the plant defense related gene β-1, 3- glucanase, it has to be isolated from tolerant and susceptible species. The information generated can be further exploited for imparting resistance to *Phytophthora* foot rot of black pepper. Hence the present study was taken up with the objective of isolating β-1, 3-glucanase gene fragments from *Piper nigrum* and *Piper colubrinum*.

REVIEW Of LITERATURE

2. REVIEW OF LITERATURE

Black pepper (*Piper nigrum* L.) is one of the most important export oriented spice crops of India with a substantial share in foreign exchange earning of the country. It occupies an area of 225000 ha with production of 78000 t during 2002-2003 (Survey of Indian Agriculture, 2005). However, in productivity, India occupies the last position among the leading black pepper producing countries in the world (Alagappan and Manoharan, 2001). One of the major reasons for this peculiar situation is the high incidence of disease especially the dreadful *Phytophthora* foot rot disease.

2.1. ECONOMIC LOSS DUE TO FOOT ROT OF BLACK PEPPER

There are several reports on economic loss due to the infection of *Phytophthora capsici* in the field. In India, Samaraj and Jose, (1966) reported 20 per cent loss due to this dreadful disease. About 25 to 30 percent loss was reported in Kerala (Nambiar and Sarma, 1977). Balakrishnan *et al* (1986) and Anandaraj *et al.* (1989) reported death of vine upto 9.4 per cent and 3.70 per cent amounting to an annual loss of 905 and 119 millon tonnes of black pepper in Kannur and Kozhikode districts of Kerala respectively. Most of black pepper nurseries in Kerala are also under the threat of foot rot disease of black pepper (Mammooty, 2003). Keeping in view of the economic loses caused by fungal pathogen; conventional breeding programs are in operation to develop disease resistant cultivars. However this approach has not been very successful. Biotechnological approaches can be adopted to compliment conventional breeding as well as to develop transgenic lines of these crops, by incorporating specific genes against foot rot. In this context, isolation of specific genes that impart resistance against foot rot is quite significant.

2.2. SOURCE OF RESISTANCE

Several *Piper spp* were screened for resistance or tolerance to the fungus *Phytophthora capsici* by many workers (Holliday and Mowat, 1963; Ruppel and Almeydar, 1965; Sarma and Nambiar., 1982 ;Vilasiani, 1982). They found that

wild species like *Piper colubrinum* and *P obliqum* showed resistance to *Phytophthora capsici*. The immunity of *P. colubrinum* from Amazon basin against *Phytophthora capsici* was also reported by Sarma *et al.*, (1991). Parab (2000) also confirmed the resistance behaviour of *Piper colubrinum* aganist *P. capsici*.

2.3. PATHOGENESIS RELATED (PR) PROTEINS

Plants attacked by pathogenic microbes respond with a number of protective biochemical changes. Such responses include hypersensitive cell death, the production of reactive oxygen species, accumulation of pathogenesis related proteins, other antimicrobial proteins and biosynthesis of low molecular weight antimicrobial compounds called 'phytoalexins' (Hamond-Kosack and Jones, 1996).

Pathogenesis related proteins are host encoded polypeptides synthesized after infection by pathogens, after treatment with various chemicals or plant hormones. They exist in acidic and basic isoforms (Van Loon, 1985). They are not only accumulated locally but also induced systemically with the development of induced resistant agents upon infection by pathogens. (Van Loon *et.al.*, 1994). Collective function of several PR may be effective in inhibiting pathogen growth, multiplication and maybe responsible for the state of induced resistance (Van Loon *et. al.*, 1997).

Mathieu *et al.* (1985) reported identification of several pathogenesis related proteins in tomato after inoculation with *Cladosporium fulvum*. Two predominant PR proteins purified were ß-1, 3-glucanases, chitinases, and these hydrolytic enzymes, were capable of degrading hyphal walls of *C. fulvum*. Their activity was high in incompatible interactions than in compatible ones. Bera and Puskayashtha, (1997) reported identification and characterization of PR proteins induced by kitazin and *Rhizoctonia solani*.

Go-Young *et al.* (1998) reported in tomatoes, the *Pto* kinase confer resistance to strains of the bacterial pathogen, *Pseudomonas syringae* pv. tomato

that carries the corresponding avirulence gene *AvrPto*. Resistance to bacterial speck disease is initiated by a mechanism involving the physical interaction of the *Pto* kinase and *AvrPto* protein. This initiation signaling events that led to responses including an oxidation burst, the hypersensitive response and expression of pathogenesis-related genes. Tamas *et al.* (1998) reported the accumulation of PR proteins in intercellular space of barley during natural and *in vitro* induced leaf senescence. Their accumulation pattern was different from those of pathogen induced senescence, which indicated different molecular bases of these processes.

2.3.1. ß-1, 3-glucanase

Plant endo ß -1, 3-glucanases (EC 3.2.1.39) are ubiquitous family of evolutionarily related proteins that are able to catalyse the hydrolytic cleavage of ß-1, 3-glycosidic linkages in cell wall of fungal pathogen. They have been described in both monocots and dicots. They are active on insoluble substrates, such as ß-glucan components of fungal cell wall, laminarin and pachyman. They act as both exo and endo hydrolyases. Phaff *et al.* (1979) reported classification of ß-glucanases as ß-1, 3-glucanase, ß-1 6 and ß-1, 4-glucanase based on the type of glycosidic linkages cleaved by them. They reported that ß-1, 3-glucanases in yeast are involved in morphogenetic events, such as cell budding, conjugation and sporulation.

Felix and Meins (1987) found that ethylene induced ß-1, 3-glucanase accumulation and auxin and cytokinin inhibit this induction process in tobacco. Simmons (1994) reported that ß-glucanases are involved in different aspects of plant physiology and development, such as germination, growth, defense against pathogens, flowering, cellular and tissue development and differentiation.

Multiple isoforms of ß-1, 3-glucanases have been isolated from plants. These various forms can be divided into four different classes based on mRNA expression profile, protein isoelectric point and sequence homology (Meins *et al.*, 1992). Class I ß-1, 3-glucanases are pathogenesis inducible, basic, vaculolar proteins that are expressed in roots and older leaves (Beerhues *et al.*, 1994;

Castresana, *et al.*, 1990). Class II, class III and class IV are acidic proteins. Class II and class III isoforms are induced upon pathogen attack but class IV isoforms are non responsive. (Ori *et al.*, 1990; Payne *et al.*, 1990).

Mauch and Stachelin (1989) found that the vacuole localized chitinase and ß-1, 3-glucanase are used as a last line of defense to be released when the attacked host cell lyse. The cell wall localized ß-1, 3-glucanase would be involved in recognition processes, releasing defense activating signaling molecules from the walls of invading pathogens. They found that ß-1, 3-glucanase activate the expression of itself and other defense genes through the production of ß-glucan elicitors.

Kombrink *et al.* (1988) reported higher activity of chitinase and ß-1, 3-glucanase in potato leaves inoculated with pathogenic fungus *Phytophthora infestans*. These PR proteins accumulated in the intercellular space of infected potato leaves and are assumed to play a role in pathogen defense. Neuhaus *et al.* (1992) confirmed that vacuolar class I ß-1, 3-glucanases are involved in defense mechanism of plants to fungal infection, by antisense transformation. Tweddell *et al.* (1994) reported that ß-1, 3-glucanase along with chitinase is responsible for mycoparasitism of *Stachybotrys elegans*, against *Rhizoctonia solani*. Jabekumar *et al.* (2001) observed that ß-1, 3-glucanase activities were significantly higher in *Phytophthora capsici* infected black pepper leaf tissues. They compared their activities in healthy and infected plant. They detected high levels of enzyme in tolerant cultivar.

2.3.1.2. Synergistic activity of chitinase and ß-1, 3-glucanase

Plant β-1, 3-glucanase and chitinase represent potential antifungal hydrolases that act synergistically to inhibit fungal growth *in vitro* (Mauch *et al.*, 1988; Nasser *et al.*, 1990). Kim *et al.* (1993) reported molecular cloning and expression of ß-1, 3-glucanase gene from *Bacillus subtilis* K4. *In vitro* assay against plant pathogenic fungi showed that ß-1, 3-glucanase alone is not sufficient

to account for the antagonistic effect of *B. subtilis* K4. It was suggested that another type of antifungal activity might exist in *B. subtilis* K4.

Jonedijik *et al.* (1995) reported that simultaneous expression of a tobacco class I chitinase and a class I ß-1, 3-glucanase gene in tomato resulted in increased fungal resistance. They found that transgenic tomato plants expressing either one of those genes were not protected against fungal infection. Chawla (2003) reported increased resistance to fungal pathogens by ß-1, 3-glucanase genes in association with chitinase gene.

2.3.1.3. Role of β-1, 3- glucanase

Abeles *et al.* (1971) were the first to report that glucan hydrolases have role in the biochemical defense against plant pathogens. Since many fungi have cell walls rich in chitin and β-1, 3-glucan, they hypothesized that these two hydrolases might help the plants defend against pathogenic fungi. Dake *et al.* (2004) reported that β-1, 3-glucanase purified from *Aureobasidium pullens*, a saprophytic mold are capable of acting on yeast β glucan and laminarin substrates. It cleaves the substrate from non reducing end, liberating glucose residues.

Mauch and Stachelin (1989) found that β-1, 3-glucanase solubilizes elicitor active glucan molecules from the walls of invading fungal pathogens, thereby inducing its own production and that of other defense enzymes involved in the synthesis of antimicrobial phytoalexins and cell wall barriers.

Kim and Hwang (1997) reported that β-1, 3-glucanase have inhibitory activity against chitin negative *Phytophthora capsici* but shown no antifungal activity against the chitin containing fungi *Alternaria mali, Collectotrichum gloeosporiodes, Magnaportha grisea* and *Fusarium oxysporum* f.sp. *cucumerinum.*

Parab (2000) reported that β-1, 3-glucanase have a positive role in the defense mechanism of black pepper in relation to *Phytophthora* foot rot disease. Achuthan *et al.* (2002) reported the accumulation of β-1, 3-glucanase in relation to

Phytophthora rot of black pepper. They found that the expression of two additional bands with molecular weight 16.5 kDa and 8 kDa in tolerant variety while it was delayed upto fifth day in the susceptible variety. One of the over expressed defense proteins in black pepper characterized was β-1, 3-glucanase. Jayaraj *et al.* (2004) reported accumulation of ß-1, 3-glucanase and thaumatin like proteins in wheat by the application of jasmonic acid and salicylic acid. This induced resistance in wheat to *Stagonospora nodorum.*

2.3.1.4. Mechanism of resistance by ß-1, 3-glucanase on P. Capsici

Hwang *et al.* (1989) reported that *P. capsici* initially grows in intracellular space of pepper plants, the fungus make contact with ß-1, 3-glucanase and chitinase molecules probably localized in the middle lamella along the air spaces. Upon contact, the ß-1, 3-glucanase is postulated to release oligosaccharide fragments from the ß-1, 3-glucan containing fungal cell wall. These oligosaccharides are demonstrated to act as elicitors of phytoalexin production. Kim and Hwang (1994) found that like other PR proteins, post-infectionally formed phytoalexin are important determinate for resistance to *P. capsici.*

Kim and Hwang, (1994) reported that *P. capsici* infection induced the synthesis and accumulation of ß-1, 3-glucanase and chitinases in the stem tissues of pepper plants. They detected differential accumulation of enzymes and found that acidic isoform of glucanase was found to associate with *P. capsici* resistance. Garcia-Perez *et al.* (1998) reported that cell suspension cultures of *Capsicum annuum* shown different degrees of sensitivity to the fungus *Phytophthora capsici.* They showed conductivity changes, browning, production of the phytoalexin capsidol and synthesis and accumulation of pathogenesis related proteins with glucanase and chitinase activity.

2.3.2. Regulation of β-1, 3- glucanase

Mauch *et al.* (1988) reported differential regulation of β-1, 3-glucanase in pea tissue. The different molecular forms of β-1,3-glucanase were differentially regulated on inoculation with compatible or incompactible strains of *Fusarium*

solani, wounded, or treated with chitosan or ethylene. One of the common effector involed in the activation of defense genes is probably plant hormone ethylene. Memelink *et al.* (1990) reported that genes encoding basic and acidic isoforms of pathogenesis related proteins are differentially regulated. Cote *et al.*, (1991) reported in tobacco, that thiamine and tobacco mosaic virus (TMV) infection resulted in a dramatic increase in the levels of β-1, 3 glucanase mRNA, protein and enzyme activities. They found that PR-2 gene expression is regulated, in part, at the level of mRNA accumulation. Eldik *et al.* (1998) reported truncated products are more abundant in silenced plants than in expressing plants.

Slakeshi and Fincher (1992) reported the developmental regulation of ß-1, 3-glucanase gene expression in barley. They found tissue specific expression of individual isoenzymes. Holtorf *et al.* (1999) suggested that post transcriptional gene silencing in tobacco plant carry class I chitinase and β-1, 3-glucanase transgene suggesting that increased RNA turnover may be a general feature of post transcriptional gene silencing. Ko *et al.* (2003) reported that alternate members of ß-1, 3-glucanase genes were expressed at different stages of development. In higher plants, ß-1, 3-glucanase activities increases in response to pathogen infection or hormonal treatment.

2.3.2.1. Induction of defense related genes in relation to pathogen attack

Linthorst *et al.* (1990) reported strong induction of acidic and basic ß-1, 3-glucanases in tobacco plant after infection with tobacco mosaic virus. After southern blot analysis, they detected that the tobacco genome contains approximately eight genes for acidic ß-1, 3 glucanase and a smaller number of genes encoding basic ß-1, 3-glucanases. They cloned and sequenced genes for both gene families of glucanases. Chang *et al.* (1992) suggested that ß-1, 3-glucanases inductions may represent part of a basic resistance response to pathogen invasion and is not necessarily specific to incompatible host fungal interactions. Rouhier *et al.* (1995) isolated ß-D, glucans from cell walls of *Phytophthora capsici* and structural analysis revealed that ß-D glucans had common ß-1, 3 linkages.

Thanseem *et al.* (2005) reported differential expression of ß-1, 3-glucanase mRNA in tolerant RRII 105 and susceptible RRIM 600 clones in response to infection by *Phytophthora meadii*. They found that the susceptible RRIM 600 clone, the tolerant RRII 105 clone had a higher rate of increase and a more prolonged induction. They noted that tolerant clone was associated with prolonged expression of the gene following infection. The antifungal activity of these hydrolyses enzymes made them rational candidates for over expression by genetic transformation to produce disease resistant crops.

Vogeli-Lange *et al.* (1998) observed a parallel increase in the chitinase and ß-1, 3-glucanase mRNAs in tobacco, following tobacco mosaic virus infection indicating that both are co-coordinately induced at the mRNA level and found that the induction in part of the hypersensitive reaction represents a highly typical defense of plants to pathogens.

2.3.2.2. Nature of ß-1, 3- glucanase gene

Bulcke *et al.* (1989) reported different isoforms of ß-1, 3-glucanases in tobacco after infection with *Pseudomonas syringae* or salicylic acid application. The protein sequence analysis found that each of the different isoforms had a unique amino acid sequence and hence encoded by a different gene. In potato leaves, chitinase and ß-1, 3-glucanase exists in multiple isoforms and activities increase strongly upon infection by the late blight fungus *Phytophthora infestans* (Kombrink *et al.*, 1988; Schroder, 1992). Beerhues *et al.* (1994) revealed that potato ß-1, 3-glucanase contains a N terminal hydrophobic signal peptide and a C terminal extension. DNA blot analysis indicated that in potato ß-1, 3-glucanase, is encoded by complex gene families.

2.4. ISOLATION OF β-1, 3 GLUCANASE GENE

β-1, 3-glucanase gene was isolated from various crops. Table. 1 represents β-1, 3-glucanase gene isolated from important crops. A number of approaches are being adopted to clone plant genes conferring resistant to fungal pathogens (Ellis

Table.1 Sequence information of ß-1, 3- glucanase of important plants deposited in the NCBI

Sl.No.	Common name	Scientific name	Accession No.	Source	Size	Author
1	Rubber	*Hevea brasiliensis*	AY325498	DNA	1468	Thanseem *et al.*, 2005
2	Rice	*Oryza sativa*	AB070742	DNA	2342	Yamaguchi *et al.*, 2002
3	Potato	*Solanum tuberosum*	AF067863	DNA	2289	Kwon *et al.*, 1999
4	Barley	*Hordeum vulgare*	AF479647	DNA	2135	Zeng *et al.*, 2002
5	Coffee	*Coffea arabica*	AY389812	mRNA	2004	Guzzo *et al.*, 2003
6	Tobacco-basic	*Nicotiana tobacum*	M59442	DNA	4766	Linthorst *et al.*, 1990
7	Tobacco-acidic	*Nicotiana tobacum*	M59443	DNA	2259	Linthorst *et al.*, 1990
9	Banana	*Musa acuminata*	AF001523	mRNA	1141	Chen *et al.*, 1997
10	Tomato	*Lycopersicon esculentum*	M80608	mRNA	1331	Van Kan *et al.*, 1992
11	Grapes	*Vitis vinifera*	AF239617	mRNA	1083	Bezier *et al.*, 2003
12	Wheat	*Triticum aestivum*	DQ078255	mRNA	1337	Cui and Kang, 2005
13	Cow pea	*Pisum sativum*	S51479	DNA	1460	Chang *et al.*, 1992
14	Strawberry	*Fragaria x ananassa*	AY989819	mRNA	1271	Shi *et al.*, 2005

et.al., 1988). Cloning of these genes is a first step toward the identification of regulatory elements involved in their co-ordinate action.

Kwon *et al.* (1999) isolated a genomic clone for potato β-1, 3-glucanase genes by plaque hybridization from potato genomic library using cDNA clone for tobacco beta-1, 3-glucanase as probe. After sequencing of the clone, it was found that β-1, 3-glucanase genes was a genomic counterpart of the β-1, 3-glucanase gene from different potato cultivar. Helleboid *et al.* (2000) reported isolation of three different ß-1, 3-glucanase cDNA fragments by reverse transcriptase - PCR approach from *Cichorium.*

Payne *et al.* (1990) reported isolation and expression of cDNA clones encoding an acidic form of β-1, 3-glucanase from tobacco. The cDNA was expressed in *E. coli* and shown to encode β-1, 3-glucanase activity. A detailed study of the cDNA indicated that this glucanase represents a third structural class of enzyme, which differs substantially from both the basic, vacuolar glucanase and the acidic extra cellular forms.

The isolation and characterization of β-1, 3-glucanase genes in apple was carried out by screening of genomic library using a β-1, 3-glucanase cDNA clone from maize as probe (Thimmapuram *et al.*, 1994). Yamaguchi *et al.* (2002) reported isolation of cDNA for β-1, 3-glucanase from rice anther and found that they belonged to monocotyledonous endo-β -1, 3-glucanase subgroup A. Zing *et al.* (2003) observed full length β-1, 3-glucanase cDNA by RT-PCR and RACE technique from Tibet hull less barley and its complete sequence obtained by DNA walking. Jung *et al.* (2000) reported on the isolation of specific cDNA genes, differentially or strongly expressed in pepper leaves infected with avirulent strain of *Xanthomona campesris* pv. *vesicatoria* using the differential hybridization technique. They cloned and sequenced different genes for pathogenesis related proteins including β-1, 3-glucanase, found pronounced identities based on sequence comparison with previously known genes

Ekinci *et al.* (1997) reported isolation a gene encoding an extra cellular β-(1,3-1,4)-glucanase from *Streptococcus bovis* B1. The isolation of specific cDNA genes, differentially or strongly expressed in pepper leaves infected with a virulent strain a of *Xanthomona campesries* pv. *vesicatoria* using the differential hybridization technique (Jung *et al.*, 2000). Two new β-glucanase encoding genes have been isolated even from plant pathogenic fungus *Cochleobolus carbonum* using polymerase chain reaction (Kim *et al.*, 2001). Dicto and Manjula (2005) identified elicitor induced PR 5 gene homologue in *Piper colubrinum* Link by suppression subtractive hybridization. β-1, 3-glucanase has also isolated and cloned from pinewood *Bursaphelenchus xylophilus*, aquired by horizontal gene transfer from bacteria (Kikuchi *et al.*, 2005). The protein is probably important in allowing the nematodes to feed on fungi.

2.4.1. Degenerate primers

A degenerate primer is a mixture of primers, all of similar sequence but with variation at one or more positions. It is commonly used when the primer sequences have to be deduced from amino acid sequence (Lee et al., 1988). It can also be employed to search for novel members of known family of genes (Wilks, 1989) or to search for homologous genes between species (Nunberg *et al.*, 1989). When a degenerate primer is designed on the basis of amino acid sequence, the degeneracy of the genetic code must be considered and avoids the degeneracy at the 3 ' terminal of the primer.

2.4.1.1. *Primer designing*

Glucanase genes from different plant species are conserved within structural domains, although degree of similarity varies (Teucker and Melligan, 1991; Lashbrook *et al.*, 1994). These conserved regions can be utilized in designing primers for the amplification of the glucanase gene from different plant species. A small gene family encodes the PR-2 (β-1, 3-glucanase) proteins in tobacco, and similar genes are present in number of plant species (Cote *et al.*, 1991).

2.4.2. Transformation in *E. coli*

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 transformable host. It has been found by several groups of workers that *E. coli* cells and plasmid DNA interact productively in an environment of calcium ions and low temperature (0-5^{0}C), and that a subsequent heat shock (3^{0}C-45^{0}C) is important. The calcium chloride affects the cell wall and might be responsible for binding DNA to the cell surface. The actual uptake of DNA is stimulated by the brief heat shock (Old and Primrose, 1994).

2.4.2.1. Confirmation tests for transformation.

Sambrook *et al.* (1989) reported restriction analysis of plasmid DNA is the method of choice when there is a high chance of finding the desired recombinant within a small number of randomly chosen transformed colonies. 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*). Embedded in this coding region is 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. Host cells of this type vectors code for carboxyl terminal portion of β-galactosidase. The host encoded or vector encoded protein are not active but during transformation, α complementation results to form enzymatically active protein (Ulmann *et al.*, 1967).

The lac+ bacteria that result from α complementation are easily recognized because they form blue colonies in the presence of the chromgenic substrate X-gal. (Horwitz *et al.*, 1964). However insertion of a fragment of foreign DNA into the polycloning site of the plasmid almost invariably results in the production of an amino terminal fragment that is not capable of complementation. Bacteria carrying recombinant plasmid form white colonies and bacteria carrying non recombinant form blue colonies. It is easily possible to screen many thousands

of colonies visually and recognize colonies that carry putative recombinant plasmids.

2.4.3. Molecular characterization of β-1, 3- glucanase gene

Characterization of rice β-1, 3-glucanase gene was sequenced and its expression at the mRNA level was reported (Simmons *et al.*, 1992). They found that shoot express glucanase *GnS1* at much higher levels when treated with ethylene, cytokinin, salicylic acid, wounding and fungal elicitors derived from the pathogen *Sclerotium oryzae* or from the non pathogen *Saccharomyces cerevisae*. The Nucleotide and deduced amino acid sequence are reported for the 4132 bp clone beta-1, 3-glucanase gene containing 1469 bp of 5^1 flanking promoter region, 2 exons interrupted by a 1099 bp intron and 451 bp of 3^1 fanking region (Chang *et al.*, 1993).

Gheysen *et al.* (1990) reported the characterization of ß-1, 3-glucanase genes encoding a vacuolar isoform in *Nicotiana plumbaginifolia*. The coding region is contained within two exons and encodes a precursor protein of 365 aminoacids containing a 32 aminoacid N- terminal signal peptide for translocation to the endoplasmic reticulum. Cote *et al.* (1991) reported the isolation and sequencing of a partial cDNA clone encoding an acidic, extra cellular form of a PR ß-1, 3- glucanase from tobacco. They characterized the structure of the acidic β-1, 3-glucanase genes and examined their expression in stressed tissue and during normal floral development in tobacco. The existence of different acidic ß-1, 3-glucanase is forms and the southern blot analysis suggest that the acidic ß-1, 3-glucanases are encoded by a small gene family. Nakumara *et al.* (1995) was reported cloning and sequencing of a cDNA for popular endo ß-1, 4 -glucanase. Hydropathy plot analysis of the deduced amino acid sequence suggested that poplar endo ß-1, 4 -glucanase have cellulose catalytic domain.

ß-1, 3-glucanase has been characterized in rice genome. (Romero *et al.*, 1998). Thirteen new beta glucanase encoding genes have been identified in the rice genome. These genes have now been classified into four subfamilies based on the

structure function of the genes. Subfamily A includes defense related subfamily, which includes two tandem clusters of genes *Gns2- Gns3- Gns4* and *Gns 5 –Gns6.*

Characterizations of ß-1, 3-glucanase gene in elongating pea epicotyls have been reported by Wu *et al.* (1996). Gueguen *et al.* (1997) reported molecular and biochemical characterization of an endo-β-1, 3-glucanase of the hyperactive thermophilic archeon *Pyrococcus furiosus.* Mateos *et al.* (1997) reported cloning and characterization of a cDNA encoding an elicitor of *Phytophthora parasitica* var. *nicotianae* that shows cellulose binding and lectin like activities. It is produced a 34 kDa glycoprotein elicitor that in localysed is the cell wall. Zanor *et al.* (2000) characterized ß-1, 3-glucanase genes from *Hordeum vulgare* and detected that amplified sequence contained the entire coding region of the isozyme II, which is interrupted by a 165-bp intron at 73 bp downstream of the starting codon.

Since ß-1, 3-glucanase gene have been isolated and characterized from different plants, fungi and bacteria, charcterisation in virus was first reported by Sun *et al.* (2000). They characterized ß-1, 3-glucanase encoded by chlorella virus PBCV-1. Sequence analysis of 330 kb chlorella virus PBCV-1 genome revealed an open reading had significant identity to ß-1, 3-glucanase. They reported that the protein is expressed in early stage and got disappeared during early stage of replication.

2.4.4. Sequence comparison

Nucleotide sequence of *Hevea* β-1, 3-glucanase showed 68 per cent similarity to that of *N. plumbaginifolia gnl* cDNA. Comparison of the predicted amino acid sequence of *Hevea* β-1, 3-glucanase with that of class I, β-1, 3-glucanase encoded by *gnl* shows 66 per cent amino acid homology (Chye and Cheung, 1995).

Chang *et al.* (1992) reported cloning and sequencing of a partial pea cDNA clone, corresponding to a β-1, 3-glucanase in pea endocarp challenged with the incompatible pathogen *Fusarium solani* f.sp. *phaseoli.* The predicted amino

acid sequence of the pea β-1, 3-glucanase has 78 per cent identity to bean β-1, 3-glucanase, 62 per cent and 60 per cent to two tobacco β-1, 3-glucanase, 57 per cent to soyabean β-1, 3-glucanase, 51 per cent barley β-1, 3-glucanase, and 48 per cent to barley β-1, 3-glucanase. They noticed sustained levels of β-1, 3-glucanase mRNA expression induced by the incompatible pathogen, suggests that the enzyme contribute to the pea plants general defense.

Kwon *et al.* (1999) reported that cloned β-1, 3-glucanase gene was 2.3 kb fragment. It was a genomic counterpart of the β-1, 3-glucanase gene from different potato cultivars and consisted of two exons and one intron encoding a protein of 315 amino acid residues. The major transcription initiation site was determined by primer extension and it appeared to be at 27 bp upstream of the translation initiation site. The canonical TATA box and AGC enhance elements were found in the promoter region of gene and the sequence comparison revealed that the relevant promoter region was more similar to that of tobacco than that of rice or barley.

Chang *et al.* (1992) reported cloning and sequencing of pea genomic clone, which corresponds to β-1, 3-glucanase in pea pods challenged with the incompatible pathogen *Fusarium solani* f.sp. *phaseoli.* The predicted amino acid sequence of the pea β-1, 3-glucanase showed 78 per cent identity to bean (*Phaseolus vulgaris*) β-1, 3-glucanase, 62 and 60 per cent of two tobacco β-1,3-, 57 per cent to soyabean β-1, 3-glucanase, 51 per cent to barley β-1, 3-glucanase and 48 per cent to barley β-1, 3-glucanase. In the incompatible reaction, mRNA accumulation remained high for 48 hr, whereas it rapidly decreased in the compatible reaction.

Ko *et al.* (2003) reported the characterization of a peach β-1, 3-glucanase gene family including two newly isolated basic beta glucanase genes and linked in a tandem array. The deduced mature proteins share 90 per cent amino acid sequence identity.

2.4.3. Structure and expression

Ohme-Takagi and Shinshi (1990) determined the primary structure of a tobacco β-1, 3-glucanase gene. It has single large intron, and the intron separates coding regions of the signal peptide and the mature enzyme. Analysis of the 5^1 flanking regions revealed an 11 bp GC rich element with perfect homology with the putative regulatory sequence of tobacco chitinase genes.

Takeuchi *et al.* (1990) reported molecular cloning and ethylene induction of mRNA encoding β-1, 3-glucanase, which is a phytoalexin elicitor-releasing factor in soybean. Harpster *et al.* (1998) reported the isolation of cDNA encoding, an endo-1, 4-beta glucanase from ripe fruits of strawberries (*Fragaria x ananassa*). The deduced protein of 496 amino acids contains a presumptive signal sequence and one potential N-glycosylation site. Khan *et al.* (2003) reported cloning of β-1, 3-glucanase gene from strawberry (*Fragaria x ananassa* Duch). This glucanase gene composed of two exons and one intron. The location of intron in the gene was confirmed by sequencing a partial cDNA clone obtained by using rapid amplification of cDNA ends (RACE).

2.5. GENETIC TRANSFORMATION

To combat fungal pathogens, many plants have been transformed with genes that code for ß-1, 3-glucanase. The genetically engineered plants over expressing PR proteins have been shown to be resistant to pathogen infection (Alexander *et al.*, 1993). Transgenic potato plants expressing soybean ß-1, 3-endoglucanase genes exhibited an increased level of resistance to *P. infestans* (Borkowska *et al.*, 1998). Ning *et al,* (2001) reported the expression of beta glucuronide under the control of 940 bp rice ß-1, 3-glucanase promoter was developmentally regulated and could only be detected in rice calluses but not in leaves, roots or seeds. This prevents the expression of a selectable marker gene product in transgenic rice seeds.

Libantova *et al.* (1998) reported transgenic tobacco (*Nicotiana tobacum* cv. Petil Havana SRI) and potato (*Solanum tuberosum* cv. Desiree) plants constitutively over expressing the ß-1, 3-glucanase from *Nicotiana plumbaginifolia* under the control of the cauliflower mosaic caulimovirus 35S promoter.

Hou *et al.* (1999) constructed plant expression vector harbouring the ß - 1, 3-glucanase gene. A plant expression vector, *Pbinh-Glu*, was constructed by inserting the modified ß-1, 3-glucanase gene into a modified plasmid *pBinh* containing a CaMV 35S promoter, a nos (Nopaline synthase) terminator and the *npt* (Neophosphotransferase) *II* gene.

Transient transformation of wheat protoplasts with the complete ß-1, 3-glucanase gene under the control of maize polyubiquitin promoter revealed high expression of gene. Chang *et al.* (2002) reported co-transformation of a pea, ß-1, 3-glucanase and chitinase genes to produce transgenic potatoes. Wang *et al.* (2003) reported co-transfer and expression of chitinase, glucanase and bar genes in bent grass. It exhibited resistance to fungal pathogens, *Sclerotina homoeocarpa* and *Rhizoctonia solani.*

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The present study was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture during the period from December 2003 to December 2005. The details of the work carried out are presented in this chapter.

Attempts were made to isolate the ß-1, 3-glucanase gene fragments from two species of *Piper* namely *Piper nigrum,* which include cultivated species of black pepper and *Piper colubrinum,* the exotic wild type which express total resistance to the *Phytophthora* foot rot disease. Since no sequence information has been reported so far for the genes encoding ß-1, 3-glucanase in *Piper* species, the work was included with the data available in the public domain from other crop species. The sequence information of plant ß-1, 3-glucanase genes available in the public domain NCBI (National Centre for Biotechnological Information) was collected. These sequences were subjected to multiple sequence alignment to detect conserved boxes of ß-1, 3-glucanase genes among different plant species. Based on the data, primers were designed and synthesized. Genomic DNA of *Piper nigrum* and *Piper colubrinum* was isolated and subjected to polymerase chain reaction with the designed primers at different thermal settings. Agarose gel electrophoresis was performed and amplicons of desired size were eluted, ligated and cloned in suitable vector. The cloned products were sequenced and further analysed using bioinformatics tools.

3.1 DATA SEARCH IN THE PUBLIC DOMAIN

The sequence information available in the public domain NCBI (www.ncbi.nlm.nihigov/) was accessed for collecting the recent information about ß-1, 3-glucanase genes.

3.1.1 Downloading sequences from NCBI

NCBI site was visited and searched for nucleotide sequences for plant ß-1, 3-glucanase. Complete mRNA or cDNA sequences encoding ß-1, 3-glucanase in tobacco, *Arabidopsis*, *Prunus*, rice, wheat and rubber were copied in FASTA format in to a notepad. Similarly, amino acid sequences of ß-1, 3-glucanase from the same plants were saved in FASTA format in another notepad.

3.1.2 Multiple sequence alignment

Multiple sequence alignment for the downloaded amino acid and nucleotide sequence of ß-1, 3-glucanase was carried out using BCM (Baylor College of Medicine) search launcher (http://searchlauncher.bcm.tmc.edu; Smith *et al.*, 1996). Multiple sequence alignment was selected for the purpose and 'start sequence launcher' was selected. The nucleotide sequence of the gene in FASTA format obtained from different plants was pasted in the displayed box and then submitted. The sequences displayed in FASTA format were selected, copied and 'box shade server' was selected. On the new web page, the following parameters were selected:

a) Format - RTF old b) Input sequences - other.

The copied sequences were pasted in the box provided and selected 'Run box shade'. Then selected 'Here is your output No.1'. Output result displayed was saved and closed the window. In the same way, multiple sequence alignment of the corresponding amino acid sequences was carried out using BCM search launcher.

3.1.3 Detection of conserved sequences

In the output of multiple sequence alignment, the base pairs are displayed in three different colours - red, blue and black. If all the sequences have the same nucleotide in a particular position it will be indicated by red colour, which shows complete alignment. If one of the nucleotides at a particular position belongs to either purines or pyramidines, it will be indicated by blue colour representing partial alignment. If the nucleotide at a particular position belongs to both purines and pyramidines it will be indicated by black colour, which shows no homology. Conserved amino acid boxes showing only red region were selected.

The location of corresponding nucleotide sequence was found based on the degeneracy of amino acid, multiplying amino acid location with 3. Thus conserved boxes of 18-24 bases were selected throughout the nucleotide sequence based on certain parameters. They are as follows:

a) The conserved sequences selected should have GC content not less than 50 per cent

b) Melting temperature (Tm) of each conserved sequence ranged between 60 and 70°C.

Melting temperature was calculated based on the formula Tm = 4 GC + 2 AT, where, Tm is the melting temperature, GC is sum of guanine and cytosine content in the sequence, AT is sum of adenine and thymine content in the sequence.

3.1.4 Primer designing

The forward and reverse primers were selected from the conserved boxes in such a way that the distance between the primers ranged from 500-800 base pairs. For designing primers, the sequence of the forward primers was taken as such and for the reverse primer, the reverse complementary sequence was taken. The following points were considered in designing the primers.

- It is preferable to have GC content at 3'end.
- The designed forward and reverse primers should not be complementary.
- Repeats of single base should not appear within primer sequence.
- There should not complementary sequences at 5' and 3' end of a single primer.
- The distance between forward and reverse primer should be greater than 500bp.

3.2. MAINTENACE OF SOURCE MATERIAL

Sixty rooted cuttings, each of *Piper nigrum* variety Panniyurl and *Piper colubrinum* were maintained in potting mixture in the green house condition (28-30°C and 80 per cent relative humidity) for the isolation of genomic DNA. (Plate 1). The plants were maintained by regular watering and manuring such that tender leaves were available throughout the research period.

3.2.2 Chemical, glassware and plasticware

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 plasticware used was obtained from Axygen and Tarson India Ltd. The primers were synthesized at Banglore Genei.

3.2.3 Equipment and machinery

The equipment available at Centre for Plant Biotechnology and Molecular biology and the Bioinformatics Centre, College of Horticulture was used for the present study. The PCR was done in PTC 200 ™ Programmable Thermocycler MJ research and horizontal gel electrophoresis system was used for agarose gel electrophoresis. Biorad imager Gel Doc XR was used for imaging the gel.

3.3 SOURCE FOR GENE AMPLIFICATION

The genomic DNA of *Piper* spp was used as the template for the amplification of ß-1, 3-glucanase gene.

3.3.1 Isolation of DNA from *Piper* spp.

The procedure suggested by Doyle and Doyle (1987) was used with appropriate modification for DNA isolation in *Piper nigrum* and *Piper colubrinum*.

Reagents

1. 4X extraction buffer

A. *Piper colubrinum*

B. *Piper nigrum*

Plate 1. Source plants used for DNA isolation

2. Lysis buffer
3. Sarcosine (5%)
4. TE Buffer (Tris 10.0 mM, EDTA 1.0 mM)
5. Chloroform: isoamyl alcohol (24:1 v/v)
6. Chilled isopropanol
7. Ethanol 70 per cent and 100 per cent.

Details of preparing all the reagents are provided in the Appendix I. The procedure followed for DNA isolation is as follows.

- 0.5 g of tender leaf tissue was ground in liquid nitrogen with the following items.

1x Extraction buffer	- 3 ml
Sodium metabisulphite	- 10 mg
ß mercaptoethanol	- 50 µl

- The homogenate transferred individually into 30 ml oakridge centrifuge tube containing prewarmed lysis buffer and added 1 ml of 5 per cent sarcosine, maintained at 65°C for 15 to 20 min in water bath.
- The contents were mixed gently and added equal volume (7 ml) of chloroform: isoamyl alcohol mixture.
- Centrifuged the homogenate at 10,000 rpm for 15 min at 4°C.
- Upper aqueous phase was saved and transferred to fresh centrifuge tube after checking the volume.
- To this 0.6 volume of chilled isopropanol was added and mixed gently, stored at -20°C for 30 min for precipitation of DNA.
- Pelleted the DNA by centrifugation at 10,000 rpm for 15 min at 4°C.
- The pellet was saved and washed with 70 per cent ethanol, followed by absolute alcohol.
- The pellet was air dried for 30 min, dissolved in 500 µl TE buffer and stored at -20°C.

3.3.2 Purification of DNA

The DNA of *Piper nigrum* and *Piper colubrinum* was purified by phenol precipitation and RNase treatment (Sambrook *et al.*, 1989).

Reagents used are as follows

1. Phenol: chloroform mixture (1:1 v/v)

   To one part of phenol, one part of chloroform was added and mixed properly.

2. Chilled isopropanol
3. 70 per cent ethanol
4. TE buffer
5. Chloroform: Isoamyl alcohol (24:1 V/V)
6. Preparation of RNase

The RNase A from sigma, USA was used to prepare RNase. 1 per cent solution was prepared by dissolving RNase A in TE buffer at 100°C for 15 min. The solution was cooled to room temperature, dispensed into aliquots and stored at -20°C.

The Procedure followed for DNA purification is as follows.

- Two µl RNase solutions was added to 100 µl DNA sample and incubated at 37°C in dry bath (Genei, Thermocon) for one hour.
- The volume was made up to 250 µl with distilled water and equal volume of phenol: chloroform mixture was added.
- Centrifuged at 12,000 x g for 10 min at 4°C.
- Collected aqueous phase of the sample in a fresh micro centrifuge tube and added equal volume of chloroform: isoamyl alcohol mixture (24:1)
- Centrifuged at 12,000 x g for 10 min at 4°C.
- Repeated the above two steps and finally precipitated DNA from the aqueous phase with 0.6 volume of chilled isopropanol
- Incubated the mixture at -20°C for 30 min and centrifuged at 10,000 rpm for 15 min at 4°C.
- The DNA pellet was washed with 70 per cent ethanol.
- Air dried the pellet and dissolved in 25 µl TE buffer.

3.3.3 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

a) Agarose: 0.7 per cent (for PCR samples)

b) Agarose: 1.0 per cent (for genomic DNA)

c) TAE buffer 50X (pH 8)

d) Loading dye Bromophenol blue

e) Intercalating dye Ethidium bromide

The procedure followed for agarose gel electrophoresis is as follows.

- The TAE buffer 50X was diluted to 1X concentration.
- The open ends of the pyrex gel casting tray were sealed with a cellotape and placed on a perfectly horizontal leveled platform with properly set comb.
- Required quantity of agarose was added to corresponding volume of 1X TAE buffer, boiled till the agarose dissolved completely and then cooled to lukewarm temperature.
- Ethidium bromide (0.5µg ml^{-1}) was added to agarose solution and poured into the gel casting tray
- After solidification of the gel the comb and cellotape were removed carefully.
- The casted gel was placed in the electrophoresis tank containing 1X TAE buffer with the wells near the cathode.
- DNA sample (5 µl) was mixed with 1 ml loading dye and loaded into the wells of the gel.
- After closing the tank, the cathode and anode were connected to power pack and the gel was run at the constant voltage (100 volts) and current (50 A).
- The power was turned off when the tracking dye reached at about two third length of the gel.
- The gel was then observed in UV transilluminator and documented in gel documentation system (Biorad, Gel Doc XR).

3.3.4 Quantification of DNA

The genomic DNA isolated and detected through agarose gel electrophoresis were further evaluated for its quantity using UV spectrophotometer (Spectronic Genesys 5). The DNA sample (3 µl) was diluted to 500 µl with sterile water. The absorbance was measured at 260 nm and 280 nm and DNA concentration was calculated using the following relationship.

1 O D at 260 nm = 50 ng/µl DNA

3.4. POLYMERASE CHAIN REACTION FOR AMPLIFICATION OF THE GENE

The genomic DNA was amplified by PCR (MJ research,USA Peltier PTC 200.) in 50 µl reaction mix using different designed primers at varying annealing temperature depending upon the GC content of primers as described in 3.1.4. The thermal settings followed are as follows.

i) Initial denaturation at 94°C for 2 min

ii) Denaturation at 94°C for 45 sec

iii) Different anealing temperature as per primer combination for 1 min

iv) Primer extension at 72°C for 10 min

v) Cooling at 4° for 5 min.

Each reaction mixture for PCR had the following reagents.

a) Genomic DNA - 5 µl (50 ng)

b) Taq assay buffer – 1X

c) dNTP mix - 2 µl (10 mM solution)

d) Forward primer - 2 µl (2.5 pmoles)

e) Reverse primer - 2 µl (2.5 pmoles)

f) *Taq* polymerase - 0.6 units

The total volume was made upto 50 µl with autoclaved distilled water. All the reaction mixture was set in 200 µl microfuge tubes chilled over ice flakes. A momentary spinning was given for the reaction and set in thermal cycler for polymerase chain amplification under suitable programme with a heated lid condition. The PCR product was checked on 0.7 per cent agarose gel and documented. The different primers designed were used for PCR amplification at

different anealing temperatures. Different concentration of template DNA, primer combinations and the enzyme were tried for obtaining optimum amplification.

3.4.1 Gel elution of PCR amplified fragments

Desired DNA fragments obtained in different PCR reactions were loaded separately on 0.8 per cent low melting agarose gel and desired amplicon in each case was eluted using gel elution minikit (Clean Genei Kit, Bangalore Genei Pvt. Ltd.). Procedure followed as per the manufacturer's guidelines:

- DNA fragment was excised from the gel using a sterile sharp scalpel UV transilluminator.
- The gel slice was weighed and 2.5 gel volumes of sodium iodide solution were added.
- Incubated at 50°C for 5 min until the gel slice get completely dissolved.
- The tube was vortexed briefly for every 3 min during incubation. Glass solution (15 µl) was added and mixed thoroughly.
- Incubated at room temperature for 20 min with occasional mixing. DNA present in solution gets adsorbed onto glass solution.
- The mixture was spinned at 12,000 rpm for 30 sec and DNA formed hard pellet with glass particles.
- Discarded the supernatant and added wash buffer to the sample (200 µl per15 µl glass solutions)
- It was vortexed and spinned (12,000 rpm for 30 sec) and discarded the supernatant.
- Above two steps was repeated twice
- After final wash, incubated the micro centrifuge tube at 37°C for 10 min to complete drain the wash buffer.
- Dissolved the DNA pellet in 30 µl 1x TE buffer, vortexed and incubated at 50°C for 5 min for complete dissolution of DNA.
- Centrifuged at 12,000 rpm for 30 sec
- The supernatant collected in a fresh tube was stored at -20°C.
- The eluted fragments were observed in gel electrophoresis and documented.

3.4.2 PCR amplification for eluted products

Since the concentration of eluted fragments was low, PCR was carried to get high concentration of gene fragments from *Piper nigrum* and *Piper colubrinum*. The programme was set as described for amplification. The PCR product was checked on 0.7 per cent agarose gel and documented.

3.5. TRANSFORMATION AND CLONING OF DNA

3.5.1 Preparation of competent cells

Competent cells for plasmid transformation were prepared following the protocol suggested by Mandel and Higa (1970).

Media prepared

LB media and LBA media.

Details of the media prepared are given in the Appendix I. The different steps followed for competent cell preparation is as follows.

Day I

- Inoculated 18 hr old *E. coli* DH-5α strain, single colony to 3 ml LB medium, in sterile condition and incubated over night at 37°C on a shaker set at 160 rpm.

Day 2

- Aseptically transferred 3 ml overnight grown culture to 50 ml LB broth and inoculated for 4 hr at 37°C on a shaker set at 160 rpm until OD_{600} reached 0.4-0.5. The growth of culture was monitored at every 30 min
- The cells were aseptically transferred to a sterile disposable ice cold 50 ml polypropylene tube.
- The culture was cooled to 0°C on ice for 10 min
- The cell suspension was centrifuged at 5,000 rpm for 10 min at 4°C.
- The supernatant obtained was carefully discarded and the pellet obtained was gently resuspended in 10 ml ice cold filter sterilized 0.1 M $CaCl_2$.
- The tubes were kept on ice for 10 min and centrifuged the cell suspension at 5,000 rpm for 10 min at 4°C.

- The supernatant was decanted and resuspended the pellet in two ml of ice cold filter sterilized 0.1 M $CaCl_2$. The tubes were kept on ice for 12-18 hrs.

Day 3

- Chilled glycerol of 400 µl was added to the cell suspension and mixed well using a chilled sterile micro tip.
- The competent cells prepared were stored at -70°C as aliquots of 100 µl chilled 1.5 ml microcentrifuge tubes.

3.5.2 Screening of competent cells

Transformation of competent cells with a plasmid having ampicillin resistance 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 -70°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 in ice for 40 min. Heat shock was given at 42°C for 2 min in a dry bath and plunged in ice for 5 min
- LB media (250 µl) was added to the cells and incubated at 37°C for 1 hour on a shaker set at 120 rpm.
- The transformed cells (100 µl) were plated on LBA/ampicillin (100 rpm) and incubated overnight at 37°C. The recombinant clones alone can grow on ampicillin plate.

3.5.3 Cloning of DNA (PCR product)

The PCR product was cloned in pGEM-T Easy vector supplied by Promega, USA. The details of pGEM-T plasmid is provided in Fig.1

3.5.4 Ligation

The pGEM-T Easy vector and control insert was centrifuged briefly to collect contents at the bottom of the tubes. Ligation reaction was set up 0.5 ml microfuge tube as follows:

Ingredients	Standard reaction	Positive control	Backward control
2x Rapid ligation buffer	5 µl	5 µl	5 µl
pGEMT Easy Vector (50 ng)	1 µl	1 µl	1µl
PCR product	3 µl	-	-
Control Insert DNA	-	2 µl	-
T_4 DNA ligase (3 Weiss Units/µl)	1 µl	1 µl	1 µl
Deionized water	-	1 µl	3 µl
Final volume	10 µl	10 µl	10 µl

The reactions were mixed and incubated for 1 hour at room temperature and then incubated at 4°C over night. Next day it was taken for transformation in competent cells of *E. Coli.*

3.5.5. Transformation of ligated PCR product and recombination selection

Reagents prepared

1. Ampicillin 10 per cent
2. IPTG - 0.5M dissolved in water.
3. X-gal - 10 mg ml^{-1} in DMSO

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

The ligated PCR product was added to 100 µl of competent cells and kept on ice for 40 min. Heat shock was given at 42°C for 2 min in a dry bath and plunged in ice for 5 min. LB media (250 µl) was added to the cells and incubated

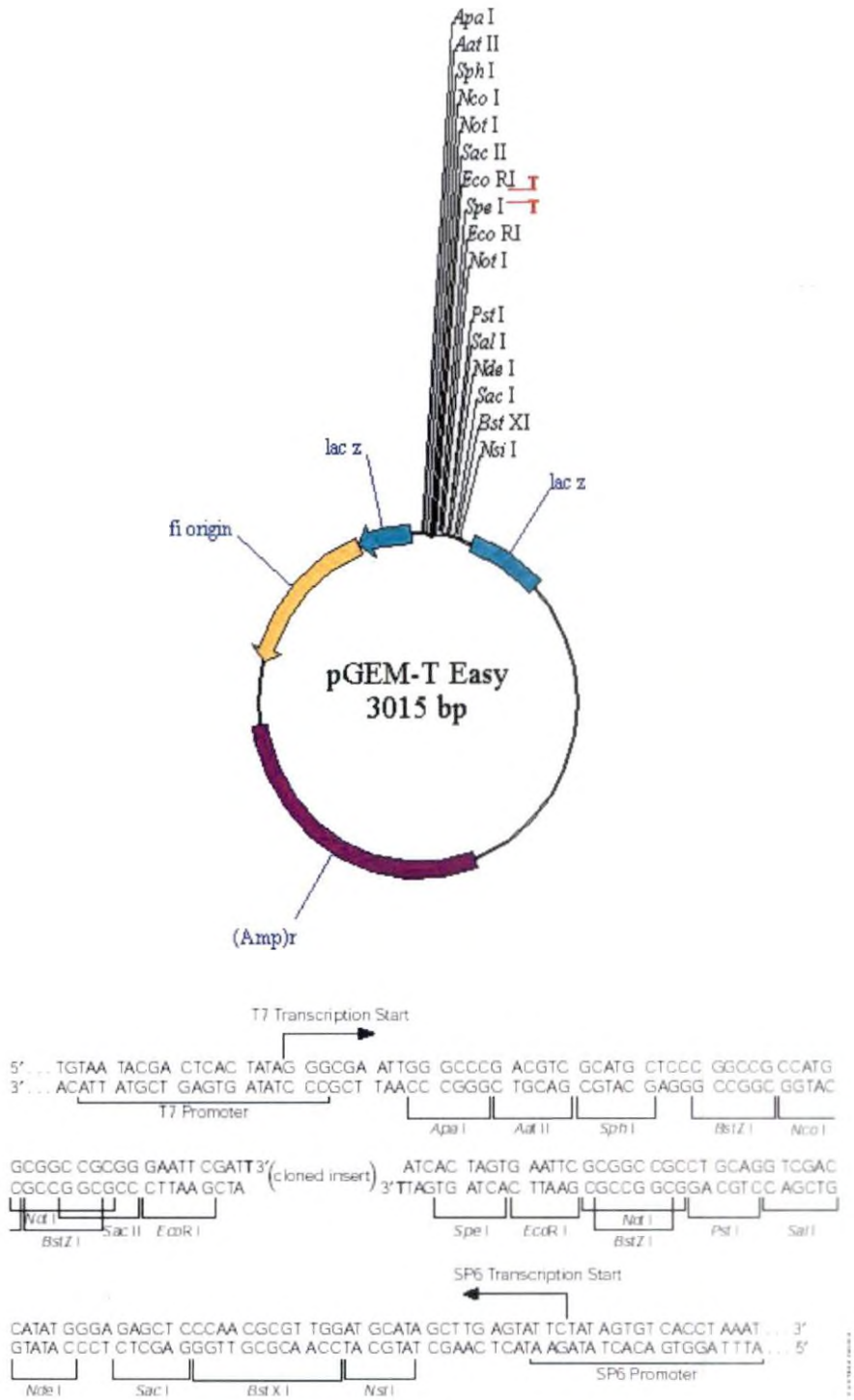


Fig.1. pGEM-T Easy vector (Promega) used for cloning PCR products. The *lac* Z region, promoter and multiple cloning sites are shown in the figure. The top strands of the sequence shown corresponds to the RNA synthesized by T7 RNA polymerase and bottom strands corresponds to the RNA synthesized by SP6 RNA polymerase.

at 37°C for 1 hour on a shaker set at 120 rpm. The aliquots cells (100 µl) were plated on LBA/ampicillin (50 mg l^{-1})/IPTG (100 mg l^{-1})/X-gal (160 mg l^{-1}) plates and incubated overnight at 37°C.

3.5. 6 Isolation of recombinant plasmid DNA

Plasmid DNA was isolated from white colonies using alkaline mini prep procedure as given by Birnboim and Doly (1979).

Reagents prepared

Resuspension buffer (Solution I)

Lysis buffer (Solution II)

Neutralization buffer (Solution III)

LB media

Ampicillin (10%).

Details of the entire reagent prepared are given in Appendix I. The procedure followed for plasmid DNA isolation is as follows.

- Cells were harvested from overnight grown recombinant *E. coli* culture from white colonies containing plasmid DNA by centrifugation at 10,000 rpm for 5 min
- The supernatant was discarded and the bacterial pellet was suspended in 100 µl ice cold resuspension buffer.
- The freshly prepared lysis buffer (200 µl) was added to the pellet and mixed gently by inverting the tubes for five times.
- Ice cold neutralization buffer (150 µl) was added, vortexed gently and kept on ice for 5 min.
- Centrifuged at 12000 g for 10 min at 4°C and transferred the supernatant into fresh tube.
- The DNA was precipitated with two volume of ethanol at room temperature and vortexed.
- Incubated at room temperature for 2 min and centrifuged at 12,000 g for 5 min at 4°C.
- Supernatant was removed and pellet was rinsed with 70 per cent ethanol.

- The pellet was air dried and dissolved in 30 µl autoclaved double distilled water.
- The plasmids isolated were observed in agarose gel electrophoresis and documented.

3.6. CONFIRMATION OF DNA CLONES

3.6.1. PCR confirmation of recombinant plasmid DNA

PCR was carried out as described in section 3.4.1 with the recombinant plasmid DNA isolated by alkali lysis method was used as template (1:10 dilution). The PCR products were analysed on 0.7 per cent agarose gel as described on section 3.3.3.

3.6.2. Restriction digestion of recombinant plasmid DNA

Purity of the recombinant plasmid DNA was checked on 0.7 per cent agarose gel before proceeding to the restriction digestion. The reaction mix was prepared as given below in a sterile 0.2 ml microfuge tube:

Contents	Quantity (µl)
Plasmid DNA	10
Restriction enzyme *Eco*RI	1
Restriction buffer	2
Autoclaved distilled water	7
Total volume	20

The reaction mix was incubated at 37°C for 3 hours. The restriction digestion was arrested by adding 1 µl 0.5M EDTA and then keeping on ice for 15 min. The restriction digestion was confirmed by running the digest in 0.7 per cent agarose gel and documented.

3.7 MAINTENACE OF CLONES

3.7.1 Preparation of pure culture of recombinant bacteria

Materials prepared

LBA medium and Ampicillin 5 per cent

In laminar flow, single white colony from the transformed plate was taken by using flame sterilized bacterial loop. This was streaked on LBA plate containing antibiotic marker ampicillin (50 mg l^{-1}). The plate was incubated overnight at 37°C and further stored at 4°C.

3.7.2 Preparation of stabs

Materials prepared

LBA medium and ampicillin 5 per cent

The LBA medium containing antibiotic ampicillin (50 mg l^{-1}) 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 to the solid medium incubated at 37 °C overnight in the culture tube. The stabs showing good growth of bacteria were further stored in refrigerator at 4 °C.

3.7.3 Glycerol culture

Materials prepared

LB Liquid and ampicillin (5%)

In laminar flow, recombinant colony from the transformed plate was taken by using flame sterilized loop. This was plunged into LB medium containing antibiotic marker ampicillin (50 mg l^{-1}). It was incubated at 37 °C in a shaker at 120 rpm overnight. The next day aliquots (800 µl) of cell culture was added to 100 per cent glycerol (200 µl), aseptically and stored at –20°C.

3.7.4 Sequencing of DNA clones

The stabs of the recombinant clones prepared were sent to DNA sequencing facility, Department of biochemistry at Delhi University South Campus. Details of the vector cloned and size of insert were provided. Sequencing was done with T7 primer to obtain the 5'-3' sequence information of the insert from the forward region.

3.8. SEQUENCE ANALYSIS

The sequence information obtained from Delhi University was further analysed for its characterization.

3.8.1. Sequence editing

The sequence of *Piper colubrinum* and *P. nigrum* was edited to remove vector region from the clones. The sequence was fed for multiple sequence alignment using CLUSTALW 1.83 (Thompson *et al.*, 1994) aligned region was deleted from the sequences.

3.8.2 Homology search

The nucleotide sequence was compared with published sequence in public database using 'BLAST' tool offered by NCBI. Homology search was carried out using Basic Local Alignment Search Tool (BLAST).

The following BLAST programmes were used:

1) Nucleotide-nucleotide BLAST (blastn)

2) Translated query vs. protein database (blastx)

3) Protein-protein BLAST (blastp)

The BLAST programme blastn (www.ncbi.nih.gov./BLAST/ Altshul *et al.*, 1997), offered by NCBI was visited. The nucleotide sequence of the insert was pasted in the BLAST web page and entered 'BLAST' at default settings. The search results were obtained after formating. The best sequence alignment of the search results were noted and saved.

3.8.3. Multiple sequence alignment

Multiple sequence alignment was performed using CLUSTALW (www.ebi.ac.uk. /clustalw/; Thompson *et al.*, 1994). The selected sequences ß-1, 3-glucanase from other crops and sequence of the insert in FASTA format was pasted in the displayed box of CLUSTALW page and the programme was run at default settings. The results would be displayed below the summary table and as a

list. Selected the option' show colours' in the output results. The results were viewed in phylogram and saved.

3.8.4 Detection of Open Reading Frame (ORF)

To find the open reading frame of the insert nucleotide sequence, the programme ORF finder of NCBI was used (www.ncbi.nlm.gov/gorf./gorf/). The nucleotide sequence was copied and pasted in the displayed box and select 'orf find'. The displayed web page shows ORF of sequence in all reading frames. Open reading frame available in all the reading frames were noted and saved. The displayed nucleotide sequences of the reading frame were pasted in a notepad and performed BLAST search. Results were saved for further interpretation.

3.8.5 Detection of exons and peptides

To predict exons and peptide in the sequences, gene prediction tools GENSCAN (www.genes.mit.edu/GENSCAN; Burge and Karlin, 1997) was used. Nucleotide sequences were copied and pasted in the box displayed and selected "Run" GENSCAN. The GENSCAN output was saved.

3.8.6 Detection of nucleotide statistics

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

3.8.7 Restriction analysis

Restriction sites available in the DNA fragment for the restriction enzymes were detected by restriction site analysis (TACG) offered by Biology Workbench. Important sites available for manipulation were selected and interpreted.

3.8.8 Amino acid analysis

Physical and chemical properties of the given protein from the deduced amino acid were determined by amino acid statistics (AASTATS) tool offered by Biology Work Bench. The site was entered and selected for protein tools. Deduced amino acids sequence for the DNA fragments of *Piper* species were saved in this workbench. The sequence was selected and chose appropriate tools for each analysis. Other analyses followed were Chou Fasman seccondary structure prediction (Chou and Fasman, 1978) and Kyte and Doolittle hydropathy plot (Kyte and Doolittle, 1982).

The conserved domains in the protein sequences were found out by conserved domain database using rps BLAST link in the NCBI BLAST page (Marchler-Bauer and Bryant, 2004). Important functional domains were located using 'InterProScan' (www.ebi.ac.uk/InterProScan/; Zdobnov *et al.*, 2001). Domain structure prediction was carried out by CATH structural database (www.biochem.ucl.ac.uk/bsm/cath/)

RESULTS

4. RESULTS

The results of the research conducted on isolation and characterization of ß-1, 3-glucanase gene from *Piper* spp are given in this chapter.

4.1 DATA SEARCH IN THE PUBLIC DOMAIN

4.1.1 Nucleotide sequence information for ß-1, 3-glucanase

Sequence information about ß-1, 3-glucanase was obtained from six different plants tobacco, *Arabdiopsis*, *Prunus*, rice, wheat and rubber. The accession numbers of the sequence used are provided in Table 2. The sequence information downloaded from NCBI site for ß-1, 3-glucanase is provided in Appendix II.

4.1.2. Multiple sequence alignment

The conserved boxes detected through multiple sequence alignment at amino acid level in BCM search launcher is provided in the Fig. 2A. Eight conserved boxes were detected among the six crops selected. Even if slight variations existed, conserved regions are unique among monocots and dicots. The corresponding sequence aligment at nucleotide level is provided in the Fig. 2B and Fig. 2C. The conserved boxes at the nucleotide level were also detected. The level of degeneracy was high among ß-1, 3- glucanase genes deposited from tobacco, *Arabdiopsis* and *Prunus*. The regions were highly conserved in the rice, wheat and rubber.

4.1.3 Primer designing

Based on the parameters described in section no.3.1.4, several primers were derived. Details are provided in Table 3. Out of the eight primers derived, three forward and three reverse primers were selected for further study. Amplicons expected from the selected primer combinations and their melting temperatures are provided in Table 4. The expected size of amplicon ranged between 590-780bp and the annealing temperature ranged between 49 °C and 65°C.

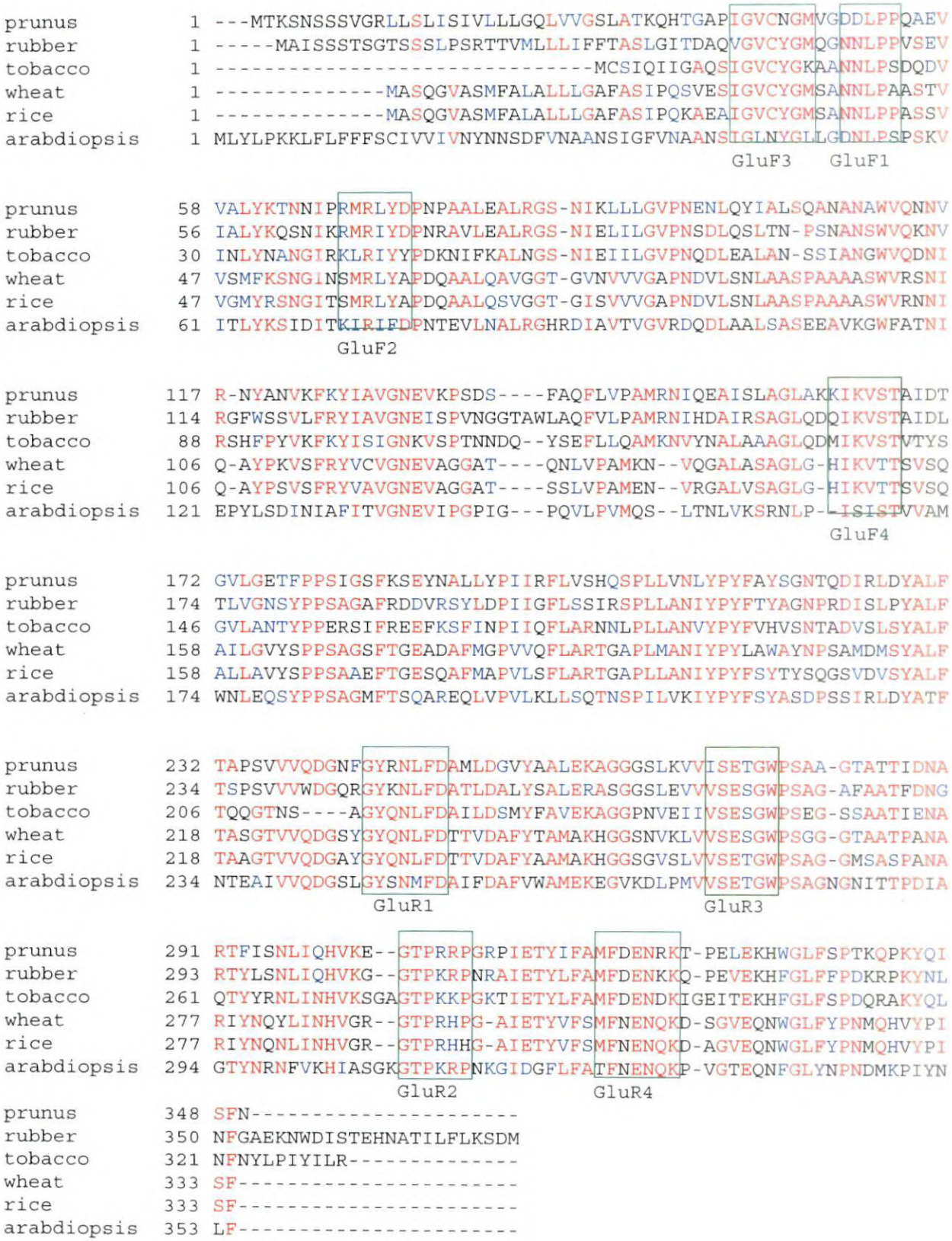


Fig. 2A. Multiple sequence alignment of the amino acids of β-1, 3-glucanase from different crops. Conserved boxes used for primer designing are shown in green coloured boxes.

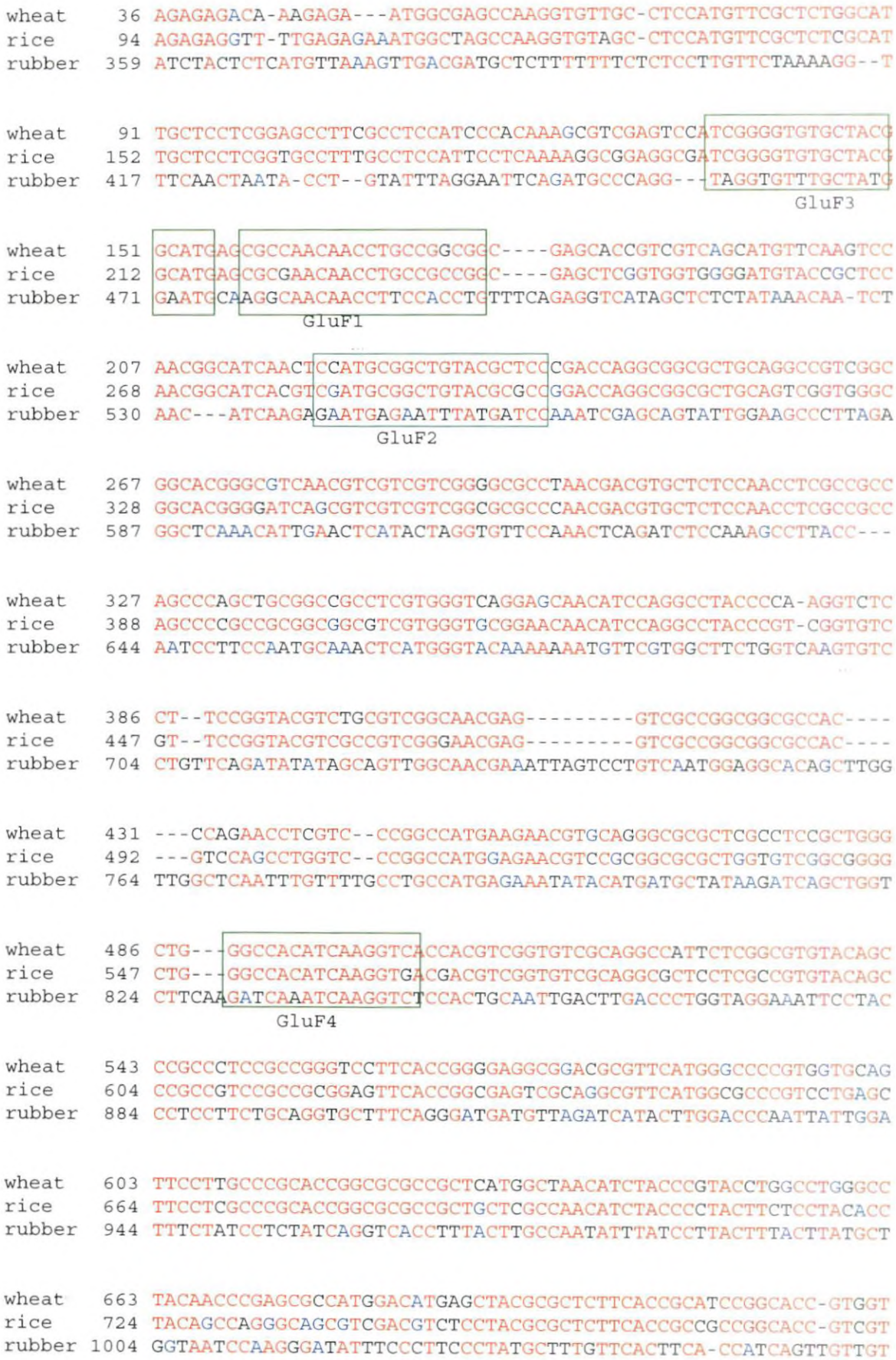
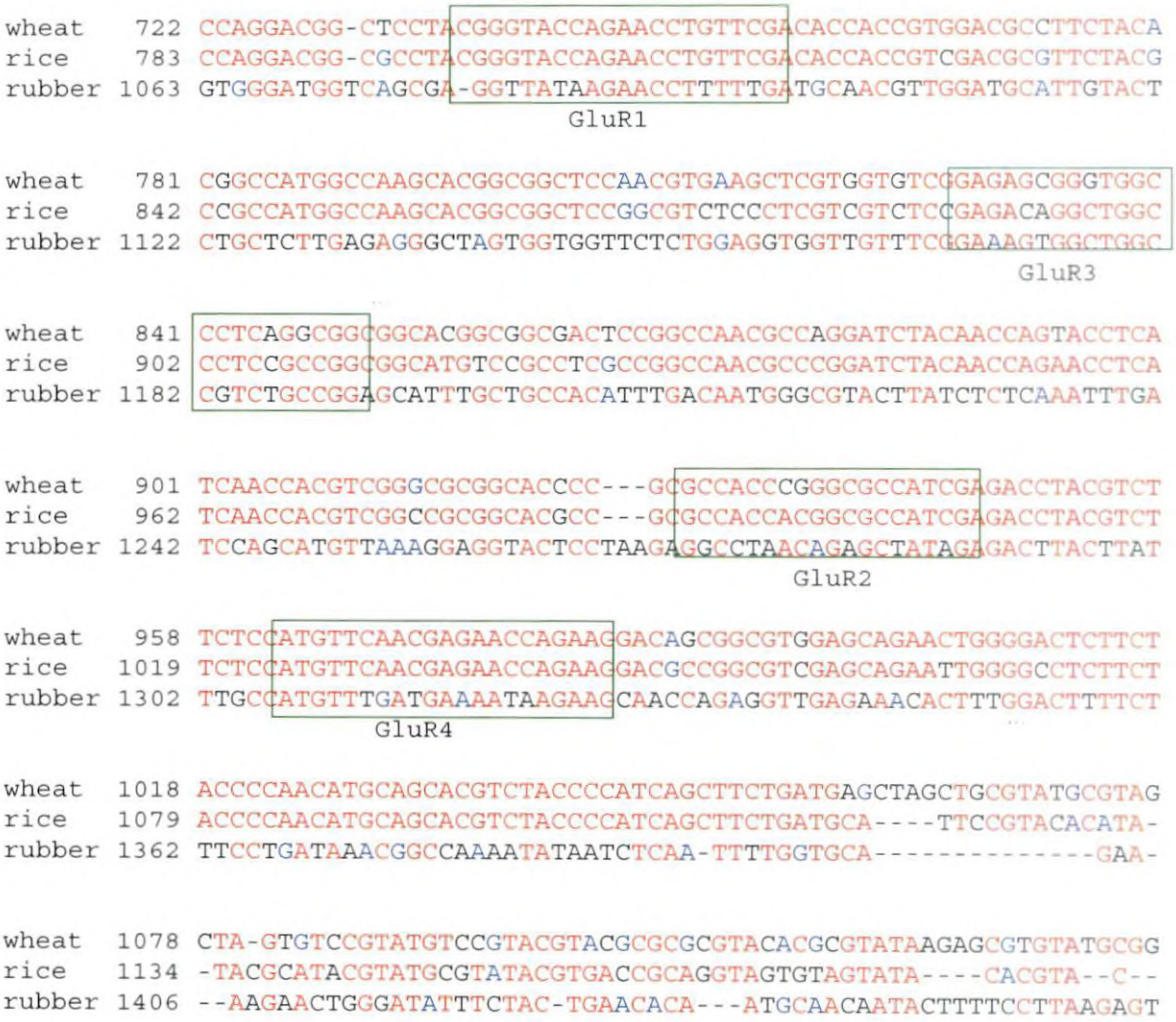


Fig. 2B. Multiple sequence alignment of the nucleotide sequence for β-1, 3- glucanase from wheat, rice and rubber. Conserved boxes used for primer designing are shown in green coloured boxes.

Contd....

Fig.2B. Contd.


wheat 722 CCAGGACGG-CTCCTACGGGTACCAGAACCTGTTCGACACCACCGTGGACGCCTTCTACA
rice 783 CCAGGACGG-CGCCTACGGGTACCAGAACCTGTTCGACACCACCGTCGACGCGTTCTACG
rubber 1063 GTGGGATGGTCAGCGA-GGTTATAAGAACCTTTTTGATGCAACGTTGGATGCATTGTACT
GluR1
wheat 781 CGGCCATGGCCAAGCACGGCGGCTCCAACGTGAAGCTCGTGGTGTCGGAGAGCGGGTGGC
rice 842 CCGCCATGGCCAAGCACGGCGGCTCCGGCGTCTCCCTCGTCGTCTCCGAGACAGGCTGGC
rubber 1122 CTGCTCTTGAGAGGGCTAGTGGTGGTTCTCTGGAGGTGGTTGTTTCGGAAAGTGGCTGGC
GluR3
wheat 841 CCTCAGGCGGCGGCACGGCGGCGACTCCGGCCAACGCCAGGATCTACAACCAGTACCTCA
rice 902 CCTCCGCCGGCGGCATGTCCGCCTCGCCGGCCAACGCCCGGATCTACAACCAGAACCTCA
rubber 1182 CGTCTGCCGGAGCATTTGCTGCCACATTTGACAATGGGCGTACTTATCTCTCAAATTTGA
wheat 901 TCAACCACGTCGGGCGCGGCACCCC---GCGCCACCCGGGCGCCATCGAGACCTACGTCT
rice 962 TCAACCACGTCGGCCGCGGCACGCC---GCGCCACCACGGCGCCATCGAGACCTACGTCT
rubber 1242 TCCAGCATGTTAAAGGAGGTACTCCTAAGAGGCCTAACAGAGCTATAGAGACTTACTTAT
GluR2
wheat 958 TCTCCATGTTCAACGAGAACCAGAAGGACAGCGGCGTGGAGCAGAACTGGGGACTCTTCT
rice 1019 TCTCCATGTTCAACGAGAACCAGAAGGACGCCGGCGTCGAGCAGAATTGGGGCCTCTTCT
rubber 1302 TTGCCATGTTTGATGAAAATAAGAAGCAACCAGAGGTTGAGAAACACTTTGGACTTTTCT
GluR4
wheat 1018 ACCCCAACATGCAGCACGTCTACCCCATCAGCTTCTGATGAGCTAGCTGCGTATGCGTAG
rice 1079 ACCCCAACATGCAGCACGTCTACCCCATCAGCTTCTGATGCA----TTCCGTACACATA-
rubber 1362 TTCCTGATAAACGGCCAAAATATAATCTCAA-TTTTGGTGCA--------------GAA-
wheat 1078 CTA-GTGTCCGTATGTCCGTACGTACGCGCGCGTACACGCGTATAAGAGCGTGTATGCGG
rice 1134 -TACGCATACGTATGCGTATACGTGACCGCAGGTAGTGTAGTATA----CACGTA--C--
rubber 1406 --AAGAACTGGGATATTTCTAC-TGAACACA---ATGCAACAATACTTTTCCTTAAGAGT

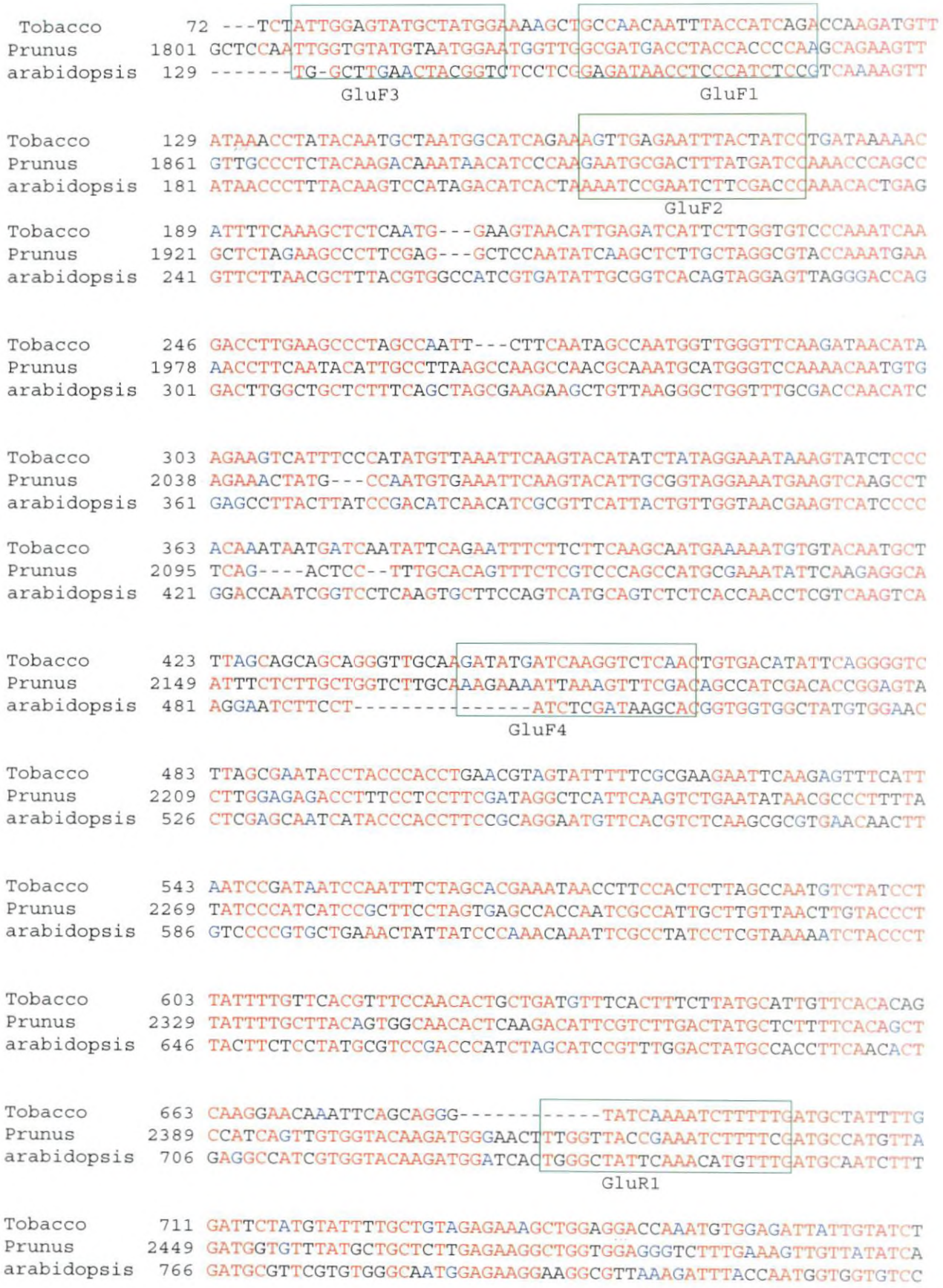


Fig. 2C. Multiple sequence alignment of the nucleotide sequence for β-1, 3- glucanase from tobacco, *Prunus* and *Arabdiopsis*. Conserved boxes selected for primer designing are shown in green coloured boxes

Contd...

Fig. 2C. Contd.


Tobacco 771 GAAAGTGGATGGCCTTCTGAAG---GAAGCTCTGCAGCAACTATTGAAAACGCTCAAACT
Prunus 2509 GAGACTGGTTGGCCATCAGCTG---CTGGAACAGCCACAACAATTGATAATGCAAGGACT
arabidopsis 826 GAGACCGGATGGCCATCTGCCGGGAATGGAAACATTACCACGCCGGATATCGCGGGTACC
GluR3
Tobacco 828 TATTACAGAAATTTGATTAATCATGTGAAAAGCGGGGCAGGAACTCCAAAGAAACCTGGA
Prunus 2566 TTTATATCAAATTTGATTCAACATGTGAAGG------AAGGGACTCCAAGGAGGCCAGGA
arabidopsis 886 TATAACAGAAATTTCGTGAAGCATATAGCAAGCGGAAAAGGTACACCTAAAAGGCCTAAC
Tobacco 888 AAGACTATAGAAACTTATTTGTTTGCCATGTTTGATGAAAATGATAAGATAGGAGAAATC
Prunus 2620 AGGCCCATAGAAACTTACATCTTTGCCATGTTTGATGAGAATAGAAAGACCCCAGAG---
arabidopsis 946 AAAGGCATCGACGGGTTTTTGTTTGCAACTTTCAATGAAAATCAAAAGCCGGTCGGG---
GluR2
GluR4
Tobacco 948 ACAGAGAAACACTTTGGACTGTTTTCTCCTGATCAAAGGGCAAAATATCAACTCAATTTC
Prunus 2677 CTTGAGAAACATTGGGGGCTCTTCTCCCCAACAAAACAGCCTAAATACCAAATCAGTTTC
arabidopsis 1003 ACTGAACAAAACTTTGGGTTATACAATCCGAATGATATG---------AAGCCCATCTAC
Tobacco 1008 AATTATTTGCCAATATATATATTGAGATG----------AGTAATAAGGACAACTGT
Prunus 2737 AATTGATTAGCACTAAAAGGGATGTTGTGGTTGCCAATAAAAGATTGCAAAGTTACCTGC
arabidopsis 1054 AATCTATTCTAA----------

Table 2. Details of ß-1, 3-glucanase sequence information used for primer designing

Crop	Accession No.
Arabdiopsis thaliana	NM11584
Prunus persica	U49454
Hevea brasiliensis	AY325498
Oryza sativa	AY768944
Triticum aestivum	Z22874
Nicotiana tabaccum	M60464

Table 3. Details of primers derived from the conserved sequences

Sl.no.	Name	Sequence	Nature	Length	Tm °C	Remarks
1	*GluF1*	GGC/TA/GAC/TA/GACT/CTG/A /CCCA/C/TG/CC/AA/TT/GC	Forward	20	66	Selected
2	*GluF2*	GATG/CA/CGG/AC/ATT/CTA/T T/CGAT/CCC	Forward	18	52	Selected
3	*GluF3*	ATAGGTGTT/CTGCTATGGA/CA TG	Forward	23	54	Selected
4	*GluF4*	GG/AC/TCAC/AATCAAGGTC/G A/TCC/G AC	Forward	20	56	Not selected
5	*GluR1*	GCTACA/GAAT/A/CAA/G/TG/AT TTC/GT/GAA/GTAT/A/GCC	Reverse	24	64	Selected
6	*GluR2*	TGGCCTC/TT/CTT/AGGA/CGTC C	Reverse	17	56	Selected
7	*GluR3*	CCAGCCGAT/CGGCCAC/ACCA CTCTC	Reverse	24	67	Selected
8	*GluR4*	C/GCTTCTGGTTC/TTCG/ATT/C G/ATAC	Reverse	20	60	Not selected

4.2. ISOLATION AND QUANTIFICATION OF GENOMIC DNA

Agarose gel electrophoresis of the genomic DNA isolated from *Piper nigrum* and *Piper colubrinum* revealed compact banding pattern for DNA. The RNase treatment could provide good quality DNA without any RNA contamination and shearing (Plate.2A). The DNA isolated from both the *Piper nigrum and Piper colubrinum* were of good quality with an absorbance ratio of 1.89 and 1.84 respectively, at 260 and 280 nm. The quantity of DNA detected in the samples was 3.675 and 3.625 $\mu g\ \mu l^{-1}$ respectively for *Piper nigrum and Piper colubrinum.* The details of results obtained are presented in Table 5. This DNA was used for gene amplification.

4.3 POLYMERASE CHAIN REACTION

Amplification observed on agarose gel after polymerase chain reaction using designed degenerate primers with different primer combinations and at various annealing temperatures is presented in Table 6. Among the five sets of primer combinations, amplicons were observed for three sets in *P. nigrum* and for four sets in *P. colubrinum*. The number of amplicons varied from one to three. The amplification pattern obtained for each primer set in the two different species is as follows.

4.3.1 Primer *GluF1R1*

The forward primer *GluF1* and reverse primer *GluR1* with the six different annealing temperatures ranging from 54 °C to 61°C did not amplify the genomic DNA of both *P. colubrinum* and *P. nigrum* in repeated trials.

4.3.2 Primer *GluF1R2*

The PCR with the forward primer *GluF1* and the reverse primer *GluR2* was carried out with the four different annealing temperatures from 53^0 C to 56^0 C. Multiple bands were observed in *Piper colubrinum* at annealing temperature 53^0C and for *P. nigrum* at 55°C (Plate 2B). Amplicons of expected size about 780 bp were eluted from the gel and observed on agarose gel electrophoresis (Plate 2D). Based on the intensity, recovery of eluted product in the gel was found to be

Table 4. Expected anealing temperature and amplicon size for the selected primer combinations

Sl. No.	Primer combination	Annealing Temperature (°C)	Expected amplicon size (bp)
1	*GluF1R1*	60	597
2	*GluF1R2*	56	780
3	*GluF2R1*	53	720
4	*GluF2R2*	49	780
5	*GluF3R3*	65	699

Table 5. Quality and quantity of genomic DNA from *Piper* spp detected using Spectrophotometer

Sl. No.	Plant sp	Absorbance		O.D260/ O.D280 ratio	Quantity ($\mu g\ \mu l^{-1}$)	Quality
		260nm	280nm			
1.	*Piper nigrum*	1.47	0.774	1.89	3.675	Good
2.	*Piper colubrinum*	1.45	0.784	1.84	3.625	Good

Table 6. Yield of amplicon with different primer combinations in *Piper* spp

Sl.No.	Primer combinations	Annealing temperature	Presence of amplicon		No. of amplicons	Size of amplicon bp
			Piper colubrinum	*Piper nigrum*		
1	*GluF1R1*	61	-	-		
		60	-	-		
		59	-	-		
		58	-	-		
		56	-	-		
		54	-	-		
2	*GluF1R2*	54	-	-		
		53	-	+	3	1376, 960, 700
		55	+	-	3	
		56	+*	+*	1	1376, 960, 700
						700
3	*GluF2R2*	49	-	-		
		50	-	-		
		51	-	-		
		52	-	-		
		53	+	+*	1	600
4	*GluF2R1*	55	-	-		
		54	-	-		
		53	-	-		
		52	-	-		
		51	+	-	3	900, 850, 560
		50	-	-		
5	*GluF3R3*	64	-	-		
		60	-	-		
		57	+*	+	1	650
		53	-	-		

* Amplicons used for cloning and sequencing

60 per cent. The eluted product from both *Piper colubrinum* and *Piper nigrum,* when reamplified at annealing temperature 56^0 C yielded single intact bands. (Plate 2E) This amplification obtained was discrete and repeatable. The PCR product was further used for cloning and sequencing study.

4.3.3 Primer *GluF2R2*

Amplification with the forward primer *GluF2* and reverse primer *GluR2* when carried out at different anealing temperatures, single intact amplicon of size 600 bp was observed in both *P. colubrinum* and *P. nigrum* at annealing temperature 53°C (Plate 2C). The amplification was reproducible and discrete in nature. The amplicon obtained from *Piper nigrum* was used for cloning and sequencing.

4.3.4 Primer *GluF2R1*

When the amplification with the forward primer *GluF2* and reverse primer *GluR1* were carried out at different annealing temperatures, multiple bands were obtained in *P.colubrinum* only, at annealing temperature 51°C (Plate 3A). They had size in range between 560 bp to 900 bp. All the amplicons obtained were eluted (Plate 3B) and on reamplification only non specific bands were obtained (Plate 3C). Amplification obtained in this combination was not reproducible. Hence it was not used for cloning and sequencing.

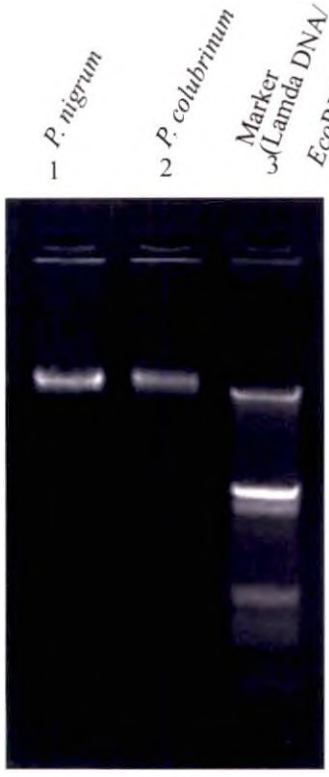
4.3.5 Primer *GluF3R3*

Amplification with the forward primer *GluF3* and reverse primer *Glu R3* when carried out at annealing temperatures ranging from 53 °C to 64 °C single amplicon of size 650 bp was obtained from both *P. colubrinum* and *P. nigrum* at annealing temperature 53 °C (Plate.3D). The amplicon obtained was discrete band in both the *Piper* spp and was used for cloning and sequencing.

4.4 TRANSFORMATION AND CLONING OF PCR PRODUCT

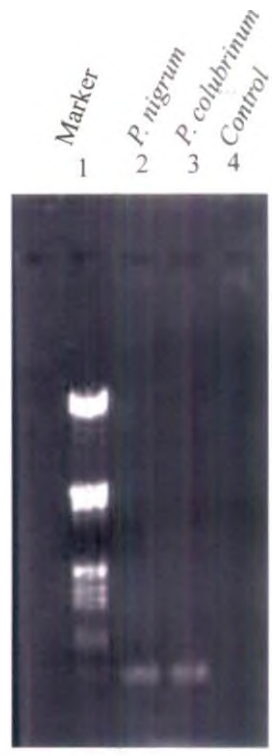
4.4.1 Preparation and screening of competent cells.

Competent cells prepared from *E. coli* DH5α strain as per procedure described on section 3.5.2 were highly competent and showed a high degree of

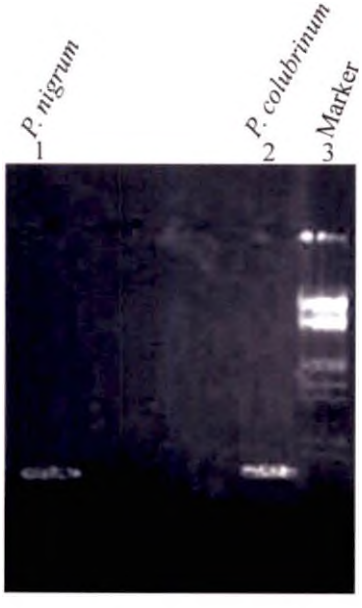


A. Genomic DNA isolation

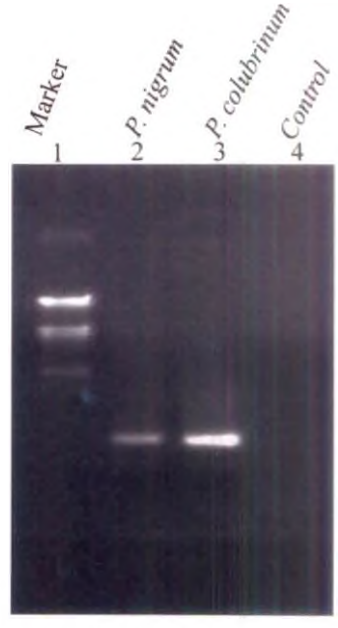
B. Amplification with *GluF1R2*



C. Amplification with primer *GluF2R2*

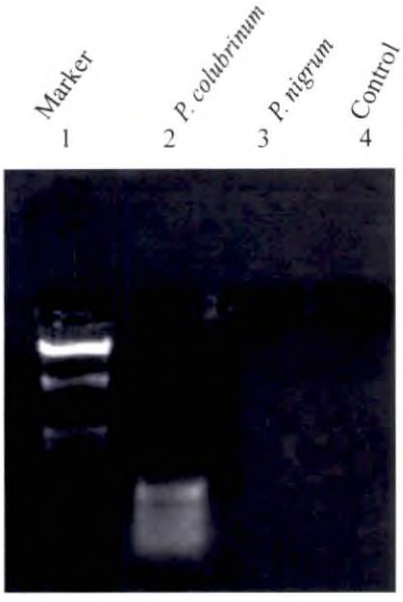


D. Amplicon (*GluF1R2*) after elution

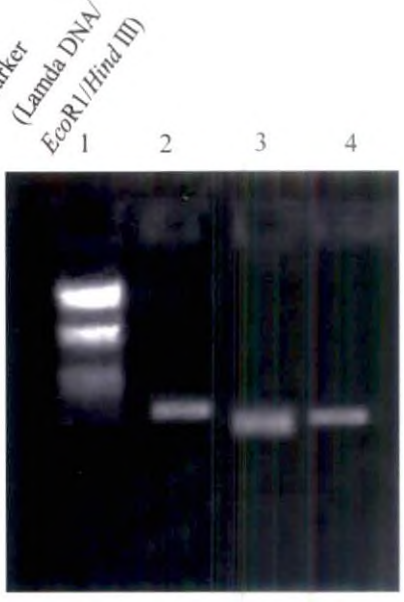


E. Amplicon (*GluF1R2*) after reamplification

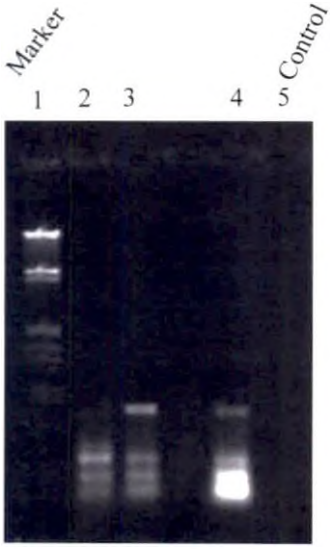
Plate 2. DNA isolation, amplification and elution in *Piper nigrum* and *P. colubrinum* with primer combination *GluF1R2* and *GluF2R2*



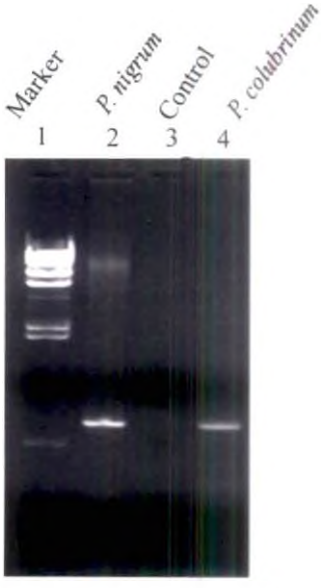
A. Amplification with primer *GluF2R1*



B. Amplicons of *GluF2R1* after elution



C. Amplicon *GluF2R1* after reamplification



D. Amplification with primer *GluF3R3*

Plate 3. Amplification and elution of DNA in *Piper nigrum* and *P.colubrinum* with primer combination *GluF2R1* and *GluF3R3*

transformation efficiency when transformed with the plasmid having ampicillin resistance. The colonies showed luxuriant growth on ampicillin plate, with no other contamination (Plate 4A). Thus the competent cells prepared were found to be efficient for transformation and further cloning works.

4.4.2. Transformation of DNA

The selected amplicons were inserted in pGEM-T easy vector and the ligated product was transferred to the competent *E. coli* (DH5α) cells by heat shock method. Both blue and white colonies were observed when the transformed *E. coli* cells were cultured in ampicillin media overlaid with X gal and IPTG confirming a successful transformation (Plate 4B). Some colonies were overlapping having both transformed and non transformed cells. The recombination efficiency of transformation is presented in Table 7. High recombination efficiency was observed ranging from 68 to 76 percent in all the four amplicons. Positive control gave 96.5 per cent recombination efficiency, none of the colonies grew on negative plate.

4.5 CONFIRMATION OF RECOMBINATION

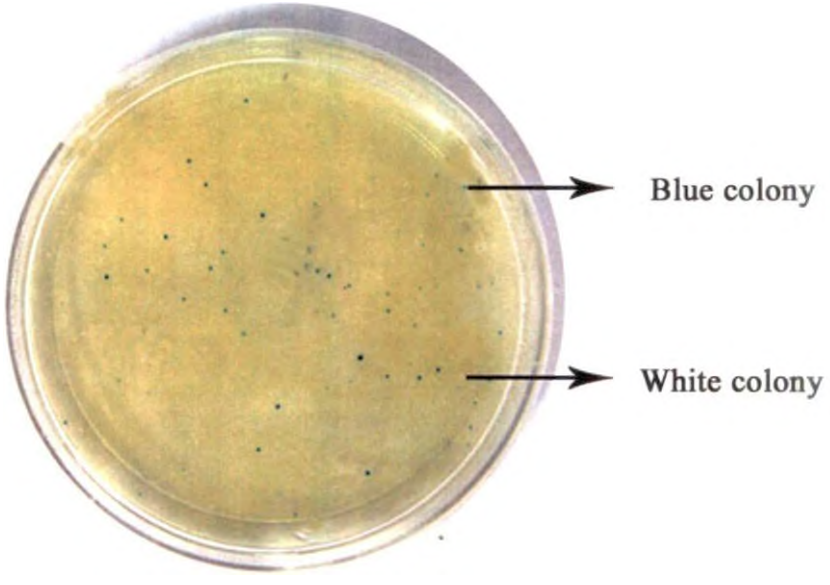
4.5.1 Checking the presence of recombinant plasmid

The blue colonies and white colonies picked up from the transformation plate were multiplied separately in the LB broth and plasmids isolated were electrophoresed. The plasmid isolated from white colonies had a higher molecular weight when compared to the plasmid isolated from blue colonies (Plate 5). This confirms the presence of insert in the plasmid.

4.5.2 Detection of the insert by PCR amplification

The PCR amplification confirmed presence of insert in the recombinant plasmid. Amplifications were observed in the plasmids isolated from the white colony, after PCR reaction with gene specific primers. Amplification was not detected in the plasmids isolated from blue colonies (negative control). Positive results were obtained for all the four fragments cloned. There was a single band of required size, in each primer combinations confirming the presence of insert in the

A. Competent cell colonies growing in ampicillin media



B. Blue and white colonies in the transformation plate

Plate 4. Screening of competent cells and transformation with PCR product

Table 7. Recombination efficiency in the *E. coli* cells transformed with different amplicons

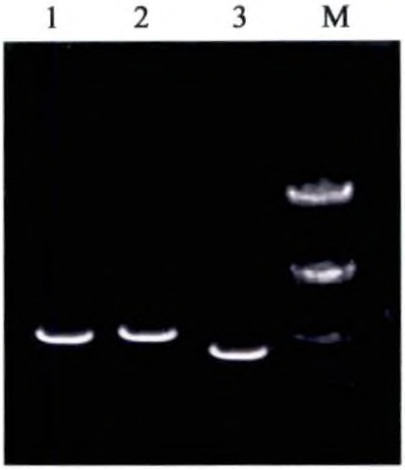
Sl.No.	Details of amplicon	No. of white colonies	No. of blue colonies	Total no of colonies	Recombination efficiency %
1	*Pc Glu F1R2*	149	63	212	70.28
2	*Pn Glu F1R2*	111	51	162	68.5
3	*Pc Glu F2R2*	176	53	229	76.5
4	*Pn Glu F3R3*	121	56	168	72.0
5	Positive plate	520	20	540	96.5
6	Negative plate	-	-	-	-

Pc GluF1R2 - *P. colubrinum* amplicon with primer combination *GluF1R2*

Pn GluF1R2 - *P. nigrum* amplicon with primer combination *GluF1R2*

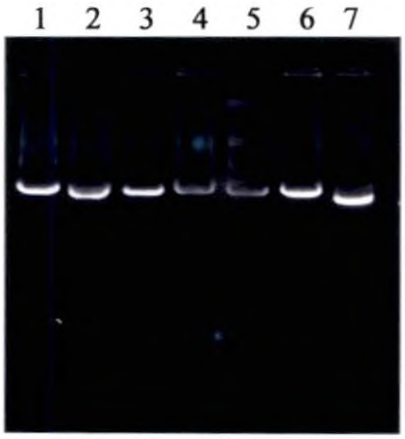
Pc Glu F2R2 - *P. colubrinum* amplicon with primer combination *GluF2R2*

Pn Glu F3R3 - *P. nigrum* amplicon with primer combination *GluF3R3*



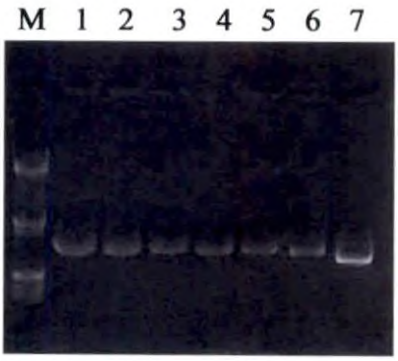
Lane 1 and 2 - White colony
Lane 3 - Blue colony
Lane M - Marker (Lamda DNA/ *Eco*R1/*Hind* III)

A. Plasmid containing insert of *GluF1R2* primer combination



Lane 1 to 6 - White colony
Lane7 - Blue colony

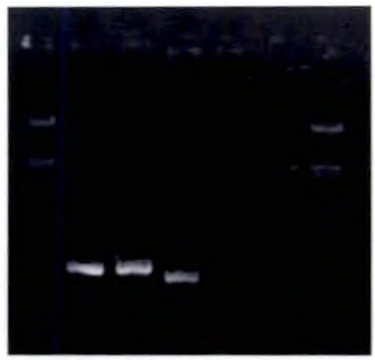
B. Plasmid containing insert of *GluF2R2* primer combination



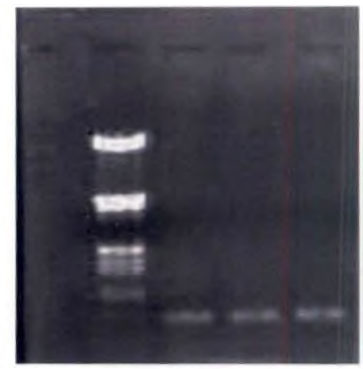
Lane M - Marker (Lamda DNA/ *Eco*R1/*Hind* III)
Lane 1 to 6 - White colony
Lane7 - Blue colony

C. Plasmid containing insert of *GluF3R3* primer combination

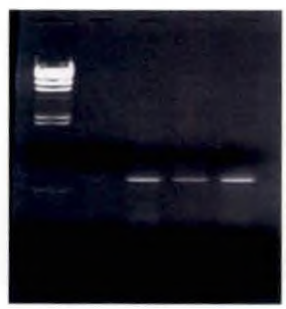
Plate 5. Plasmid DNA isolation from the transformed colonies



A. PCR of recombinant plasmid with *GluF1R2*

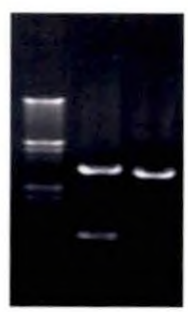


B. PCR of recombinant plasmid with *GluF2R2*



C. PCR of recombinant plasmid with *GluF3R3*

D. Restriction digestion of plasmid containing insert from *GluF1R2* primer combination



E. Restriction digestion of plasmid containing insert from *GluF2R2* primer combination

F. Restriction digestion of plasmid containing insert from *GluF3R3* primer combination

Plate 6. Confirmation of recombination in plasmid DNA

>*Pcglu*
TGGCGGCCGCGGGAATTCGATTTGGCCTTTTTGGAGTCCCCTGCCCAAC
ATGCCTGATCAGATTCTGGTTGTAAGTCCTTGCATTGTCAATGGTGGCC
GCAGAATGCCCCGCCGACGGCCAACCACTCTCCGATACAACTATCTAA
CATTGGGTGTCCCAACCTCCTCCAAGGCAGCGTACATAGCATCCAAGAT
GGCATCAAAGAGGTTCCTATATTGGTTAGCCCCATCCTGCACTCTAACC
GATGGGTACGTGAACAGTGCATATGACAATGGGATGTCGGCAGGATTG
CCCTTGTAGCTAAAATACGGGTAAACGTTGGCTAGAAGGGGAGCTCCG
GTGTCGGCGAGGAACCGTGCTATTGGCCGCAAGTACCCGTCAGCCGCG
CCGGAGAATGCACCGGCAGAGGGAGGGTAGGATGTGCCTAGGACCCC
GGTGTCCACGGCAGTCGACACCTTGATGTCATTTTGTAGGCCGGCGGCA
ACAATGGCGTTGTAAACATTTCGCATGGCCGGGAGCACTGCCTGAGCA
TTGCCTCGGGGGATGACCTCGTTGCCGACGGCGATGTACCGGAATCGA
ACCGCCGGCCAGTATGCCC//

The graphical outputs of each sequence are represented in Fig. 3

4.6 SEQUENCE ANALYSIS OF CLONED SEQUENCES

4.6.1 Nucleotide analysis

4.6.1.1 Homology search in P. nigrum fragments

Homology of nucleotide sequences of cloned products from *P. nigrum* with other reported sequences was done. There was no homology with ß–1, 3-glucanase gene for the amplicon derived from *P. nigrum* with the primer combination *GluF1R2* (Fig. 4A). However, the sequences had significant homology (E value 0.002) with the non coding sequences reported from *Lotus* species. Results of homology search for cloned insert from *P. nigrum* amplified by *GluF2R2* primer combination are displayed in Fig. 4B. No homology was observed with ß-1, 3- glucanase gene fragments deposited in the public domain database to date. The sequences had significant homology with the non coding sequences reported from *Lotus* spp, *Medicago* and *Physalis* sp.

The sequence from *Piper nigrum* obtained with primer combination *GluF3R3, Pnglu* showed significant homology with ß-1, 3- glucanase of *Piper colubrinum*, *Chicorium* sp, *Hordeum vulgare* and *Triticum aestivum* deposited in the public domain through nucleotide-nucleotide BLAST (Fig. 4C). Homology

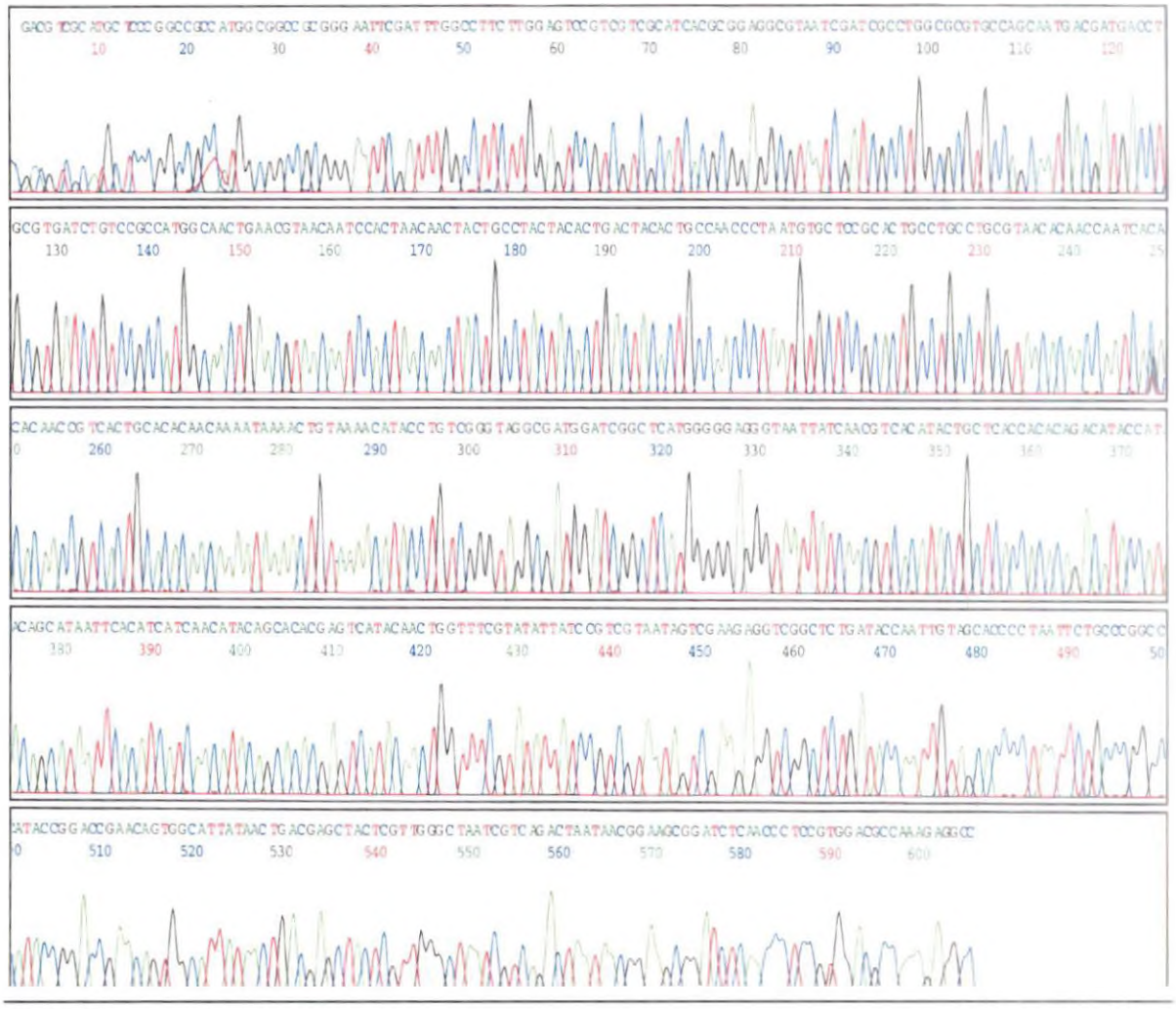


Fig. 3A. Graphical output of sequence in *P. nigrum* obtained with *GluF1R2* primer combination.

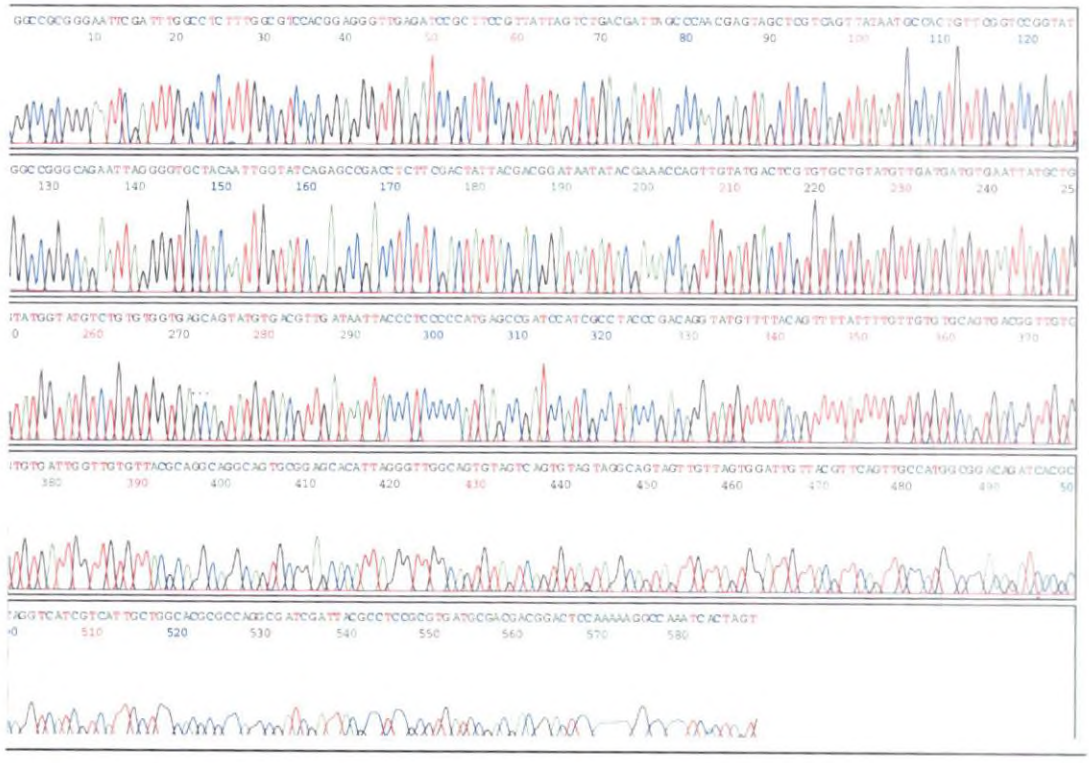


Fig. 3B. Graphical output of sequence in *P. nigrum* obtained with *GluF2R2* primer combination.

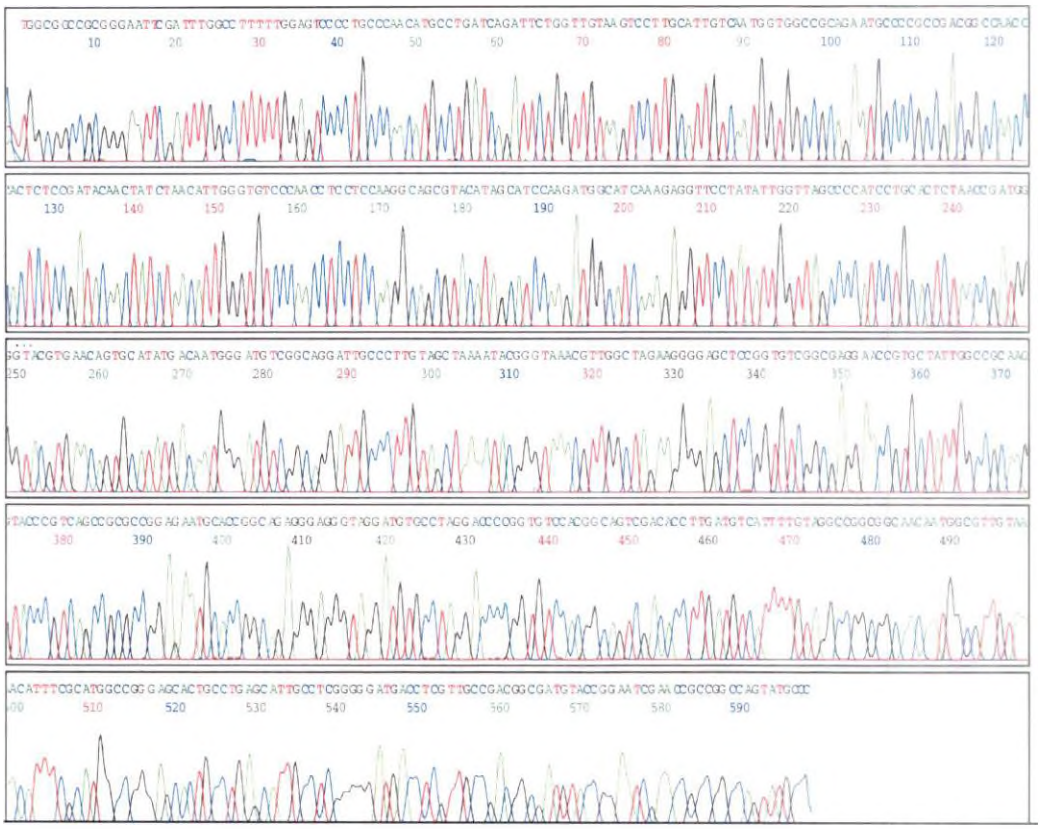
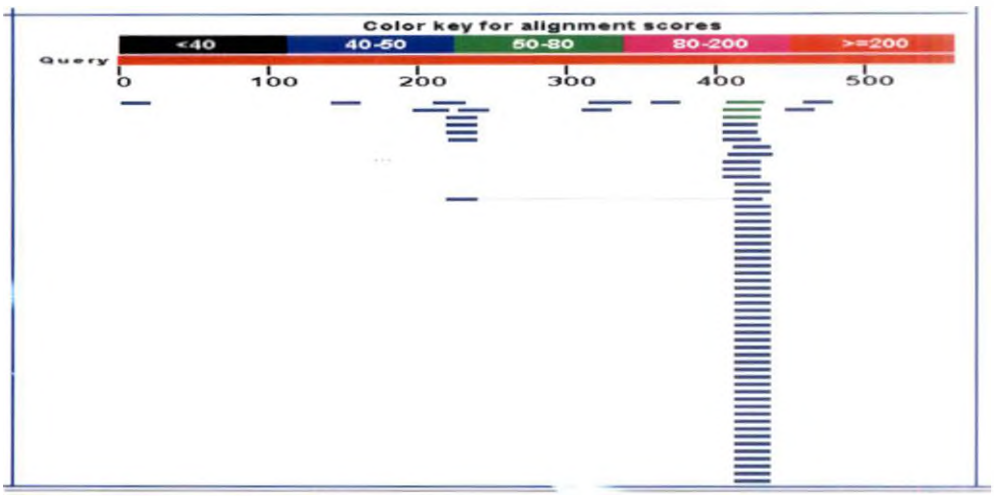
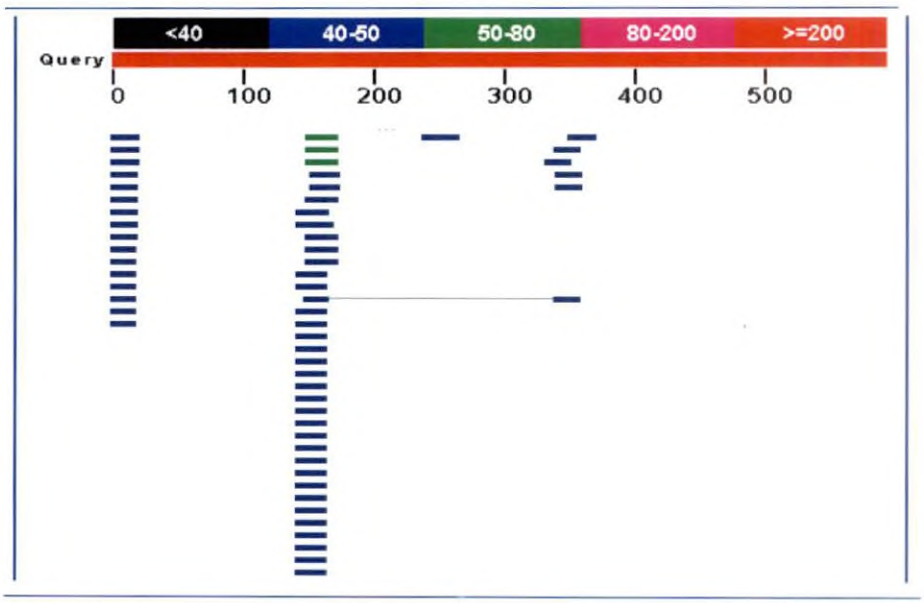


Fig. 3C. Graphical output of sequence in *P. colubrinum* obtained with *GluF1R2* primer combination.



Sequences producing significant alignments:		Score (Bits)	E Value
gi|56805611|dbj|AP007295.1|	*Lotus corniculatus var. japonicus*...	52.0	0.002
gi|21907911|dbj|AP004895.1|	*Lotus corniculatus var. japonicus*...	52.0	0.002
gi|17736850|dbj|AP004483.1|	*Lotus corniculatus var. japonicus*...	52.0	0.002
gi|31581034|dbj|AP006403.1|	*Lotus corniculatus var. japonicus*...	46.1	0.11
gi|31580997|dbj|AP006366.1|	*Lotus corniculatus var. japonicus*...	46.1	0.11
gi|57334912|gb|AC139600.16|	*Medicago truncatula* clone mth2-20m4,	44.1	0.45
gi|51699619|gb|AC146805.17|	*Medicago truncatula* clone mth2-12n20	44.1	0.45
gi|46879218|gb|AC124951.19|	*Medicago truncatula* clone mth2-7p23,	44.1	0.45
gi|33331391|gb|AF538608.1|	Phleum pratense isolate TIMOTHY_63...	44.1	0.45
gi|41688320|dbj|AP006635.1|	*Lotus corniculatus* var. *japonicus*...	44.1	0.45

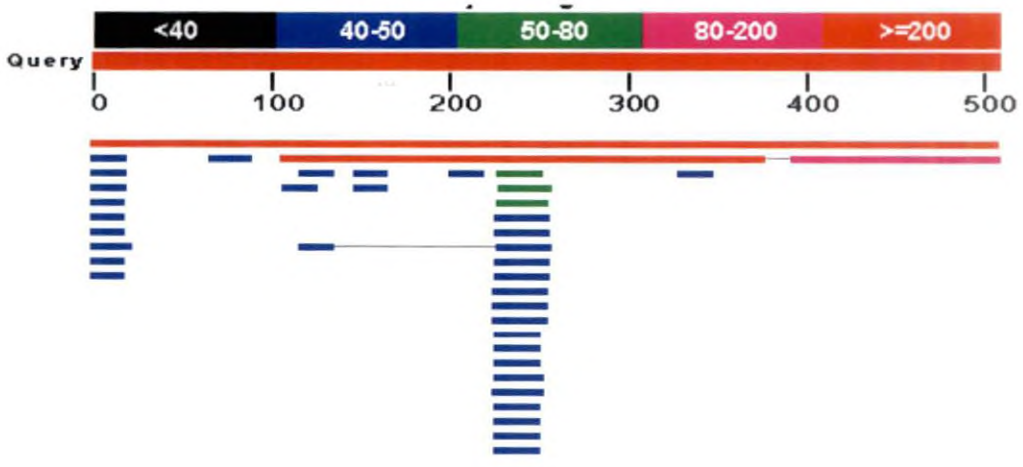
Fig. 4A. Homology search in cloned fragment in *P. nigrum* obtained using primer combination *GluF1R2* through blastn



Sequences producing significant alignments:

		Score (Bits)	E value
gi|56805611|dbj|AP007295.1|	*Lotus corniculatus var. japonicus*...	52.0	0.002
gi|21907911|dbj|AP004895.1|	*Lotus corniculatus var. japonicus*...	52.0	0.002
gi|17736850|dbj|AP004483.1|	*Lotus corniculatus var. japonicus*...	52.0	0.002
gi|31581034|dbj|AP006403.1|	*Lotus corniculatus var. japonicus*...	46.1	0.12
gi|31580997|dbj|AP006366.1|	*Lotus corniculatus var. japonicus*...	46.1	0.12
gi|57334912|gb|AC139600.16|	*Medicago truncatula* clone mth2-20m4,	44.1	0.47
gi|51699619|gb|AC146805.17|	*Medicago truncatula* clone mth2-12n20	44.1	0.47
gi|46879218|gb|AC124951.19|	*Medicago truncatula* clone mth2-7p23,	44.1	0.47
gi|77627619|dbj|AB211186.1|	*Physalis alkekengi* nii2 mRNA for nit	44.1	0.47
gi|33331391|gb|AF538608.1|	*Phleum pratense* isolate TIMOTHY_63..	44.1	0.47
gi|14039753|gb|AF373414.1|AF373414	*Ipomoea* nil PNZIP gene, pr...	44.1	0.47
gi|41688320|dbj|AP006635.1|	*Lotus corniculatus var. japonicus*...	44.1	0.47
gi|29122715|dbj|AP006076.1|	*Lotus corniculatus var. japonicus*...	44.1	0.47
gi|61675753|gb|AC125389.33|	*Medicago truncatula* clone mth2-12a18	44.1	0.47

Fig. 4B. Results of homology search in cloned fragment obtained in *P. nigrum* using primer combination *GluF2R2*.



Sequences producing significant alignments:

		Score (Bits)	E Value
gi|84657342|gb|DQ248887.1|	*Piper colubrinum* beta-1,3-glucanase-l	252	9e-64
gi|3702408|emb|AJ011769.1|CIN011769	*Cichorium intybus x Cicho...*	52.0	0.002
gi|167054|gb|M96939.1|BLYGLCNHV	*Hordeum vulgare* glucan endo-1...	50.1	0.007
gi|347943|gb|M91814.1|BLYGLU2X	Barley beta-1,3-glucanase (GLU2)	48.1	0.027
gi|34906501|ref|NM_189709.1|	*Oryza sativa* (japonica cultivar-gro	46.1	0.11
gi|4741845|gb|AF112965.1|	*Triticum aestivum* beta-1,3-glucanas...	46.1	0.11
gi|58530787|dbj|AP008207.1|	*Oryza sativa* (japonica cultivar-g...	46.1	0.11
gi|18844943|dbj|AP004031.3|	*Oryza sativa* (japonica cultivar-g...	46.1	0.11

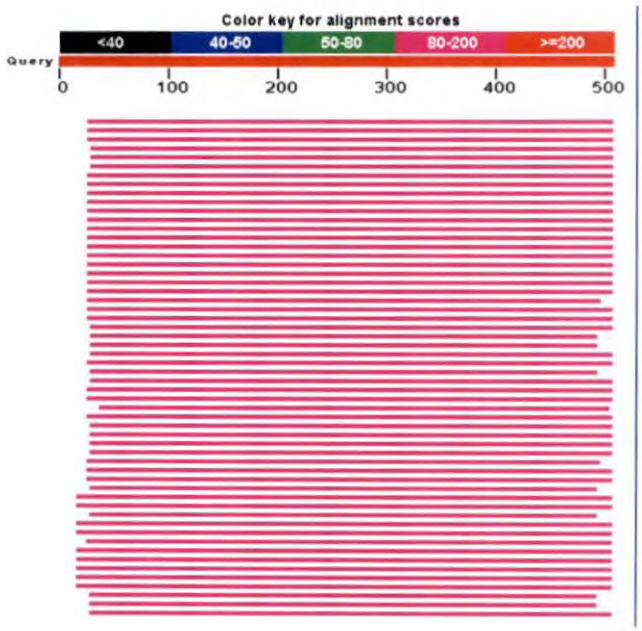
Fig. 4C. Homology search results of *Pnglu* using blastn tool.

search through translated query vs. protein database (blastx) showed that *Pnglu* is showing 55 per cent identity to ß-1, 3-glucanase gene cloned and characterized in *Musa accuminata,* 54 per cent identity to *Vitis vinifera* and 52 per cent identity to *N. plumbaginifolia* (Fig. 4D). Homology search through protein-protein BLAST (blastp) showed that *Pnglu* had 55 per cent identity with ß-1, 3-glucanase gene cloned and characterized in *Musa accuminata,* 54 per cent identity with the gene from *Vitis vinifera* and 50 per cent with ß -1, 3-glucanase characterized from *Hevea brasiliensis* (Fig. 4E).

4.6.1.2 Homology search in P. colubrinum fragments

The sequence of cloned fragment derived from *P. colubrinum* obtained with primer combination *GluF1R2,* named as *Pcglu* observed siginificant homology to ß-1, 3-glucanase genes deposited in the public domain database using search tool blastn (Fig. 5A). *Pcglu* showed 98 per cent identity to ß -1, 3-glucanase gene cloned and characterized from *P. colubrinum,* 93 per cent identity to *P. nigrum* and 84 per cent identity to ß-1, 3-glucanase characterized from *Musa accuminata.* The results of homology searches through blastx are presented in Fig. 5B. It displayed 87 per cent identity to ß -1, 3-glucanase characterized from *P.colubrinum,* 80 per cent identity to *Musa accuminata* and 68 per cent identity to ß -1, 3-glucanase characterized from *Vitis vinifera.*

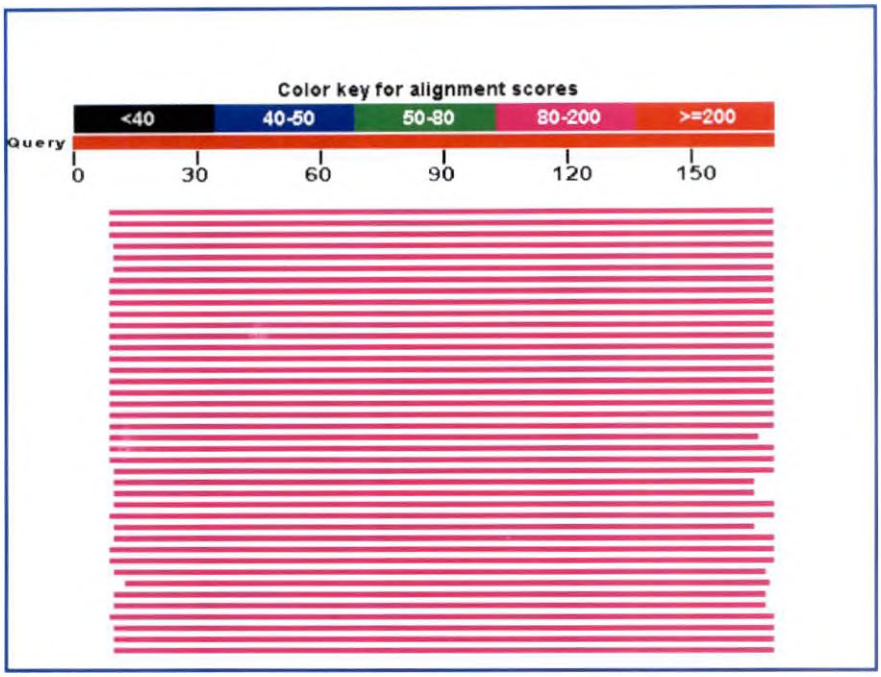
The cloned fragment from *P. colubrinum, Pcglu* had shown significant homology to ß -1, 3-glucanase deposited in the public domain database through protein-protein BLAST (Fig. 5C). It has shown 87 per cent identity to *P. colubrinum,* 80 per cent identity to ß-1, 3-glucanase characterized from *Musa accuminata* and 68 per cent identity to *Vitis vinefera.* Since good homology was obtained for the fragment amplified with primer combination *GluF1R2* in *P.colubrinum,* the other cloned fragment obtained in *P. colubrinum* with *GluF2R2* were not sequenced.



Sequences producing significant alignments:

		Score (Bits)	E Value
gi|6073860|gb|AAB82772.2|	beta-1, 3-glucananse [*Musa acuminata*]	179	5e-44
gi|6448757|gb|AAF08679.1|	beta-1,3-glucanase [*Musa acuminata*]	179	5e-44
gi|83754908|pdb|2CYG|A	Chain A, Crystal Structure At 1.45- Re...	179	5e-44
gi|22550395|gb|AAF44667.2|	beta-1,3-glucanase [*Vitis vinifera*]	176	4e-43
gi|829281|emb|CAA30261.1|	beta-glucanase precursor [*Nicotiana pl*	174	1e-42
gi|31442891|gb|AAA51643.3|	beta-glucanase precursor [*Nicotian...*	174	1e-42
gi|170243|gb|AAA34078.1|	beta(1,3)-glucanase regulator	174	1e-42
gi|4469175|emb|CAB38443.1|	beta-1,3-glucanase [*Hevea brasiliensi*	162	5e-39
gi|10946499|gb|AAG24921.1|	beta-1,3-glucanase [*Hevea brasiliensi*	162	5e-39
gi|32765543|gb|AAP87281.1|	beta-1,3-glucanase [*Hevea brasiliensi*	159	3e-38
gi|68360040|gb|AAY96764.1|	1,3-beta-D-glucanase [*Phaseolus vulga*	159	5e-38
gi|41584319|gb|AAS09829.1|	endo-beta-1,3-glucanase [*Glycine taba*	156	4e-37
gi|1184668|gb|AAA87456.1|	beta-1,3-glucanase	156	4e-37
gi|44889026|sp|P52407|E13B_HEVBR	Glucan endo-1,3-beta-glucosi...	156	4e-37

Fig. 4D. Homology search results in *Pnglu* using blastx tool.

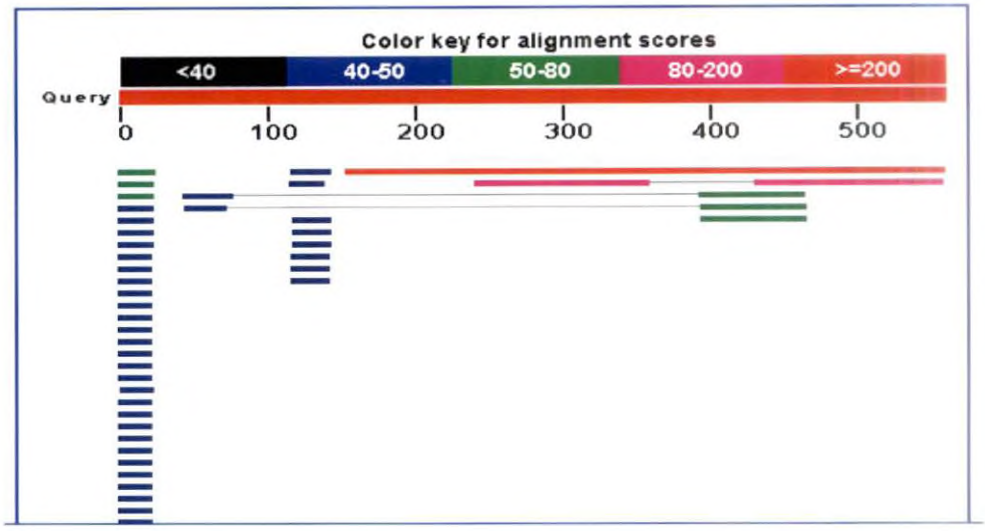


Sequences producing significant alignments:

		Score (Bits)	E Value
gi|6073860|gb|AAB82772.2|	beta-1, 3-glucananse [Musa acuminata]	179	4e-44
gi|6448757|gb|AAF08679.1|	beta-1,3-glucanase [Musa acuminata]	179	4e-44
gi|22550395|gb|AAF44667.2|	beta-1,3-glucanase [Vitis vinifera]	176	4e-43
gi|31442891|gb|AAA51643.3|	beta-glucanase precursor [Nicotian...	174	8e-43
gi|170243|gb|AAA34078.1|	beta(1,3)-glucanase regulator	174	8e-43
gi|829281|emb|CAA30261.1|	beta-glucanase precursor [Nicotiana pl	174	8e-43
gi|4469175|emb|CAB38443.1|	beta-1,3-glucanase [Hevea brasiliensi	162	4e-39
gi|10946499|gb|AAG24921.1|	beta-1,3-glucanase [Hevea brasiliensi	162	4e-39
gi|32765543|gb|AAP87281.1|	beta-1,3-glucanase [Hevea brasiliensi	159	3e-38
gi|68360040|gb|AAY96764.1|	1,3-beta-D-glucanase [Phaseolus vulga	159	4e-38
gi|41584319|gb|AAS09829.1|	endo-beta-1,3-glucanase [Glycine taba	156	2e-37
gi|1184668|gb|AAA87456.1|	beta-1,3-glucanase	156	3e-37
gi|44889026|sp|P52407|E13B_HEVBR	Glucan endo-1,3-beta-glucosi...	156	3e-37
gi|1197520|emb|CAA37289.1|	1,3,-beta-D-glucanase [Phaseolus v...	154	8e-37
gi|41584350|gb|AAS09844.1|	endo-beta-1,3-glucanase [Glycine s...	154	1e-36
gi|41584364|gb|AAS09851.1|	endo-beta-1,3-glucanase [Glycine s...	154	1e-36
gi|1706546|sp|Q03773|E13A_SOYBN	Glucan endo-1,3-beta-glucosid...	154	1e-36
gi|41584323|gb|AAS09831.1|	endo-beta-1,3-glucanase [Glycine falc	153	2e-36

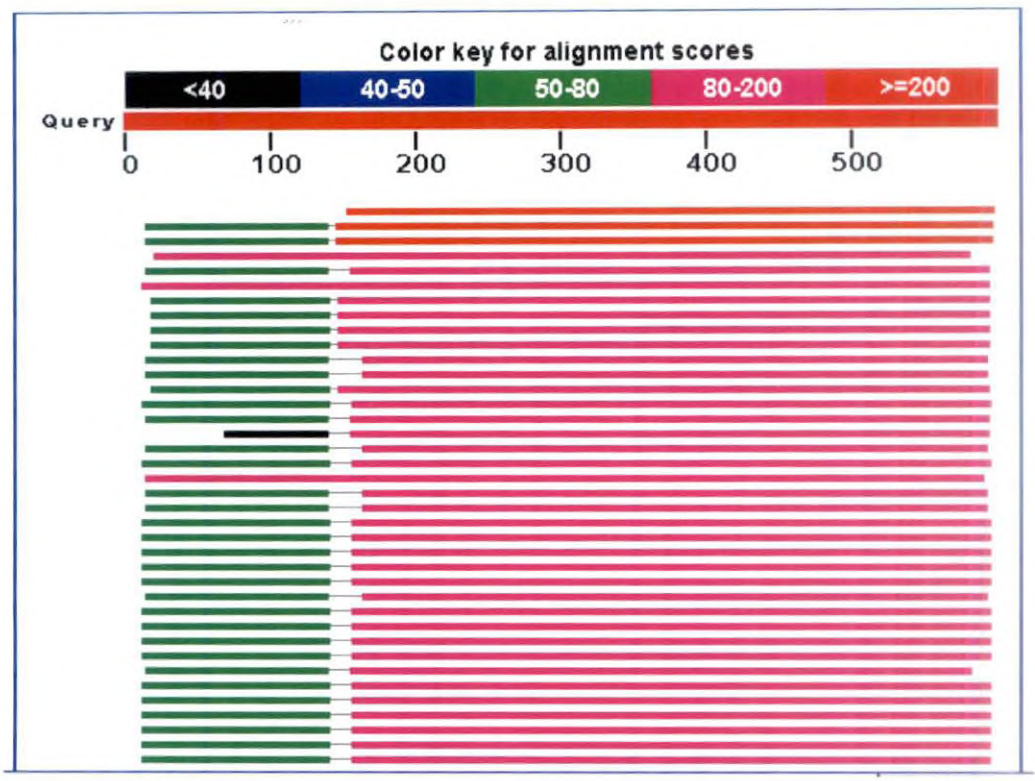
Fig. 4E. Results of homology search in *Pnglu* through blastp tool

Distribution of 103 Blast Hits on the Query Sequence



Sequences producing significant alignments:		Score (Bits)	E value
gi|51863322|gb|AY710290.1|	*Piper colubrinum* beta-1,3-glucanase g	733	0.0
gi|53801273|gb|AY683478.1|	*Piper nigrum* beta 1,3-glucanase-like	180	3e-42
gi|6073859|gb|AF001523.2|AF001523	*Musa acuminata* beta-1, 3-gluca	56.0	1e-04
gi|6448756|gb|AF004838.1|AF004838	*Musa acuminata* beta-1,3-glucan	56.0	1e-04
gi|6249514|emb|Z99986.1|MAZ99986	*Musa acuminata* mRNA for puta...	56.0	1e-04
gi|14039753|gb|AF373414.1|AF373414	*Ipomoea* nil PNZIP gene, pr...	52.0	0.002
gi|62867594|emb|AJ879490.2|	Cucumber mosaic virus segment RNA 1	50.1	0.007
gi|45934504|gb|AY548363.1|	*Malus x domestica* ribonuclease-lik...	50.1	0.007
gi|57116623|gb|AY849555.1|	*Humulus lupulus* chitinase mRNA, parti	48.1	0.027
gi|498923|emb|X74905.1|LEQA	*L.esculentum* TomQ'a mRNA for beta(1,	48.1	0.027

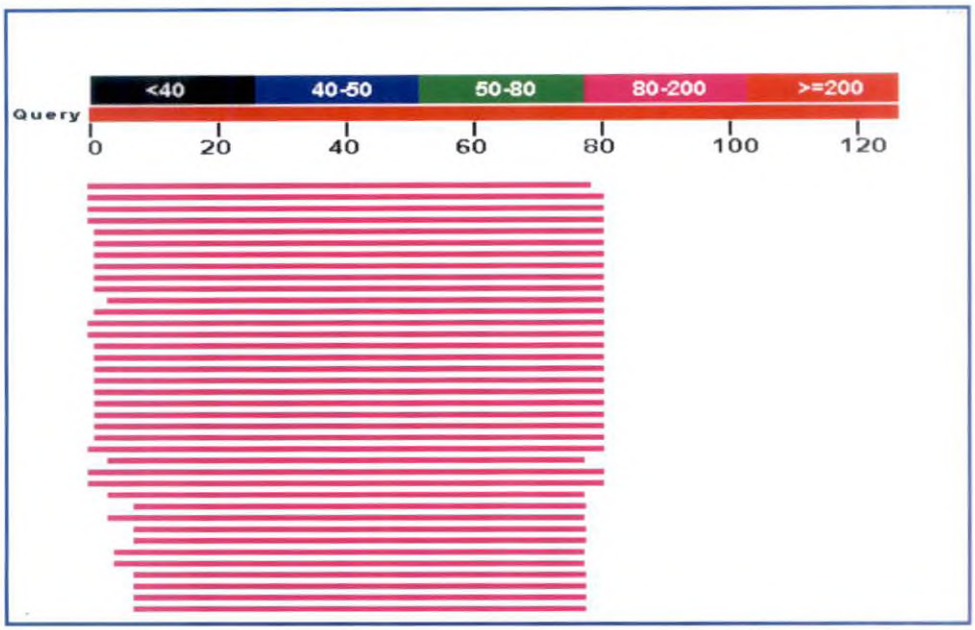
Fig.5A. Homology search results for the cloned fragment *Pcglu* using blastn tool



Sequences producing significant alignments:

		Score (Bits)	E Value
gi|51863323|gb|AAU11817.1|	beta-1,3-glucanase [*Piper colubrinum*]	239	3e-62
gi|6073860|gb|AAB82772.2|	beta-1, 3-glucananse [*Musa acuminata*]	189	3e-47
gi|6448757|gb|AAF08679.1|	beta-1,3-glucanase [*Musa acuminata*]	189	3e-47
gi|7801384|emb|CAB91554.1|	beta 1-3 glucanase [*Vitis vinifera*]	171	1e-41
gi|37992763|gb|AAR06588.1|	beta-1,3-glucanase [*Vitis riparia*]	169	4e-41
gi|29243202|dbj|BAC66186.1|	beta-1,3-glucanase [*Fragaria x anana*	166	4e-40
gi|29243200|dbj|BAC66185.1|	beta-1,3-glucanase [*Fragaria x anana*	166	4e-40
gi|29243198|dbj|BAC66184.1|	beta-1,3-glucanase [*Fragaria x an...*	166	5e-40
gi|62362436|gb|AAX81589.1|	beta-1,3-glucanase [*Fragaria x ananas*	164	2e-39
gi|62362438|gb|AAX81590.1|	beta-1,3-glucanase [*Fragaria x ananas*	163	2e-39
gi|22550395|gb|AAF44667.2|	beta-1,3-glucanase [*Vitis vinifera*]	163	3e-39
gi|4469175|emb|CAB38443.1|	beta-1,3-glucanase [*Hevea brasiliensi*	159	6e-38
gi|10946499|gb|AAG24921.1|	beta-1,3-glucanase [*Hevea brasiliensi*	159	6e-38
gi|32765543|gb|AAP87281.1|	beta-1,3-glucanase [*Hevea brasiliensi*	155	5e-37
gi|14329816|emb|CAC40755.1|	putative prepo-beta-,3-glucanase pre	154	1e-36
gi|16903144|gb|AAL30426.1|	beta-1,3-glucanase [*Prunus persica*]	154	1e-36

Fig. 5B. Homology search result for the cloned fragment *Pcglu* using blastx



Sequences producing significant alignments:

		Score (Bits)	E Value
gi|51863323|gb|AAU11817.1|	beta-1,3-glucanase [Piper colubrinum]	157	8e-38
gi|29243202|dbj|BAC66186.1|	beta-1,3-glucanase [Fragaria x anana	107	9e-23
gi|29243200|dbj|BAC66185.1|	beta-1,3-glucanase [Fragaria x anana	107	9e-23
gi|29243198|dbj|BAC66184.1|	beta-1,3-glucanase [Fragaria x an...	107	9e-23
gi|77862319|gb|ABB04452.1|	pathogenesis-related protein 6 [Zea m	107	9e-23
gi|77862313|gb|ABB04449.1|	truncated pathogenesis-related pro...	107	9e-23
gi|77862309|gb|ABB04447.1|	pathogenesis-related protein 6 [Zea m	107	9e-23
gi|7687419|gb|AAB47177.2|	PRm 6b [Zea mays]	107	1e-22
gi|77862321|gb|ABB04453.1|	pathogenesis-related protein 6 [Ze...	107	1e-22
gi|77862311|gb|ABB04448.1|	pathogenesis-related protein 6 [Zea m	107	1e-22
gi|77862301|gb|ABB04443.1|	pathogenesis-related protein 6 [Zea m	107	1e-22
gi|77862299|gb|ABB04442.1|	pathogenesis-related protein 6 [Zea m	107	1e-22
gi|6073860|gb|AAB82772.2|	beta-1, 3-glucananse [Musa acuminata]	106	2e-22
gi|6448757|gb|AAF08679.1|	beta-1,3-glucanase [Musa acuminata]	106	2e-22
gi|77862323|gb|ABB04454.1|	pathogenesis-related protein 6 [Ze...	106	2e-22
gi|77862315|gb|ABB04450.1|	pathogenesis-related protein 6 [Zea m	106	2e-22

Fig. 5C. Results of homology search in the cloned fragment *Pcglu* through blastp tool

4.6.1.3 Multiple sequence alignment

Pnglu- Multiple sequence alignment of *Pnglu* clone with the ß-1, 3-glucanase genes isolated from different crops in the public domain are presented in Fig. 6. Evolutionary relation of *Pnglu* clone with the other ß-1, 3-glucanase of different crops is depicted in phylogram (Fig 7). Here, ß-1, 3-glucanase is evolutionarily related to barley and wheat.

Pcglu- Multiple sequence alignment of sequence derived from *P.colubrinum, Pcglu* with the ß-1, 3-glucanase genes isolated from different crops in the public domain are presented in Fig. 8. Significant levels of conserved regions existed for ß-1, 3-glucanase from among different plant species and *Pcglu*. Evolutionary relation of *Pcglu* clone with the ß-1, 3-glucanase of different crops is depicted in phylogram (Fig. 9). *Pcglu* had showed close similarity with another fragment ß-1, 3- glucanase of *P.colubrinum* earlier isolated and deposited in public domain.

4.6.1.4 Detection of Open reading frame

Pnglu- Open reading frames of the sequences isolated from *P. nigrum* detected using NCBI 'ORF finder' are presented in Fig. 10. *Pnglu* had largest ORF of 450 bp and code for 149 aminoacids in length. All the significant open reading frames of *Pnglu* when subjected to BLAST search detected the identity percent with other ß-1, 3- glucanase sequences. Details are presented in the Table 8. The identity of the isolated fragment varied from 93 to 100 percent with ß-1, 3-glucanase genes from other plants species like *Chichorium intybus, P. colubrinum*, barley, rice and wheat.

Pcglu- Open reading frames of the sequences derived from *P. colubrinum, Pcglu* identified using NCBI 'ORF finder' are presented in Fig. 11. *Pcglu* had largest ORF of 372bp and code for 123 aminoacids in length. All the significant open reading frames of *Pnglu* when subjected to BLAST search revealed significant homology to ß-1, 3- glucanase (Table 9). The identity of *Pcglu* ranged

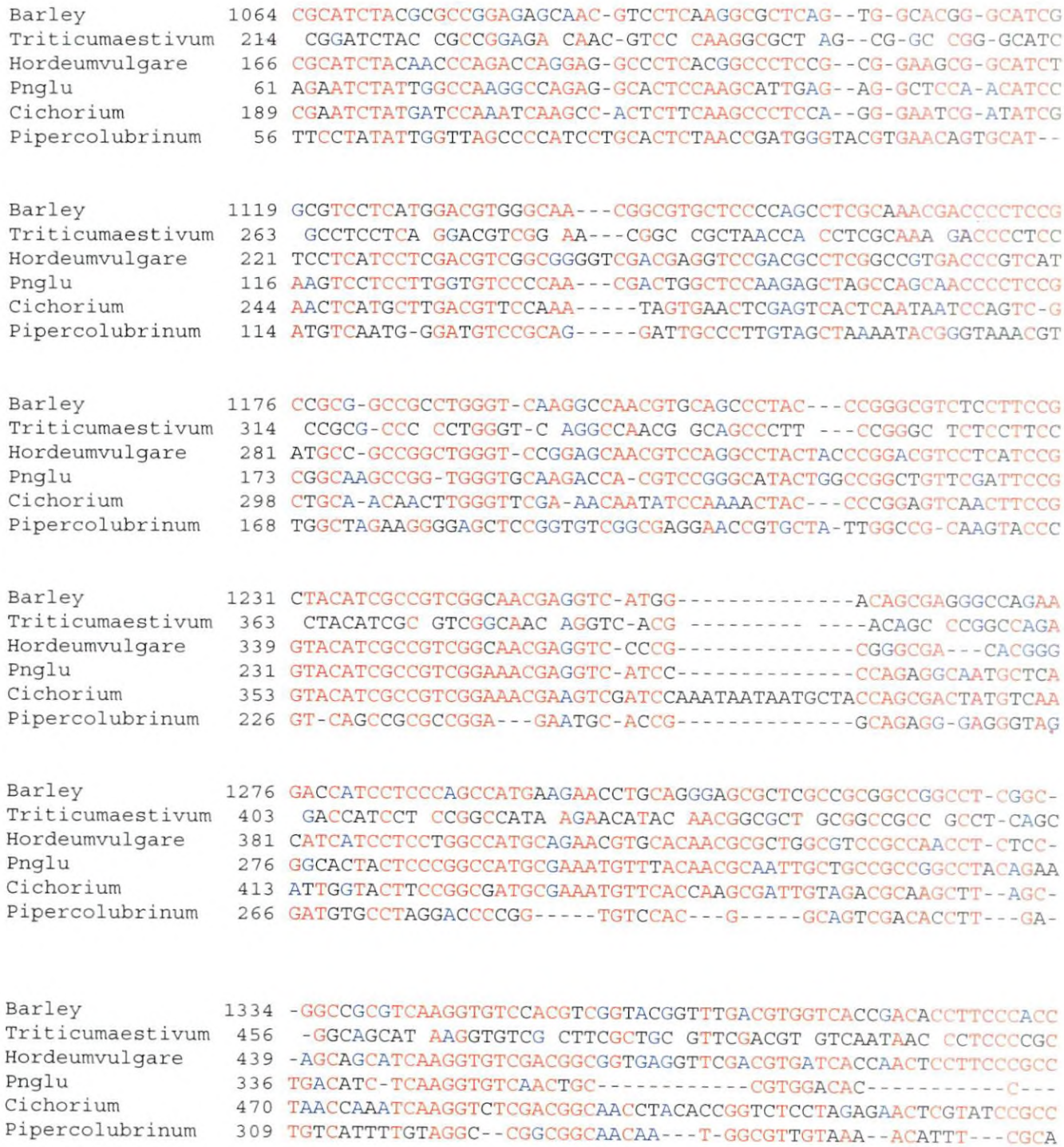


Fig.6. Multiple sequence alignment of *Pnglu* with β-1, 3-glucanase sequences in the databank. Nucleotide location with red colour indicate conserved region

Fig. 6. Contd.

```
Barley              1393 CTCCAACGGCGTGTT-CGCGGAC-CTTGACTAC----ATGGGGCCCATCCTGGACTTCCT
Triticumaestivum     509  CTCCAACGG GTGTT-CGC GACACATCA T-C----AT GGGCCGATC TGGACTTCC
Hordeumvulgare       498 TTCCAGCGGCGTGTT-CAGGGACCCATCTGG-A----TTAGTGCCCATCGCGCGGTTCCT
Pnglu                369 -------GGGGTCCT-C-----C---TCCCT------------CCGAGCGCGG--TTCCT
Cichorium            530 AAGTGACGGCGTATTTCACGAAAACGTGAAGGCGTTTATCGAGCCAATAATCGCATTTCT
Pipercolubrinum      358 TGGC--CGGAGCACTGCCTGAGCATTTGCCTCGGGGGAT-GACCTCGTTGCCGACGGCTT

Barley              1447 GGTGAGCACCGATGCGCCGCTGCTGGCCAACGTGTACCCCTACTTCGCCTACAAGGGTGA
Triticumaestivum     557  GGCGAGCAC GGCGCACCG TGCTGGTCA CGTGTACCC TACTTCGCC ACAAGGGCG
Hordeumvulgare       552 GGACTCGACCGGCGCCCCGTTCCTGGCCAACGTGTACCCTTACTTCGCCTACCGGGACGA
Pnglu                399 GGCCAACACCGGAGCTCCCCTCCTAGCCAACGTCTACCCGTATTTTAGCTACAAGGGCAA
Cichorium            590 AGTTCAAAACAATTTGCCGATGCTTGCCAATATTTACCCCTACTTCGCTGCTCAAGGC-A
Pipercolubrinum      415 ATTGT--ACCGGAATCGAACCGCCGGCCA--GTATGCCCGGACGTTGTTTTGCACCCCCG

Barley              1507 CCCG---AAGAACATCAAGCTCAACTACGCCACCTTCGCGCCGGGCA---CCACCGTGAA
Triticumaestivum     611  CCAG---CA AACATCAAG TCGACTTCG CACCTTCGT CCAGGCAGC CCACCGTGA
Hordeumvulgare       612 CCGTGGCCAGAACATCCGGCTCAACTACGCCACGCTCCAGCCGGGCA---CCACGGTGAG
Pnglu                459 TCCT---GCGGACATCCCACTGTCGTATGC-ACTGTTTACTTACCCA-----TCGGTTA-
Cichorium            649 GCAT-----GCAAGTGAATCTGTCGTATGCATTGTTACAACCGGACGCACCAGTTGTGAA
Pipercolubrinum      471 GCTT---GCGGCAAGGGGTTGCTGCTACTC---TTTGGAGCCTGTCCG----TTGGGGAC
```

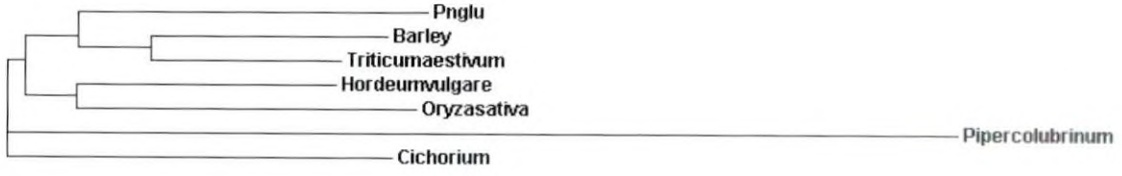


Fig.7. Phylogram of *Pnglu* showing evolutionary relationship among β-1, 3-glucanase genes

Fig. 6. Contd.

```
Barley              1393 CTCCAACGGCGTGTT-CGCGGAC-CTTGACTAC----ATGGGGCCCATCCTGGACTTCCT
Triticumaestivum     509  CTCCAACGG GTGTT-CGC GACACATCA T-C----AT GGGCCGATC TGGACTTCC
Hordeumvulgare       498 TTCCAGCGGCGTGTT-CAGGGACCCATCTGG-A----TTAGTGCCCATCGCGCGGTTCCT
Pnglu                369 -------GGGGTCCT-C-----C---TCCCT------------CCGAGCGCGG--TTCCT
Cichorium            530 AAGTGACGGCGTATTTCACGAAAACGTGAAGGCGTTTATCGAGCCAATAATCGCATTTCT
Pipercolubrinum      358 TGGC--CGGAGCACTGCCTGAGCATTTGCCTCGGGGGAT-GACCTCGTTGCCGACGGCTT

Barley              1447 GGTGAGCACCGATGCGCCGCTGCTGGCCAACGTGTACCCCTACTTCGCCTACAAGGGTGA
Triticumaestivum     557  GGCGAGCAC GGCGCACCG TGCTGGTCA CGTGTACCC TACTTCGCC ACAAGGGCG
Hordeumvulgare       552 GGACTCGACCGGCGCCCCGTTCCTGGCCAACGTGTACCCTTACTTCGCCTACCGGGACGA
Pnglu                399 GGCCAACACCGGAGCTCCCCTCCTAGCCAACGTCTACCCGTATTTTAGCTACAAGGGCAA
Cichorium            590 AGTTCAAAACAATTTGCCGATGCTTGCCAATATTTACCCCTACTTCGCTGCTCAAGGC-A
Pipercolubrinum      415 ATTGT--ACCGGAATCGAACCGCCGGCCA--GTATGCCCGGACGTTGTTTTGCACCCCCG

Barley              1507 CCCG---AAGAACATCAAGCTCAACTACGCCACCTTCGCGCCGGGCA---CCACCGTGAA
Triticumaestivum     611  CCAG---CA AACATCAAG TCGACTTCG CACCTTCGT CCAGGCAGC CCACCGTGA
Hordeumvulgare       612 CCGTGGCCAGAACATCCGGCTCAACTACGCCACGCTCCAGCCGGGCA---CCACGGTGAG
Pnglu                459 TCCT---GCGGACATCCCACTGTCGTATGC-ACTGTTTACTTACCCA-----TCGGTTA-
Cichorium            649 GCAT-----GCAAGTGAATCTGTCGTATGCATTGTTACAACCGGACGCACCAGTTGTGAA
Pipercolubrinum      471 GCTT---GCGGCAAGGGGTTGCTGCTACTC---TTTGGAGCCTGTCCG----TTGGGGAC
```

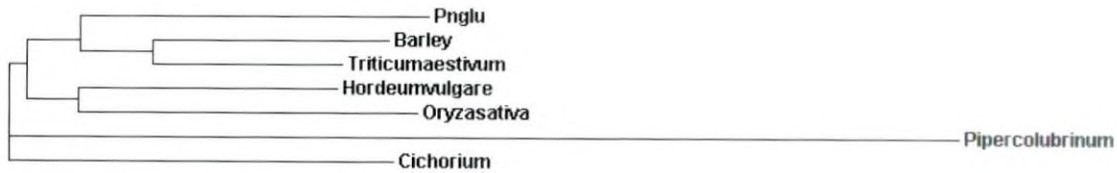


Fig.7. Phylogram of *Pnglu* showing evolutionary relationship among β-1, 3-glucanase genes

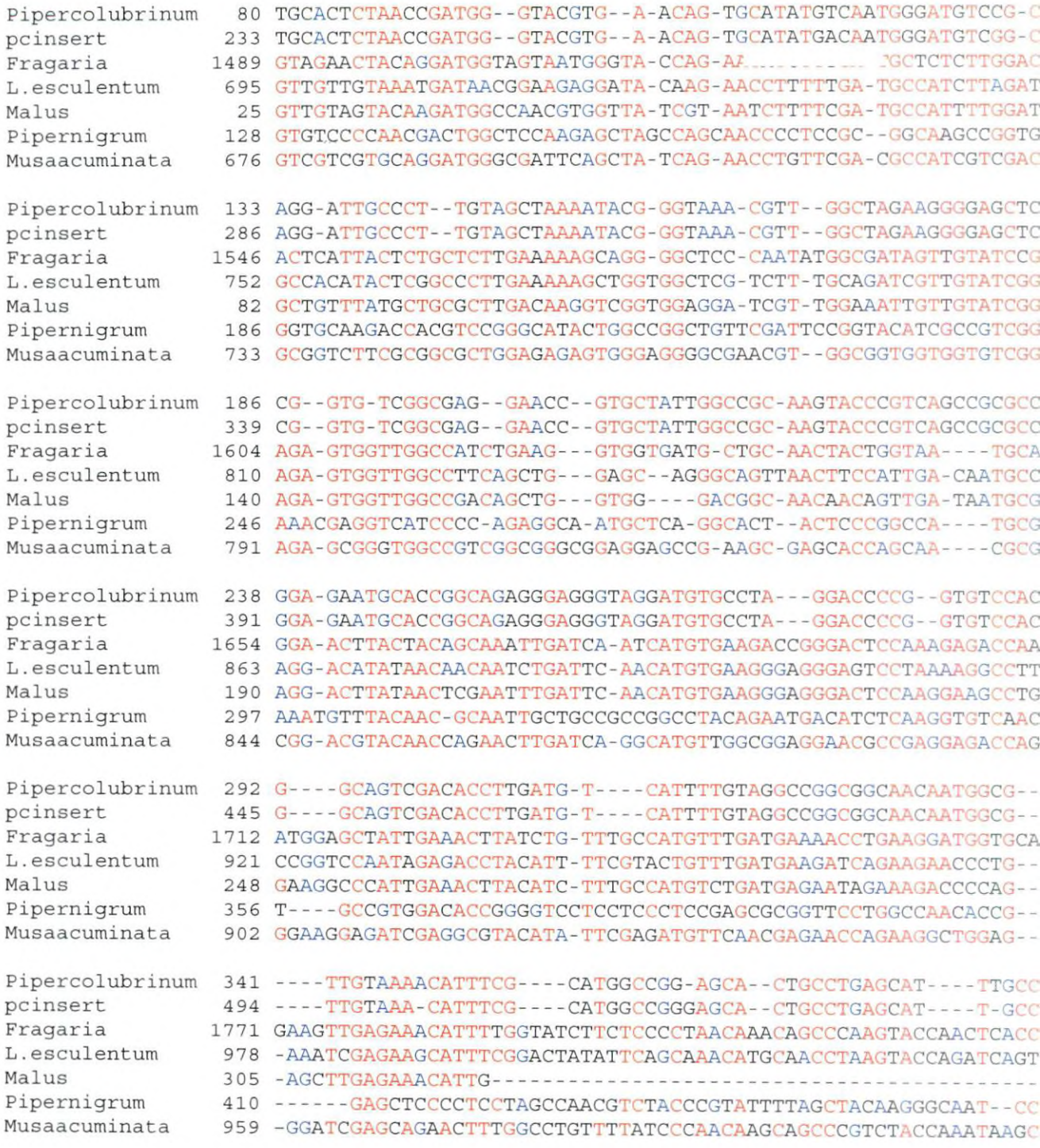


Fig. 8. Multiple sequence alignment of *Pcglu* with β-1, 3-glucanase sequences in the databank. Nucleotide location with red colour indicate conserved region

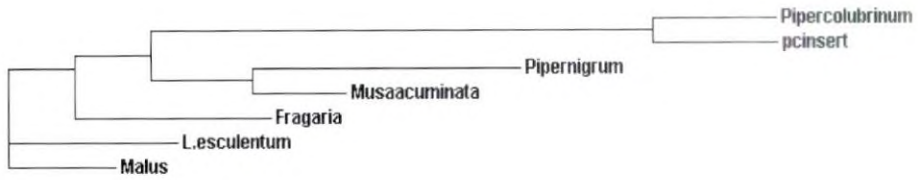
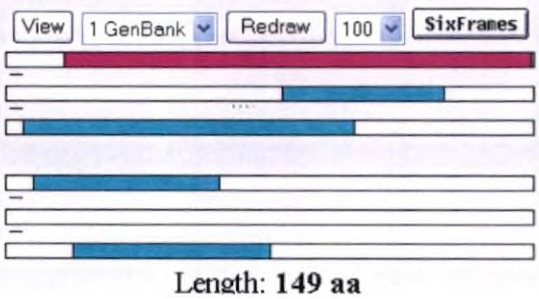


Fig. 9. Phylogram of *Pcglu* showing evolutionary relationship with β-1, 3-glucanase genes deposited in the databank.



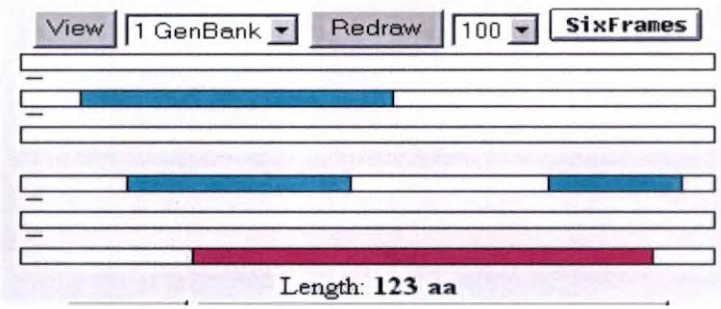
Frame	from	to	Length
+1	58	507	450
+3	18	338	321
-3	66	257	192
-1	29	208	180
+2	269	424	156

```
58 atgagaatctattggccaaggccagaggcactccaagcattgaga
    M  R  I  Y  W  P  R  P  E  A  L  Q  A  L  R
103 ggctccaacatccaagtcctccttggtgtccccaacgactggctc
    G  S  N  I  Q  V  L  L  G  V  P  N  D  W  L
148 caagagctagccagcaacccctccgcggcaagccggtgggtgcaa
    Q  E  L  A  S  N  P  S  A  A  S  R  W  V  Q
193 gaccacgtccgggcatactggccggctgttcgattccggtacatc
    D  H  V  R  A  Y  W  P  A  V  R  F  R  Y  I
238 gccgtcggaaacgaggtcatccccagaggcaatgctcaggcacta
    A  V  G  N  E  V  I  P  R  G  N  A  Q  A  L
283 ctcccggccatgcgaaatgtttacaacgcaattgctgccgccggc
    L  P  A  M  R  N  V  Y  N  A  I  A  A  A  G
328 ctacagaatgacatctcaaggtgtcaactgccgtggacaccgggg
    L  Q  N  D  I  S  R  C  Q  L  P  W  T  P  G
373 tcctcctccctccgagcgcggttcctggccaacaccggagctccc
    S  S  S  L  R  A  R  F  L  A  N  T  G  A  P
418 ctcctagccaacgtctacccgtattttagctacaagggcaatcct
    L  L  A  N  V  Y  P  Y  F  S  Y  K  G  N  P
463 gcggacatcccactgtcgtatgcactgtttacttacccatcggtt 507
    A  D  I  P  L  S  Y  A  L  F  T  Y  P  S  V
```

Fig.10. Open reading frames of *Pnglu* with its largest ORF

Table 8. Homology of *Pnglu* ORF with the β-1, 3 -glucanase sequences in the public domain

ORF position			Plants showing identity		% Identity	Function
+strand	-strand	Length (bp)	Name	Accession no.		
+1 (58--507)		450	*Piper colubrinum*	AY710290	95	β-1, 3-glucanase
			Chicorium intybus x Chicorium endivia	AJ011769	100	,,
			Hordeum vulgare	M96939	96	,,
			Barley	M91814	93	,,
			Oryza sativa	AB027430	93	,,
+3 (18--338)		321	*Piper colubrinum*	AY710290	95	,,
			Chicorium intybus x Chicorium endivia	AJ011769	100	,,
			Hordeum vulgare	M96939	96	,,
			Barley	M91814	93	,,
			Oryza sativa	AB027430	93	,,
			Triticum aestivum	AF112965	93	,,



Frame	from	to	Length
-3	139	510	372
+2	50	301	252
-1	87	266	180
-1	426	533	108

```
510 atgcgaaatgtttacaacgccattgttgccgccggcctacaaaat
    M  R  N  V  Y  N  A  I  V  A  A  G  L  Q  N
465 gacatcaaggtgtcgactgccgtggacaccggggtcctaggcaca
    D  I  K  V  S  T  A  V  D  T  G  V  L  G  T
420 tcctaccctccctctgccggtgcattctccggcgcggctgacggg
    S  Y  P  P  S  A  G  A  F  S  G  A  A  D  G
375 tacttgcggccaatagcacggttcctcgccgacaccggagctccc
    Y  L  R  P  I  A  R  F  L  A  D  T  G  A  P
330 cttctagccaacgtttacccgtattttagctacaagggcaatcct
    L  L  A  N  V  Y  P  Y  F  S  Y  K  G  N  P
285 gccgacatcccattgtcatatgcactgttcacgtacccatcggtt
    A  D  I  P  L  S  Y  A  L  F  T  Y  P  S  V
240 agagtgcaggatggggctaaccaatataggaacctctttgatgcc
    R  V  Q  D  G  A  N  Q  Y  R  N  L  F  D  A
195 atcttggatgctatgtacgctgccttggaggaggttgggacaccc
    I  L  D  A  M  Y  A  A  L  E  E  V  G  T  P
150 aatgttagatag 139
      N  V  R  *
```

Fig.11. Open reading frames of *Pcglu* with its largest ORF

Table 9. Homology of *Pcglu* ORF with the β-1, 3 - glucanase sequences in the public domain

ORF position		Plants showing identity		% Identity	Function
+strand	-strand	Name	Accession no.		
	-3 (139-510)	*Piper colubrinum*	AY710290	99	B-1, 3-Glucanase
		Piper nigrum	AY683478	92	,,
		Musa accuminata	AF001523	84	,,
		Musa accuminata	AF004838	84	,,
		Musa accuminata	Z99986	84	,,
		Nicotiana glutinosa	U49242	85	,,
+2 (50-301)		*Piper colubrinum*	AY710290	97	,,
		Piper nigrum	AY683478	91	,,
		Lycopersicon esculentum	X74905	96	,,
		Malus x domestica	AF494405	100	,,
		Fragraria x ananosa	AY170375	96	,,
	-1 (87-266)				,,
		Piper colubrinum	AY710290	98	,,
		Lycopersicon esculentum	X74905	96	,,
		Malus x domestica	AF494405	100	,,
		Fragraria x ananosa	AY989819	96	
		Fragraria x ananosa	AB106653	96	,,
		Piper colubrinum	AY710290	100	,,
		Piper nigrum	AY683478	93	,,

from 84 to 100 per cent with other ß-1, 3-glucanase sequences like *P. colubrinum, P. nigrum,* banana, tomato and tobacco.

4.6.1.5 Nucleic acid statistics

Nucleic acid statistics of the isolated sequences obtained in the Biology Workbench is presented in the Table 10 Both the fragments *Pnglu* and *Pcglu* has high GC content about 56.1 per cent and 56.9 per cent respectively.

4.6.1.6 Restriction analysis

Pnglu-Major restriction sites of common restriction enzymes for the fragment *Pnglu* are provided in the Table 11. The frequent cutter *Alu*I had three sites and the rare cutter *N*aeI had two sites. *Eco*RI had single restriction site at position 9 of the *Pnglu* fragment. However it lacks restriction site for the common restriction enzymes including *Hind*111, *BamH1*, *Sma*1 and *Xma* 1.

Pcglu Major restriction sites deduced for the fragment *Pcglu* are provided in the Table 12. *Pcglu* had two sites for the frequent cutter *Alu* and two sites for the rare cutter *Nae* I. It had single restriction site for *EcoR*1 at position 14. It lacks restriction site for restriction enzymes including *Bam* H1, *Xba*1, and *Hind* III.

4.6.1.7 Gene prediction analysis

Gene prediction analysis predicted 423 bp exons coding for 141 aminoacids in *Pnglu* .The graphical output are displayed in the Fig. 12A. It predicted 348 bp exons and 141 aminoacids for *Pcglu* (Fig. 12B)

4.6.2 Amino acid sequence analysis

4.6.2.1 Amino acid composition

Deduced amino acid sequence of protein analysed and proportion of each amino acid calculated using AASTATS from biology workbench are presented in Table 13.The major amino acids deduced from the cloned fragment *Pnglu* were

Table 10. Nucleotide statistics of *Pnglu* and *Pcglu* obtained using NASTATS

Sl.No.	Nucleotide	*Pnglu*		*Pcglu*	
		Total	%	Total	%
1	A	122	23.9	130	21.7
2	T	100	19.6	128	21.4
3	C	163	32.0	165	27.5
4	G	123	24.0	176	29.4
5	A + T	222	43.5	258	43.1
6	C + G	286	56.1	341	56.9

Table 11. Restriction sites of *Pnglu* from *Piper nigrum* with common restriction enzymes

Sl.No.	Name of restriction enzymes	Recognition sequence	No. of cuts	Position of restriction	Position of restriction fragments (bp)
1.	*Alu*1	AG'CT	3	154, 413, 447	34, 61, 154,259
2.	AvaI	C'yCGr_G	0	0	0
3.	*Bcc*1	CCATnnnn^{1}n	1	508	508, 0
4.	*Bmt*1	G_CTAGG^{1}C	1	158	158, 350
5.	*Dpn*1	GA'TC	1	40	40, 468
6.	*Eco*R1	G'AATT_C	1	9	9, 499
7.	*Nae*1	GCC'GGC	2	216, 325	109, 183, 216
8.	*Not*1	GC'GGCC_GC	0	0	0
9.	*Mbo*1	'GATC_	1	38	38, 470
10.	*Taq*1	T'CG_A	2	13, 223	13, 210, 285

Table 12. Restriction sites of *Pcglu* with common restriction enzymes

Sl.No.	Name of restriction enzymes	Recognition sequence	No. of cuts	Position of restriction	Position of restriction fragments (bp)
1	*Alu*1	AG'CT	2	302, 336	34, 221, 302
2	*Ava*1	C'yCGr_G	1	538	19, 538
3	*Bcc*1	CCATnnnn^{1}n	3	189,236,241	5, 47, 189, 358
4	*Dpn*1	GA^{1}TC	1	58	58, 499
5	*EcoR*1	G'AATT_C	1	14	14, 543
6	*Mbo*I	'GATC_	1	56	56,543
7	*Nae*1	GCC'GGC	2	478, 587	12, 109, 478
8	*Not*1	GC'GGCC_GC	1	5	5, 552
9	*Sal*1	G'TCGA_C	2	450	107, 450
10	*Taq*1	T'CG_A	3	18, 451, 578	18, 21,127, 433

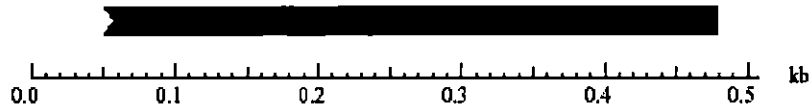


Fig.12A. Exons predicted from *Pnglu* using GENSCAN

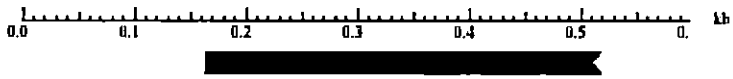


Fig.12B. Exons predicted from *Pcglu* using GENSCAN

Table 13. Amino acid composition of deduced sequences from *Pnglu* and *Pcglu*

Amino acid group		Amino acid	*Pnglu* (*Piper nigrum*)		*Pcglu* (*Piper colubrinum*)	
			No.	Mol%	No.	Mol %
Non polar		*Gly*	9	5.33	19	9.64
		Ala	23	13.61	25	12.69
		Val	10	5.92	16	8.12
		Leu	17	10.06	18	9.14
		Ile	10	5.92	10	5.08
		Met	2	1.18	4	2.03
		Pro	14	8.28	17	8.63
		Phe	4	2.37	7	3.55
		Trp	6	3.55	1	0.00
Polar	Uncharged	*Ser*	13	7.69	8	4.06
		Thr	3	1.78	9	4.57
		Cys	1	0.59	0	0.00
		Tyr	10	5.92	13	6.60
		Asn	12	7.10	10	5.08
		Gln	9	5.33	7	3.55
	Basic	*Lys*	1	0.59	3	1.52
		Arg	16	9.47	18	9.14
		His	1	0.59	0	0.00
	Acidic	*Asp*	4	2.37	8	4.06
		Glu	4	2.37	4	2.03

4.6.2.1 Secondary structure prediction

The secondary structure of proteins predicted by CHOFAS tool offered by Biology workbench is presented in Fig. 13A and Fig. 13B respectively. The amino acids deduced from *Pnglu* had 56.8 per cent helices, 44.4 per cent sheets and 13.6 per cent turns. The deduced amino acids from *Pcglu* had 52.3 per cent helixes, 41.6 sheets and 10.2 per cent turns.

4.6.2.2 Detection of conserved domains

The conserved domains in the deduced amino acid sequences derived from *Pnglu* and *Pcglu* are presented in Fig. 14A and Fig. 14B respectively. Both belonged to single domain family of glycosyl hydrolases family 17. The functional domains located through 'InterProScan' of the deduced proteins derived from *Pnglu* and *Pcglu* are presented in Fig.15A and Fig.15B respectively. The important functional domain also classed the peptide deduced from both the fragment to glycoside hydrolase family 17.

The domain structure obtained through CATH structural database is presented in Fig. 16. Both belonged to glycosidase, which is a sequence family of ß-1, 3 glucanase. Hydropathy plot of sequence was constructed by means of Kyte-Doolittle Hydropathy Profile and showed that both the fragment *Pnglu* and *Pcglu* lack transmembrane regions (Fig.17A and Fig.17B).

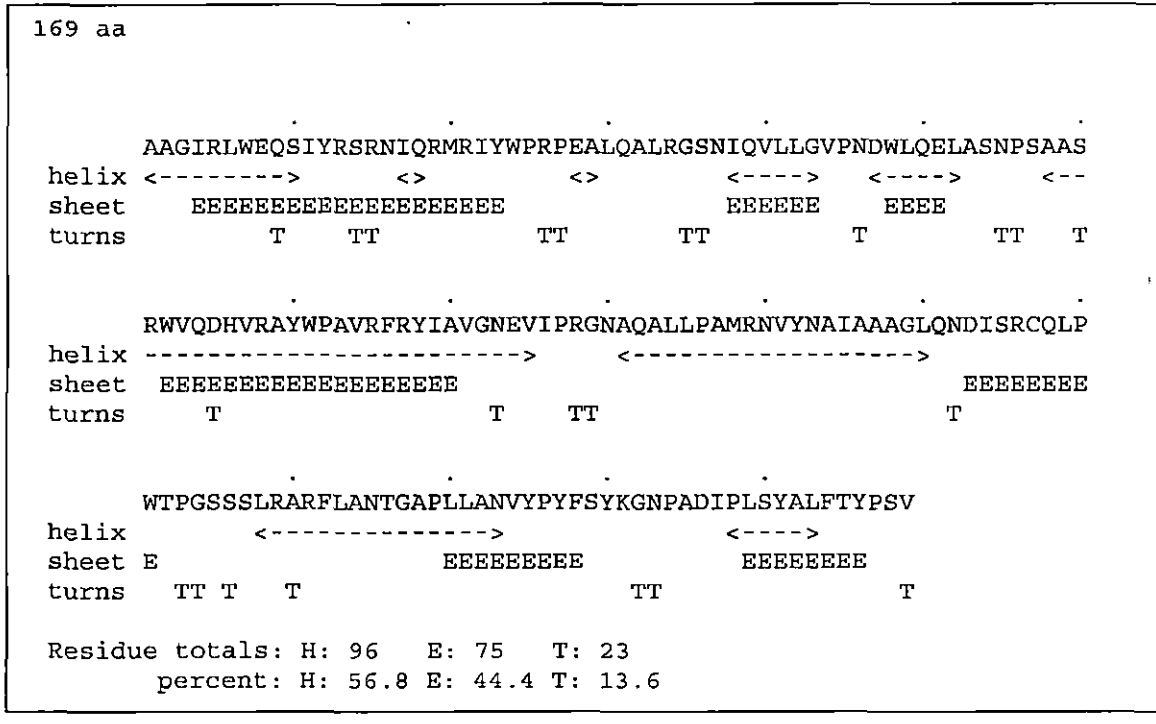


Fig. 13A. The secondary structure of protein *Pnglu* predicted by CHOFAS tool

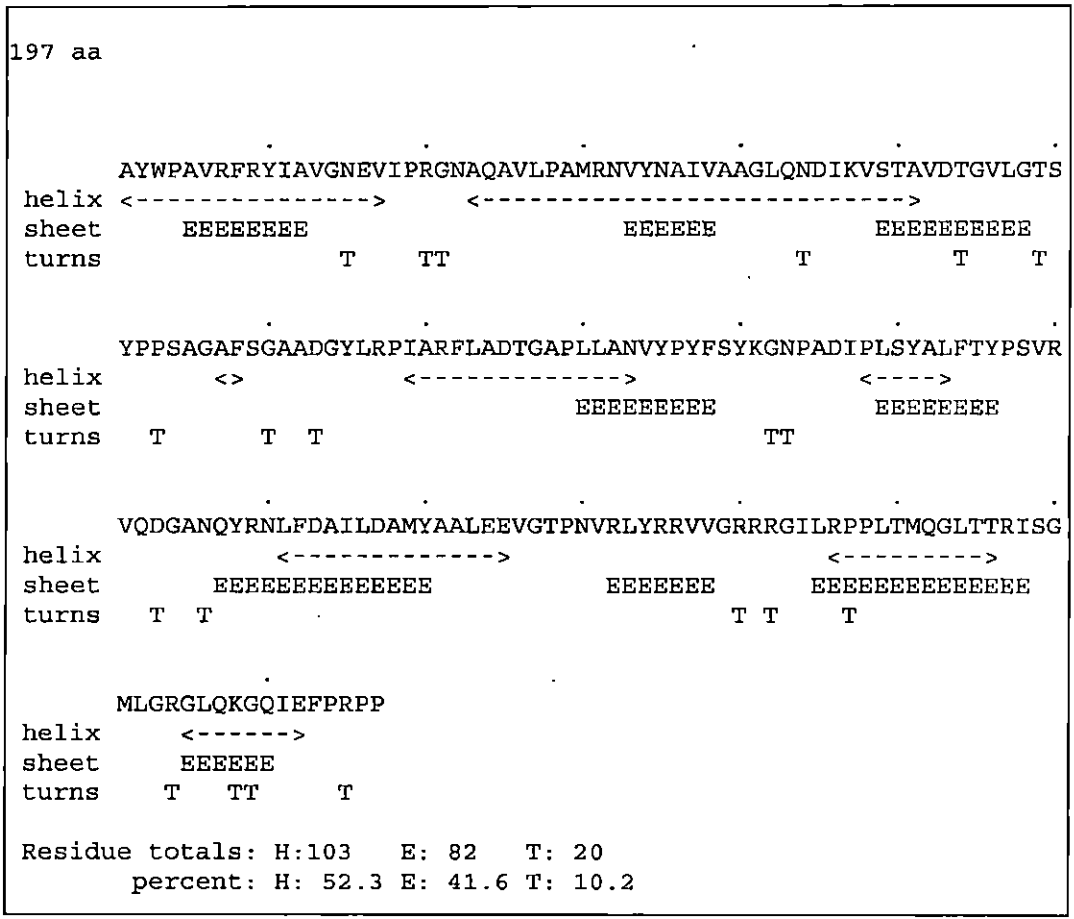


Fig. 13B.The secondary structure of protein *Pcglu* predicted by CHOFAS tool

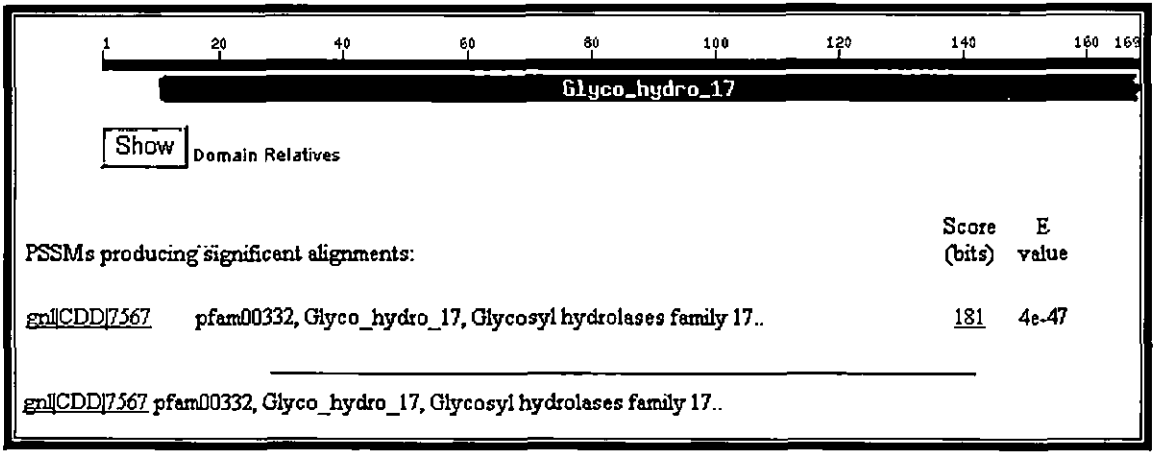


Fig.14A. Results of conserved domain in the deduced aminoacids from *Pnglu*

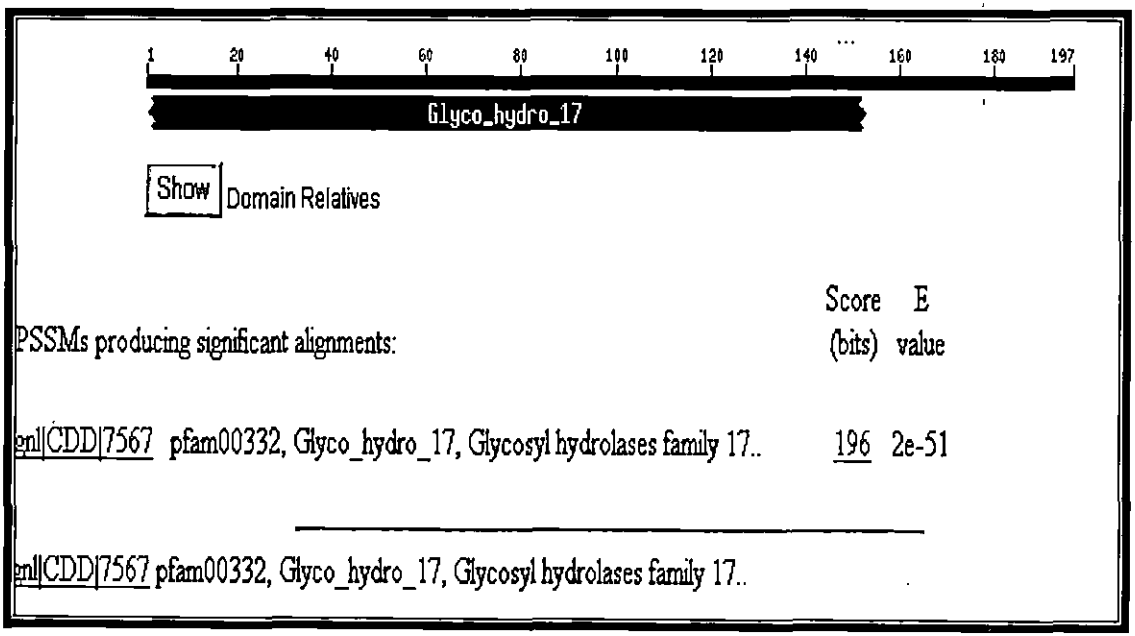


Fig.14B. Results of conserved domain in the deduced aminoacids from *Pcglu*

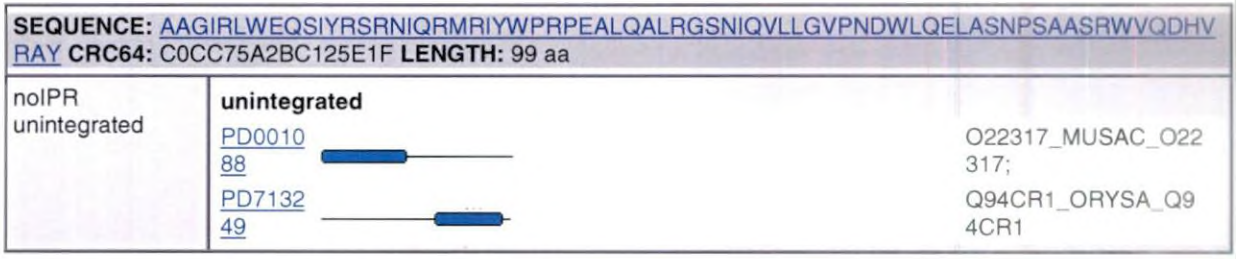


Fig.15A Determination of functional domains in *Pnglu* using InterProScan

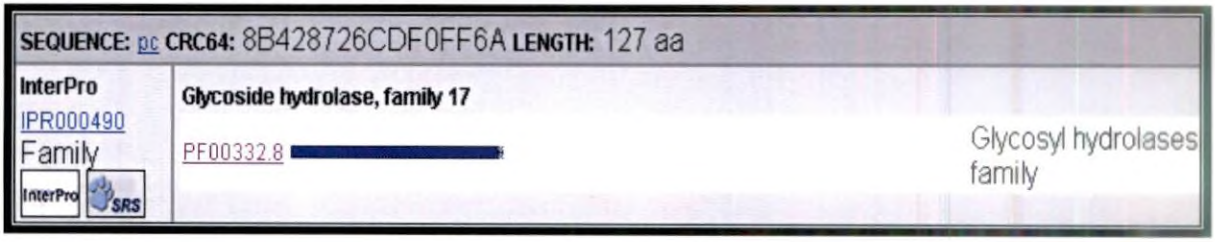


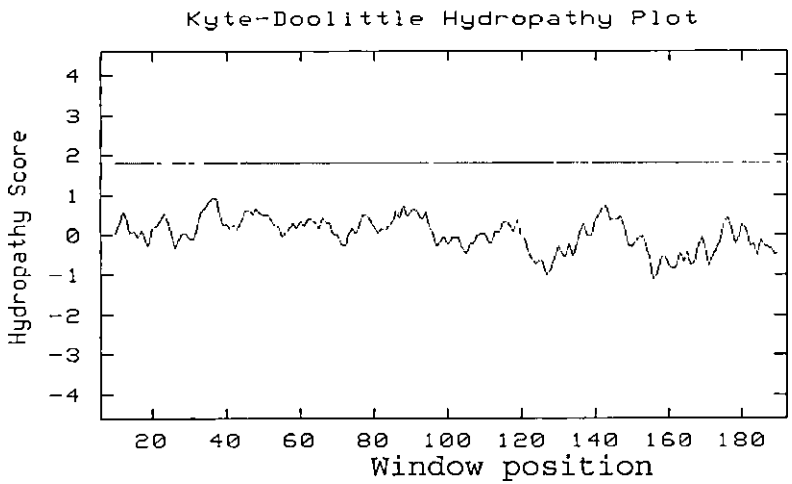
Fig.15B Determination of functional domains in *Pcglu* using InterProScan

3.20.80.80.39.1
Glycosidases

3.20.80.80.39.1
Glycosidases

Fig.16 Domain structure of Glycosyl hydrolases family

A. Kyte Doolittle Hydropathy Plot for deduced proteins *Pnglu*



B. Kyte Doolittle Hydropathy Plot for deduced proteins *Pcglu*

Fig. 17 Detection of transmembrane regions

DISCUSSION

5. DISCUSSION

The genus *Piper* (*Piperacea*) is known to have wide distribution in tropical and pan tropical regions (Ravindran *et al.*, 1992). The genus includes the renowned spice as the "king of spices", the black pepper. Even though India is the largest producer and consumer of black pepper, productivity level is comparatively low due to various reasons including *Phtyophthora* foot rot disease. Increased resistance to this dreadful disease could be provided by biotechnological approach, by transferring disease resistance genes to susceptible varieties. The most effective gene that can act against chitin negative *Phytophthora capsici* is found to be β-1, 3-glucanase. Earlier studies have confirmed overexpression of the gene in resistant genotypes as compared to the susceptible ones. (Parab, 2000)

The enzyme β-1, 3- glucanase belongs to pathogenesis related protein under the group PR2. They can hydrolyse β -1, 3 glucan present in the fungal wall of *Phytophthora capsici* and thereby inhibit the growth of the pathogen. *P. colubrinum* is an exotic wild species, which exhibit total resistance to the fungal pathogen. The β-1, 3-glucanase activities in this species has been reported to be two fold higher than the high yielding susceptible varieties of *P. nigrum* (Achuthan *et al.*, 2002). Therefore, isolation and characterization of the gene encoding β-1, 3-glucanase from *Piper* spp namely *P. nigrum* and *P. colubrinum* can be used to transform susceptible varieties of black pepper. 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. PRIMER DESIGNING

The conserved regions were observed throughout the sequences when the sequences of ß-1, 3-glucanase gene reported from different plants were compared through multiple sequence alignment. It was observed that variation in ß-1, 3- glucanase gene existed among plants but they are conserved within their structural domains. This was in accordance with Teucker and Melligen (1991). The conserved regions constitute the functional domains of ß-1, 3- glucanase genes.

Most of the conserved region in the sequence of these enzymes observed in the present study is located in the central section (Fig. 2). This region contains a conserved tryptophan residue, which could be involved in the interaction with the glucan substrates (Ori *et al.*, 1990). They also have a conserved glutamate residue, which has been shown to act as the nucleophile in the catalytic mechanism (Varghese *et al.*, 1994). 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. The primers designed and synthesized have melting temperature (Tm) greater than 52°C. Lowest annealing temperature was noticed for the primer combination *GluF2R2*. All the other primer combinations used have annealing temperature greater than 50 °C.

5.2. ISOLATION AND QUANTIFICATION OF DNA

DNA isolated from *Piper* spp using modified Doyle and Doyle (1989) protocol, yielded DNA of good quality. Quantity and quality of DNA was high when liquid nitrogen was used for grinding leaf sample. The major problem during DNA isolation was degradation of DNA. It was mainly due to high DNase activity and can be controlled by providing low temperature. Babu (2000) also has the same findings during the genomic DNA isolation from *Piper nigrum*. Since black pepper contains high quantity of phenolic compounds, use of antioxidants like ß mercaptoethanol and sodium metabisulphite has great significance.

During DNA isolation and purification, there exists chance of shearing of the DNA molecule during chloroform: isoamyl alcohol extraction. This was in accordance with the observation made by Rogers and Bendich (1994). Isolation of DNA from small quantity of leaf yielded good quality when compared to large quantity of leaf sample. Spectrophotometer reading showed that protein and RNA contamination disturbance was nil. The O.D ratio obtained was 1.89 for *Piper nigrum* and 1.84 for *Piper colubrinum*. These suggest that DNA isolation protocol was uniformly effective in *Piper* spp.

5.3. POLYMERASE CHAIN REACTION

Polymerase chain reaction facilitates amplification of specific gene efficiently and less expensively. The main disadvantage in PCR was its sensitivity to DNA amplification. The PCR parameters are very critical to amplify DNA fragments with gene specific primers. This was also in accordance with the observations reported by Muralidharan and Wakeland (1993). Therefore various PCR parameters like DNA concentration, *Taq* DNA polymerase concentration, annealing temperature, and primer concentration were standardized for the specific amplification of ß-1, 3- glucanase gene.

It was found that amplification varies even with template lot. This is due to presence of minute traces of ethanol content in the genomic DNA sometimes will degrade gene specific regions. Optimization of template DNA dilution was therefore to be carried for each template lot. Good quality DNA template was found to be necessary for amplification of specific genes. *Taq* DNA polymerase concentration was found to be effective at 0.6 units. Amplification pattern varied with the annealing temperature. Primer combination $GluF_1R_2$ yielded amplification at varied annealing temperature for *Piper* spp particularly 53°C for *P. nigrum* and 55 °C for *P. colubrinum*. This may be due to variation in GC content among *Piper* spp. If the template DNA contains high GC content higher degree of denaturation temperature and corresponding level of annealing temperature has to be given for the smooth amplification of the gene.

Gene amplification with gene specific primer combination $GluF_1R_2$ yielded multiple bands in both *P. colubrinum* and *P. nigrum*. It was also found that primer combination *GluF2R1* yielded multiple bands in *P. colubrinum*. Since ß-1, 3-glucanase belongs to complex gene families (Linthorst *et al.*, 1990; Memelink *et al.*, 1990; Beerhues *et al.*, 1994) and different isoforms have a unique amino acid sequence that was therefore encoded by a different gene. These genes shares conserved regions and might have amplified with this primer combination. There is also a 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.

Polymerase chain reaction with *GluF2R2* yielded single crisp band from *Piper nigrum* and *P. colubrinum*. The same phenomenon was observed with primer combination *GluF3R3*. This amplification was found to be repeatable. It concluded that specific region corresponding to the primer combination has amplified.

The primer combination *GluFIR1* did not give amplification in both the species at varying parameters tried. Since most of the primers designed were derived from complete coding sequences of mRNA, there exists the possibility of introns in the priming site. It is supposed that mismatch of template and primer particularly had occurred particularly at the 3' end of the primer or the occurrence of large introns immediately after priming region and hence amplification got disrupted.

5.4. TRANSFORMATION AND CLONING OF DNA

5.4.1 Preparation and screening of competent cells

The high frequency of transformation was obtained with cultures grown directly from a stock maintained in a freezing medium at -70°C. The entire procedure for competent cells preparation was carried out in aseptic condition, since contamination in competent cells can produce white colonies after blue white screening. If any other ampicillin resistant bacteria thrive in transformation plate they will give white colonies and this can be misinterpreted as transformed cells. Since calcium chloride treated *E. coli* cells was effective for plasmid DNA, they are said to be competent (Cohen *et al.*, 1972). The calcium chloride affected the cell walls and might be responsible for binding of DNA to the cell surface (Old and Primrose, 1994)

5.4.2 Ligation of PCR product

Amplicons obtained by PCR ligated with pGEM-T (Promega) Easy Vector System I was found to be very useful for cloning of PCR products. The vector was prepared by cutting Promega's pGEM$^{(R)}$ 5Zf (+) and pGEM-T Easy vectors with *Eco*R V and adding a 3'terminal thymidine to both ends. 3'T overhangs at the insertion site improve efficiency of ligation of PCR product, by preventing recirculation of the vector and providing a compatible overhang for PCR products generated by *Taq* polymerases often add a single deoxyadenosine, in a template independent fashion, to the 3' ends of the amplified fragments (Clark, 1988). This vector contained T_7 and SP_6 RNA polymerase promoter flanking a multiple cloning region within the lac Z encoding region (Fig. 1). This facilitates blue-white screening of recombinants by insertional inactivation of ß-galactosidase. It also posses multiple cloning sites within this lac z gene. It was observed that ligation reaction set at room temperature for one hour followed by incubating at 4°C was effective for transformation.

5.4.3 Transformation of DNA

Competent cells of *E. coli* were transformed with ligated PCR product both blue and white colonies were observed. It was due to α complementation results blue colonies. During transformation, the host encoded and vector encoded protein regions of β galactosidase undergo α complementation to form enzymatically active protein. Such transformed *E. coli* cells with non recombinant plasmid developed into blue colonies in the presence of chromogenic substrate X-gal (Ullman *et al.*, 1967; Hortwitz *et a.*, 1964). The insertion of foreign DNA into the polycloning site of the plasmid was not capable of α complementation and such transformed *E. coli* cells, carrying recombinant plasmid developed into white colonies.

High number of white colonies and less number of blue colonies were observed in the transformed plate. It was found that number of transformants increased in proportion to the amount of DNA added to the system until it becomes saturated. It was also observed that colonies having ß galactosidase activity

(untransformed plasmid) grew poorly relative to cells lacking this activity. After overnight growth, the blue colonies were observed to be smaller than the white colonies, which were approximately one millimeter in diameter. High frequency of transformation was noted in the positive control plate transformed with the insert supplied by pGEM-T kit indicating that transformation was carried effectively.

5.5 CONFIRMATION OF RECOMBINATION

Polymerase chain reaction carried out with gene specific primers yielded amplicon of desired size 780bp approximately. No amplicon was obtained in negative control. This confirms presence of insert in the vector.

Restriction analysis was carried out in the plasmid DNA isolated from randomly selected white colonies and observed high rate of transformation (Sambrook *et al.*, 1989). Since pGEMT vector possessed restriction sites for different restriction enzymes within the multiple cloning sites, the release of insert was possible by digestion with single restriction enzyme *Eco*RI.

5.5.1 Sequencing of the clones

The cloned fragments when sequenced by automated sequencing with T7 primer provided the information of the region starting from forward primer. The service (*AI*) offered by DNA sequencing facility offered by Delhi university south campus facilitated the sequencing service upto 600bp. Further sequencing with SP6 primer has to be done for obtaining details of region starting from the reverse primer of the cloned insert.

5.6 SEQUENCE ANALYSIS

5.6.1 Nucleotide analysis

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.*, 1997). This can determine the sequence homology to predict the identity and function of query sequence. Sequences obtained from *P. colubrinum* with the primer combination $GluF_1R_2$ and from *P.*

nigrum with the primer combination *GluF3R3* yielded high homology to ß -1, 3-glucanase genes from other crops such as *Musa accuminata, Cichorium* sp, *Vitis vinifera, Nicotiana, Hordeum vulgare, P. colubrinum* and *Hevea brasiliensis.*

The multiple sequence alignment of *Pnglu* shared conserved regions with ß-1, 3-glucanase sequences of barley, wheat, *Piper colubrinum*, *Hordeum vulgare* and *Oryza sativa.* (Fig. 7). The cloned fragment *Pcglu* from *P. colubrinum* amplified by *GluF1R2* primer combination, shared conserved region with ß-1, 3-glucanase sequences of *Fragaria,* tomato, *Malus*, *Musa accuminata* and *P. colubrinum* (Fig. 8). This confirms that the clone *Pcglu* and *Pnglu* are the fragments of the gene encoding for ß-1, 3-glucanase in *Piper* spp.

Phylogram is a branching diagram assumed to be an estimate of a phylogeny; branch lengths are proportional to the amount of inferred evolutionary change. Thus it indicates the evolutionary time separating taxa. Phylogram analysis in *Pnglu* observed that it was evolutionary more related to barley, wheat and *Oryza sativa* and least related to the nucleotide sequences of ß-1, 3-glucanase gene from *P. colubrinum.* Sequence divergence observed for the protein encoded by *Pnglu* and *Pcglu* reflects the evolutionary relationship (Fig. 9). Phylogram analysis in *Pcglu* found that it is evolutionary more related to *P. colubrinum* and least related to *Malus* (Fig. 8).

Open reading frame (ORF) is the part of protein coding gene that is translated into protein. It starts with an initiation codon and ends with a termination codon (Old and primrose, 1994). The clone *Pnglu* has an ORF 450 bp length and codes for peptide of 149 amino acids in length (Fig. 10). The clone *Pcglu* has an ORF 372bp length and codes for a peptide of 123 amino acids (Fig. 11). All the ORFs displayed for *Pcglu* and *Pnglu* where subjected to BLAST tools revealed that given sequence code for β -1, 3-glucanase gene family (Table 8 and Table 9)

The sequence obtained from *P. nigrum* at primer combination $GluF_1R_2$ and *GluF2R2* showed no homology to ß-1, 3- glucanase. This non homology can

occur due to several reasons. Most probable reason is the presence of other competitive regions in the genomic DNA. Homology search revealed that these sequences are non coding regions. The introns inserted between the priming sites made the region to amplify and had given amplicon of expected size. The number of introns is reported to vary widely within different genes of the same species and within the same gene in different species (Minocha, 2000). The size of introns is also quite variable ranging from about 70 bases to as much as 7 kb and typical intron size is reported to vary 100 to 200 bp long (Simpson and Filipowicz, 1996).

The gene fragment for ß-1, 3-glucanase when subjected to nucleotide statistics tools revealed that both *Pcglu* and *Pnglu* have high G+C content compared to A+T content. This suggests that it has characteristics of eukaryotes, where G+C content is high compared to A+T content. High content of G+C indicate the stability of the sequences.

The sequences of *Pnglu* and *Pcglu* were analysed for commonly used restriction endonucleases sites and it produced a comprehensive and precise map (Fig. 18A and Fig. 18B). The cloned fragment *Pcglu* had only two sites restriction enzymes *Alu*I where as in *Pnglu* had three sites restriction enzyme *Alu*I. Restriction sites for *Ava* I, *Not* I, *Sal* I were absent in *Pnglu* but corresponding sites were present in *Pcglu.*

Gene prediction analysis from *Pnglu* and *Pcglu* determined internal exons of one each from both the clones *Pcglu* and *Pnglu. Pcglu* has an exon of size 348bp and *Pnglu* has exon of size 423bp. Since *Pcglu* and *Pnglu* are only fragments of β-1, 3-glucanase exons predicted from these sequences will not represent the entire coding sequences of the gene.

5.6.2 Amino acid analysis

Amino acids statistics carried out with deduced amino acid found that *Pcglu* has only one tryptophan residues, whereas *Pnglu* has only six tryptophan residues (Table 13). Tryptophan residues are involved in the interaction with

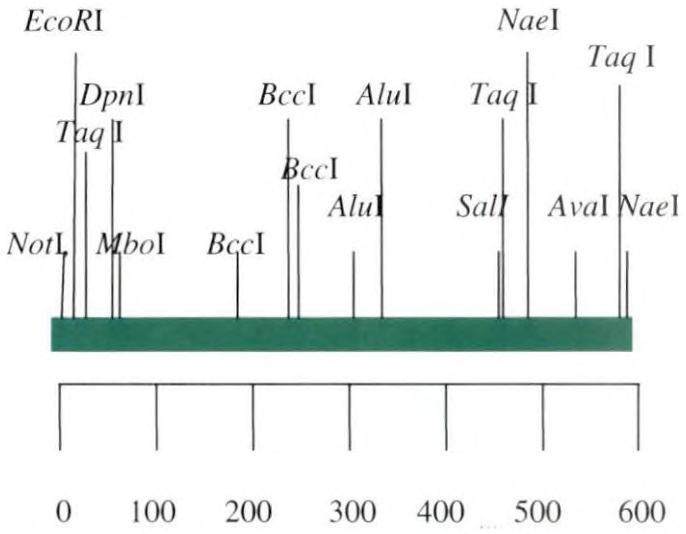


Fig.18A Restriction map of *Pcglu* with common restriction enzymes

Scale
1cm=100bp

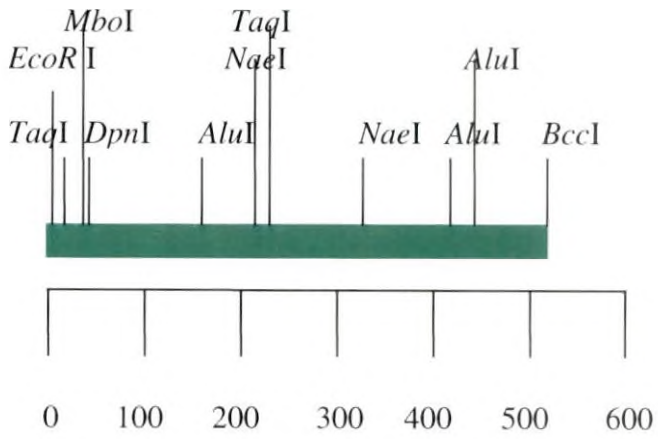


Fig.18B Restriction map of *Pnglu* using commonly used restriction enzymes

glucan residues (Ori *et al.*, 1990). Glutamic residues present in both *Pcglu* and *Pnglu* were four in number, they act as nucleophile in the catalytic mechanism (Varghese *et al.*, 1994). Alanine content was high in both predicted peptide of *Pcglu* and *Pnglu*. Histidine residue was absent in the peptide of *Pcglu* and present in peptide from *Pnglu*.

Sequence alignment of deduced amino acids of *Pcglu* and *Pnglu* was in supporting with the observation of Sun *et al.* (2000). The amino acid *glu* in β-1, 3-glucanase is usually preceded by *gly* rather than residue and β -1, 3-glucanase have an extra amino acid, typically a *met* preceding the second g*lu*.

Tobacco, pea and bean ß-1, 3-glucanases are postulated to be synthesized as large precursors with an N terminal signal peptide (Shinshi *et al.*, 1988; Vogeli *et al.*, 1988). They have a single putative N glycosylation site, *Asn-Xaa-Ser/The*, starting at *Asn* 330 in the C terminal extension (Chang *et al.*, 1992). Since the ß-1, 3- glucanase clones *Pcglu* and *Pnglu* were amplified neither from N terminal nor from C terminal, both lack the signal peptide and single putative N glycosylation site.

The conserved domains in the protein sequences of *Pcglu* and *Pnglu* suggest that both have functional domains of glycosyl hydrolase family 17 (Fig. 14). Glycosyl hydrolases are a widespread group of enzymes that hydrolyse the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. Glycosyl hydrolase are classified into 85 different families (Henirssatt, 1999). Because the fold of proteins is better conserved than their sequences, some of the families are grouped under 'clans'. Under 'clan GA' comes glycosyl hydrolases 17. Their family contains glucan endo-1, 3-beta-glucosidases (EC 3.2.1.39) and lichenases (EC 3.2.1.73). Henrissat (1999) and Ori *et al* (1990) have given the description of glycosyl hydrolases family 17 signatures.

The signature of glycosly hydrolase is (LIVMKS) -x- (LIVMYFWA) (3)- STAG-E- (STACVI) -G- (WY) -P- (ITN) -x- (SAGQ). Multiple sequence

alignment revealed that the sequences are seen among plants at position 218 to 237 range. Since the predicted peptide *Pcglu* and *Pnglu* protein regions are not complete, they have amino acids of length 188 and 169 amino acids in length respectively the glycosly hydrolase signature are fully not present.

Functional domain determination in *Pnglu* and *Pcglu* further confirmed that their domains belonged to Glycosyl hydrolase 17. The domain architecture underlying a particular protein sequence is important because it gives hint about possible 3D structure and suggests its potential biochemical functions. Domain structural comparison through CATH structural database revealed that both belongs to glycosidase, which is a sequence family of β 1, 3 glucanase.

Secondary structure of polypeptide tells about the potential interaction between the amino acids in the sequence. *Pcglu* protein have 52.3 per cent helix region where as in *Pnglu* has 56.8 helix region (Fig.19). Hydrophobicity is the most popular analysis because it gives a good indication of transmembrane segments or core regions within a protein. No transmembrane region was detected from both *Pcglu* and *Pnglu* proteins (Fig.17A and Fig.17B). Each curve shows the average of residue specific hydrophobicity for a window size of 19. Hydrophobic sequences are indicated by positive value and hydrophilic sequences by negative values. Numbers below the graph indicates residue positions in the sequences.

The sequence information of *Pnglu* and *Pcglu* confirms that they are components of β-1, 3-glucanase gene in *Piper* spp. This sequence information can be further utilized for the isolation of full length gene from *Piper* spp.

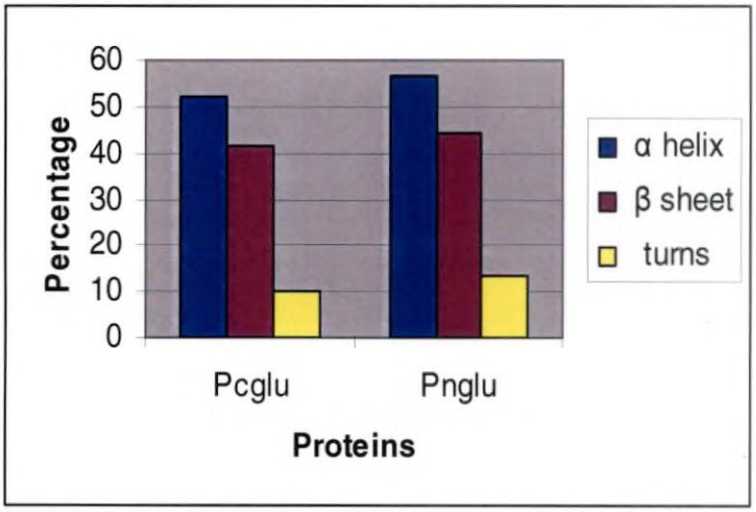


Fig.19
Depiction of secondary structure of the deduced aminoacids from *Pcglu* and *Pnglu*

SUMMARY

SUMMARY

An investigation on isolation and characterization of β- 1, 3- glucanase gene from *Piper* spp was undertaken in the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara from December 2003 to September 2005. Two species of *Piper* namely *P. nigrum* and *P. colubrinum* were included in the study. The salient findings are summarized below.

1. The method suggested by Doyle and Doyle (1987) with slight modifications was found effective in isolating good quality genomic DNA from *Piper* spp. Homogenisation in liquid nitrogen and incorporation of β-mercaptoethanol reduced the phenolic interference and increased the recovery of DNA.

2. Multiple sequence alignment of sequences for β-1, 3- glucanase gene from different crop species showed that most of the conserved regions are in the mid sequence regions.

3. Eight (four forward and four reverse) primers were derived based on the conserved region and out of this three sets of forward and reverse primers were selected for gene isolation.

4. The PCR amplification with primer combination *GluF1R2* yielded multiple bands in *P. nigrum* and *P. colubrinum*. Discrete bands were obtained for primer combination *GluF2R2* and *GluF3R3* in both the species.

5. The amplicons were effectively ligated in pGEM-T vector

6. Competent cells of *E. coli* were prepared and transformed with the ligated pGEM-T vector and transformant was screened through blue white screening.

7. High recombination efficiency (68 % to 76 %) was observed for the four amplicons cloned.

8. The presence of recombinant plasmid was confirmed by PCR amplification and restriction analysis.

9. Sequencing of the amplicons with T7 primer gave the sequence data for 508 to 606 bp for the fragments amplified with different primers.

10. The homology search for fragments *Pnglu*, cloned from *Piper nigrum* with primer combination *GluF3R3* and the fragment *Pcglu,* cloned from *P. colubrinum* with primer combination *GluF1R2* showed significant levels of homology to β-1, 3- glucanase gene deposited from several plant species such as *Musa accuminata, Vitis, Nicotiana, Hordeum vulgare, P.colubrinum* and *Hevea brasiliensis* in the public domain database.

11. The fragments amplified with the primer *GluF1R2* and *GluF2R2* may be introns, as they did not show homology with β-1, 3-glucanase gene sequence.

12. Multiple sequence alignment of nucleotide of *Pnglu* and *Pcglu* with the selected nucleotides of β-1, 3- glucanase gene sequence from different plant species indicated several conserved regions.

13. The cloned fragment *Pcglu*, from *Piper nigrum* had largest open reading frame of size 450 that coded 149 amino acids. The cloned fragment *Pcglu*, from *P. colubrinum* had largest open reading frame of size 372 and coded for 123 amino acids.

14. Internal exons of size 423 bp and 348 bp respectively were detected in the fragment *Pnglu* and *Pcglu* respectively.

15. Restriction analysis revealed that both *Pnglu* and *Pcglu* have restriction sites for the frequent cutter *Alu* I and the rare cutter *Nae* I.

16. The fragments had high GC content of 56.1 per cent and 56.9 per cent for *Pnglu* and *Pcglu* respectively.

17. The major amino acid composition deduced from the cloned DNA fragment *Pnglu* were alanine, leusine, arginine and proline, while in the fragment *Pcglu* major amino acids were alanine, glycine, arginine, leusine and valine. The secondary structures predicted for the polypeptides with the deduced amino acids of *Pnglu* and *Pcglu* had a high proportion of helices.

18. Conserved domains detected from deduced amino acids of *Pnglu* and *Pcglu* suggested that both belong to glycosyl hydrolase family 17, which is a single domain family.

19. Domain structure comparison with CATH structural database indicated that domains of *Pnglu* and *Pcglu* had similarity to glycosidases, which is the sequence super family of β-1, 3-glucanases.

20. Kyte-Doolittle Hydropathy profile showed that both *Pnglu* and *Pcglu* lack transmembrane regions.

21. The sequence information of *Pnglu* and *Pcglu* confirmed that they are genomic counter part of β -1, 3-glucanase gene from *Piper* spp and can be further utilized for the isolation of full-length gene from *Piper* spp.

REFERENCES

REFERENCES

Abeles, F.B., Bosshart, R.P., Forrence, L.E. and Habig, W.C. 1971. Preparation and purification of glucanase and chitinase from bean leaves. *Pl. Physiol.* 47: 129-134

Achuthan, C.R., Nazeem, P.A., Babu, T.D., Girija, D. and Keshavachandran, R. 2002. Expression of β-1, 3-glucanase in relation to foot rot disease of *Piper nigrum* L. *International symposium on molecular approaches for improved crop productivity and quality*, May 22-24, 2002. TNAU, Coimbatore, p. 14

Alagappan, V. and Manoharan, V. 2001. Production of pepper in India. A global perspective. *Spice India* 14: 4-8

Alexander, D., Goodmann, R.M., Gut-Rello, M., Glasock., Weyman, K., Friedich, L., Maddox, D., Ahl-Goy, P., Luntz, T., Ward, E. and Ryals, J. 1993. Increase in tolerance to two oomycetes pathogens in transgenic tobacco expressing pathogenesis related proteins. *Proc. nat. Acad. Sci. USA.* 90: 7327-7331

*Altschul, S.F., Thomas, L.M., Schäffer, A, A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25: 3389-3402

Anandaraj, M., Abraham, J. and Balakrishnan, R. 1989. Crop loss due to foot rot (*Phytophthora palamivora*, MF_4) disease of black pepper (*Piper nigrum* L.) in Canannoore district of Kerala. *Indian Phytopath.* 41: 473-476

Babu, T.P.H. 2000. RAPD analysis to assess the genetic stability in tissue culture derived black pepper (*Piper nigrum* L.) MSc. (Ag.) thesis, Kerala Agricultural University, Thrissur, 83 p.

Balakrishnan, R., Anandaraj, M., Nambiar, K.K.N., Sarma, Y.R., Brahma, R.N. and George, M.V. 1986. Estimates on the extent of loss due to quick wilt disease of black pepper (*Piper nigrum* L.) in Calicut district of Kerala. *J. Plantation Crops* 14: 15-18

Beerhues, L. and Kombrink, E. 1994. Primary structure and expression of mRNAs encoding basic chitinase and β-1, 3-glucanase in potato. *Pl. mol. Biol.* 24: 353-367

Bera, S. and Purkayastha, R.P. 1997. Identification and characterization of some PR proteins induced by kitazin and *Rhizoctonia solani* causing sheath blight of rice. *Indian J. Exp. Biol.* 35: 644-649

Bezier, A., Lambert, B. and Baillieul, F. 2003. Molecular cloning of a beta-1, 3-glucanase mRNA induced by grapevine in response to *Botrytis cinerea* infection [Online]. Available:www.ncbi.nlm.nihgov/ [2005, Oct.21]

Birnboim, H.C. and Doly, J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic. Acid Res.* 1513-1523

Borkowska, M., Kizymowska, M., Talaeczyk, A., Awan, M.F.M., Yalkouleva, L., Klerizkowski, K. and Wiegat, B. 1998. Transgenic potato plants expressing soyabean β-1, 3-endo glucanase gene exhibit an increased resistance to *Phytophthora infestans. Biosciences* 53: 1012-1016

Bulcke, M.V., Baw, G., Castresana, C., Van Montagu, M. and Vanderkerckhove, J. 1989. Characterization of vacuolar and extracellular β-1, 3-glucanase of tobacco: Evidence for a strictly compartmentalized plant defense system. *Proc. nat. Acad. Sci. USA.* 86: 2673-2677

Burge, C. and Karlin, S. 1997. Prediction of complete gene structures in human genomic DNA. *J. mol. Biol.* 268: 78-94

Castresana, C., Carvalho, F., Gheysen, G., Habets, M., Inze, D. and Montagu, V.M. 1990. Tissue specific and pathogen induced regulation of a *Nicotiana plumbaginifolia* β-1, 3-glucanase gene. *Pl. Cell* 2: 1131-1143

Chang, M., Culley, D.E. and Hadwiger, L.A. 1993. Nucleotide sequence of a pea (*Pisum sativum* L.) β-1,3-glucanase gene. *Pl. Physiol.*101: 1121-1122

Chang, M., Hadwiger, L.A. and Horovitg, D. 1992. Molecular characterization of a pea β-1, 3-glucanase induced by *Fusarium solani* and chitosan challenge. *Pl. mol. Biol.* 20: 609-618

*Chang, M.M., Culley, D., Choi, J.J. and Hadwiger, L.A. 2002. *Agrobacterium* mediated co-transformation of a pea beta-1, 3-glucanase and chitinase genes of a potato (*Solanum tuberosum* L.c.v. Russet Burbank) using a single selectable marker. *Pl. Sci.* 163: 83-89

Chawla, H.S. 2003. *Introduction to Plant Biotechnology.* Oxford and IBH Publishing Co. Pvt. Ltd., New Delhi. 538 p.

Chen, Y.C., Do Y.Y. and Huang, P.L. 1997. Isolation and nucleotide sequence of a fruit ripening-related cDNA encoding glucan endo-1, 3-beta-glucosidase homolog from banana. *Pl. Physiol.* 115: 864-871

*Chou, P.Y. and Fasman, G.D. 1978. Prediction of the secondary structure of proteins from their amino acid sequence. *Adv. Enzymol.* 47: 45-148

Chye, M. and Cheung, K. 1995. β-1, 3-glucanase is highly expressed in laticifers of *Hevea brasiliensis. Pl. mol. Biol.* 29: 397-402

*Clark, J.M. 1988. Novel non-templated nucleotide addition reactions catalyzed by prokaryotic and eukaryotic DNA polymerases. *Nucleic Acids Res.* 16: 9677-9686

Cohen, J. D., Chang, A.C.Y. and Hsu, L. 1972. Non chromosomal antibiotic resistance in bacteria: genetic transformation of *E.coli* by R factor DNA. *Proc. nat. Acad. Sci. USA.* 69: 2110-2114

Cote, F., Cutt, J.R., Asselin, A. and Klessig, D.F. 1991. Pathogenesis related acidic β-1,3-glucanases genes of tobacco are regulated by both stress and developmental signals. *Mol. Pl. Microbe Interaction* 4: 173-181

Cui, S. and Kang, Z. 2005. The expression pattern of defense response gene transcript accumulation and cloning in wheat leaves infected by *Puccinia striiformis* f. sp. *Tritici* [Online]. Available:www.ncbi.nlm.nihgov/ [2005, Aug. 21]

Dake, M.S., Jadhav, J. P. and Patil, N. B. 2004. Induction and properties of (1,3)-β-D glucanase from *Aureobasidium pullens. Indian J. Biotech.* 3: 58-64

Dicto, J. and Manjula, S. 2005. Identification of elicitor induced PR 5 gene homologue in *Piper colubrinum* Link by suppression substrative hybridization. *Curr. Sci.* 88: 624-627

Doyle, J.J. and Doyle, J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* 19: 11-15

Ekinci, M.S., McCrae, S.I. and Flint, H.J. 1997. Isolation and over expression of a gene encoding an extracellular ß-(1, 3-1,4)-Glucanase from *Streptococcus bovis* JBI. *Appl. Environ. Microbiol.* 63: 3752-3756

Eldik, G.J.V., Litiere, K., Jacobs, J.M.R., Montagu, M.V. and Cornelissen, M. 1998. Silencing of β-1, 3-glucanase gene in tobacco correlates with an increased abundance of RNA degradation intermediates. *Nucleic Acids Res.* 26: 5176-5181

Ellis, J.G., Lawerance, G.J., Peacock, W.J. and Peyor, A.J.1988. Approaches to cloning plant genes conferring resistance to fungal pathogen. *A. Rev. Phytopathol.* 25: 245-263

Felix, G. and Meins, F.J. 1987. Ethylene regulation of β-1, 3-glucanase in tobacco. *Planta.* 172: 386-392

*Garcia-Perez, M.D., Egea, C. and Candela, M.E. 1998. Defense response of pepper (*Capsicum annum*) suspension cells to *Phytophthora capsici*. *Pl. Physiol.* 103: 527-533

Gheysen, G., Inze, D., Soetaert, P., Montagu, M.M., Castresana, C. and Van-Montagu, M.1990. Sequence of a *Nicotiana plumbiginifolia* beta (1,3)-glucanase gene encoding a vacuolar isoform. *Nucleic Acids Res.* 18: 6685-6691

Go-Young, Q., Martin, G. B. and Gu, Y.Q. 1998. Molecular mechanisms involved in bacterial speck disease resistance of tomato. *Molecular bases of signal transduction in plants* (eds. Hetherington, A.M., Hooley, R. and Irvine, R.F.). Proceedings of a discussion meeting held on 18-19 February 1998, London, pp. 1455-1461

Gueguen, Y., Voaehorst, W.G.B., Oost, J.V. and Vos, W.M. 1997. Molecular and biochemical characterization of an endo-β-1,3-glucanase of the hyper thermophilic archeon *Pyrococcus furiosus*. *J. biological Chem.* 272: 31258-31267

Guzzo, S .D., Harakava, R. and Tsai, S. M. 2003 Identification of defense-related coffee genes by suppression subtractive hybridization [Online]. Available:www.ncbi.nlm.nihgov/ [2004, Dec. 21]

Hamond-Kosack, K.E. and Jones, J.D.G. 1996. Resistance gene dependent plant defense response. *The Pl. Cell* 8: 1773-1791

Harpster, M.H., Brummell, D.A., and Dunsmuir, P. 1998. Expression analysis of a ripening specific, auxin repressed endo-1, 4-beta glucanase gene in strawberry. *Pl. Physiol.*118: 1307-1316

Helleboid, S., Chapman, A., Hendriks, T., Inze, D., Vasseur, J. and Hilbert, J.L. 2000. Cloning of β-1, 3-glucanases expressed during *Cichorium* somatic embryogenesis. *Pl. mol. Biol.* 42: 377-386

Henrissat, B.1999. A classification of glycosyl hyrolases based on amino acid sequence similarities. *Biochem. J.* 280: 309-316

Holliday, P. and Mowat, W.P. 1963. Root rot of *Piper nigrum* L. (*Phytophthora palmivora*). *Phytopathology* 5: 62-65

Holtorf, H., Schof, H., Kanz, C., Waldvogel, R. and Meins, F.J. 1999. Stochastic and non stochastic post-transcriptional silencing of chitinase and beta,1, 3-glucanase genes involves increased RNA turnover possible role of ribosome-independent RNA degradation. *Pl. Cell* 11: 471-483

Horwtiz, J. P., Chua, R. J., Curby, A.J., Tomson, M. A., DaRooge, B. E., Fisher, J., Mauricio, J. and Klundt, I. 1964. Substrates for cytochemical demonstration of enzyme activity. *J. Med. Chem.*7: 574-578

*Hou, L., Luo, X.Y., Shen, H., Pel, Y. and Cal, Y.F. 1999. Construction of a plant expression vector harbouring the beta-1, 3-glucanase gene. *J. South West. agric. Univ.* 21: 251-253

Hwang, B.K., Kim, W.B. and Kim, W.K. 1989. Ultrastructure at the host-parasite interface by *Phytophthora capsici* in roots and stems of *Capsicum annum*. *J. Phytopathol.* 127: 305-315

Jabekumar, R.S., Anandaraj, M. and Sarma, Y.R. 2001. Induction of PR proteins and defense related enzymes in black pepper due to inoculation with *Phytophthora capsici. Indian Phytopath.* 54: 23-28

Jayaraj, J., Muthukrishnan, S., Liang, G.H. and Velazhaban, R. 2004. Jasmonic acid and salisylic acid induce accumulation of ß-1, 3-glucanase and thaumatin-like proteins in wheat and enhance resistance against *Stagonospora nodarum. Biol. Pl.* 481: 425-430

Jonedijik, F., Tigelaar, H., Roeckel, S. C., Bress-Vloemans, S. A., Dekker, I., Elzen, P. J. M., Cornelissen, J.C., Melchers, L. S., Van-Roeckal, S. C., Van-den-Elzen, P. J .M and Jones, P. W. 1995. Synergistic activity of chitinases and beta-1, 3-glucanases enhances fungal resistance in transgenic tomato plants. *Euphytica* 85: 173-180

Jung, H.W. and Hwang, B.K. 2000. Isolation, partial sequencing and expression of pathogenesis related cDNA genes from pepper leaves infected by *Xanthomonas capestris* pv. *vesicatoria. Mol. Pl. Microbe Interaction* 13: 136-142

*Khan, A.A., Shi, Y.L. and Shih, D.S. 2003. Cloning and characterization of a beta-1, 3-glucanase gene from strawberry. *DNA sequence* 14: 406-412

Kikuchi, T., Shibuya, H., and Jones., J. T. 2005. Molecular and biochemical characterization of an endo-beta-1, 3-glucanase from the pinewood nematode *Bursaphelenchus xylophilus* acquired by horizontal gene transfer from bacteria. *Biochem J.* 389: 117-125

Kim, H., Ann, J., Gorlach, J.M., Caprari, C., Scott-craig, J.S. and Walton, J.D. 2001. Mutational analysis of β-glucanase genes from the plant-pathogenic fungus *Cochliobolus carbonum. Mol. Pl. Microbe Interactions* 14: 1436-1443

Kim, Y.H., Koo, B.S.,Oh, S.S., Park, K.S., Lim ,S.Y., Ryu, J.C. and Eun, M.Y. 1993. Molecular cloning of a gene coding for beta-1, 3-glucanase from *Bacillus subtilis* K4, antagonist to plant pathogenic fungi. *J. Agri. Sci. Biotech.* 35: 213-218

Kim, Y.J. and Hwang, B.K. 1994. Differential accumulation of β-1, 3-glucanase and chitinase isoforms in pepper stems infected by compatible and incompatible isolates of *Phytophthora capsici. Physiol. mol. Pl. Path.* 45: 195-209

Kim, Y.J. and Hwang, B.K. 1997. Isolation of basic 34 kilo dalton β-1, 3-glucanase with inhibitory activity against *Phytophthora capsici* from pepper stems. *Physiol. mol. Pl. Path.* 50: 103-115

Ko, T.S., Lee, S.M., Schaefer, S.C. and Karban, S.S. 2003. Characterization of a tissue specific and developmentally regulated β-1, 3-glucanases gene family in *Prunus persica. Pl. Physiol Biochem.* 41: 955-963

Kombrink, E., Schroder, M. and Hahlbrock, K. 1988. Several 'pathogenesis-related' proteins in potato are β-1, 3-glucanase and chitinases. *Proc. nat. Acad. Sci. USA.* 85: 782-786

Kwon, T.H., Kim, T.G., Kum, D. H. and Yang, M.S. 1999. Molecular cloning of a genomic DNA encoding the potato beta-1, 3-glucanase. *J. Pl. Physiol.* 155: 520-526

*Kyte, J. and Doolittle, R.F. 1982. A simple method for displaying the hydropathic character of a protein. *J. mol. Biol.* 157: 105-32

Lashbrook, C., Gonzales-Bosch, C., Bennet, A. 1994. Two divergent endo-β-1,4-glucanase genes exhibit overlapping expression in ripening fruit and absicissing flowers. *Pl. Cell* 6: 1485-1490

Lee, C.C., Wu, X., Gibbs, R.A., Coot, R.G., Muzey, D.M. and Caskey, C.T. 1988. Generation of cDNA probes directed by amino acid sequence: cloning of cerate oxidase. *Science* 239: 1288-1291

*Libantova, J., Bauer, M., Mlynarova, l., Moraucikova, J and Bekesiova, I. 1998. Transgenic tobacco and potato plants expressing basic vacuolar beta-1, 3-glucanase from *Nicotiana plumbaginifolia. Biol. Brastislava* 53: 739-748

Linthorst, H.J.M., Melchers, L.S., Mayer, A., Jeroen, S.C., Roekel, V., Cornelissen, B.J.C. and Bol, J.F. 1990. Analysis of gene families encoding acidic and basic β 1, 3 glucanase of tobacco. *Proc. nat. Acad. Sci. USA.* 87: 8756-8760

Mammooty, K.P. 2003. Nursery diseases of black pepper (*Piper nigrum* L.) in Kerala with special reference to the management of *Phytophthora capsici* Leonian, emend. Alizedeh and Tsao. Ph.D. thesis, Kerala Agricultural University, 176 p.

Mandel, M. and Higa, A. 1970. Calcium-dependent bacteriophage DNA infection. *J. mol. Biol.* 53: 159-162

Marchler-Bauer, A. and Bryant, S.H. 2004. CD-Search: protein domain annotations on the fly. *Nucleic Acids Res.* 32: 327-331

Mateos, F.V., Rickaue, M. and Esquerro-Tugaye, M. 1997. Cloning and characterization of a cDNA encoding an elicitor of *Phytophthora parasitica* var. *nicotianae* that shows cellulose binding and lectin like activities. *Mol. Pl. Microbe Interactions* 10: 1045-1053

Mathieu, H.A., Joosten, A.J. and Dewit, P.J.M. 1985. Identification of several pathogenesis related proteins in tomato leaves inoculated with *Cladosporium fulvum* (syn. *Fulvia fulva*) as 1, 3 β-glucanase and chitinases. *Pl. Physiol.* 89:945-951

Mauch, F. and Stachelin, C.A. 1989. Functional implications of the subcellular localization of ethylene-induced chitinase and ß-1, 3-glucanase in bean leaves. *The Pl. Cell* 1: 447-457

Mauch, F., Hadwiga, L.A. and Boller, T. 1988. Antifungal hydrolases in pea tissue. *Pl. Physiol.* 87: 325-333

Mauch, F., Mauch-Mani, B. and Boller, T. 1988. Antifungal hydrolases in pea tissue II. Inhibition of fungal growth by combinations of chitinase and β-1, 3-glucanase. *Pl. Physiol.* 88:936-942

Meins, J. R., Neuhhaus, J. M., Spersen, C. and Ryals, J. 1992. The primary structure of plant pathogenesis related glucanohydrolases and their genes. *Genes involved in Plant Defense.* (eds. Boller, T. and Meins, F.) Springer Verlag, Wein, pp. 245-282

Memelink, J., Linthorst, H.J.M., Schilperoost, R.A. and Hoge, H.C. 1990. Tobacco genes encoding acidic and basic isoforms of pathogenesis related proteins display different expression pattern. *Pl. mol. Biol.* 14: 119-126

Minocha, S.C. 2000. Optimization of the expression of transgene in plant (eds. Jain, M. and Minocha, S.C). *Molecular Biology of Woody Plants*. Vol I. Kluwer Academic Publishers, London, pp. 30

Muralidharan, K. and Wakeland, E.K. 1993. Concentration of primer and template qualitatively affects products in random amplified polymorphic DNA PCR. *Biotechniques* 14: 362-364

Nakumura, S., Mori, H., Sakai, F. and Hayash, F. 1995. Cloning and sequencing of a cDNA for polar Endo-1, 4-β-glucanase. *Pl. Cell Physiol.* 36: 1229-1235

Nambiar, K.K.N. and Sarma, Y.R. 1977. Wilt disease of black pepper. *J. Plantation Crops* 5: 92-103

Nasser, W., DeTapia, M. and Burkard, G. 1990. Maize pathogenesis related proteins: Characterisation and cellular distribution of β-1, 3-glucanase and chitinase induced by brome mosaic injection or mercuric chloride treatment. *Physiol. mol. Pl. Pathol.* 36: 1-14

Neuhaus, J., Flores, S., Keeje, D., Ahl-Goy, P. and Meins, F. 1992. The function of vacuolar β-1, 3-glucanases investigated by antisense transformation. Susceptibility of transgenic *Nicotiana sylvestris* plants to *Cercospora nicotianae* infection. *Pl. mol. Biol.* 19: 803-813

*Ning, H., Liying, W., Nandi, S., Bowman, E., Jiamin, H., Sutliff, T., Rodriguez, R. L., Huang, N. and Huang, J.N. 2001. The tissue specific activity of a rice beta glucanase promoter (*Gns9)* is used to select rice transformants. *Pl. Sci.* 161: 589-595

Nunberg, J. H., Wright, D. K., Cole, G. E., Petrovskis, E. A., Post, L. E., Compton, T. and Gilbert, J. H. 1989. Identification of the thymidine kinase gene of feline herpesvirus: use of degenerate oligonucleotides in the polymerase chain reaction to isolate herpesvirus gene homologs. *J. Virol.* 63: 34240-34249

Ohme-Takagi, M. and Shinshi, H. 1990. Structure and expression of a tobacco β-1, 3-glucanase gene. *Pl. mol. Biol.* 15: 941-946

Old, R.W. and Primrose, S.B. 1994. *Principles of Gene Manipulation An Introduction to Genetic Engineering*. Oxford Black well Scientific Publications, London, 474 p.

Ori, N., Sessa, G., Lotan, T., Hemmelhoch, S. and Fluhr, R. 1990. A major stylar matrix polypeptide (sp41) is a member of the pathogenesis related proteins superclass. *EMBO J.* 9:3429-3436

Parab, G.V. 2000. Evaluation of pathogenesis related proteins in relation to phytophthora foot rot in black pepper (*Piper nigrum* L.) M.Sc. (Ag.) thesis, Kerala Agricultural University, Thrissur, 97 p.

Payne, G., Ward, E., Gaffney, T., Ahl-Goy, P., Moyee, M., Hasper, A. and Meins, F.J. 1990. Evidence for a third structural class of beta-1, 3 glucanase in tobacco. *Pl. mol. Biol.* 6: 797-808

*Phaff, H.J.A. 1979. A retrospective and current view on endogenous ß-glucanases in yeast. *Proceedings of the Fifth International Symposium on Advances in Protoplast Research Hungarian Academy of Science,* Budapest, Hungary, pp.171-182

Rajan, P.P., Sarma, Y.R. and Anandaraj, M. 2002. Management of foot rot disease of black pepper with *Trichoderma* spp. *Indian Phytopath.* 55: 34-38

Ravindran, P.N., Ashokan, N.R., Nirmalbabu, K., Chandran, K. and Nair, M.K. 1992. Ecological and morphological notes on *Piper* species from the silent valley forest, Kerala. *J. Bombay nat. Hist. Society.* 870: 421-426

Rogers, S.O and Bendich, A.J. 1994. Extraction of total cellular DNA from plants, algae and fungi. *Pl. mol Biol. Manual* 91: 1-18

Romero, G.O., Simmons, C., Yaneshita, M., Doan, M., Thomas, B.R., Rodriguez, R.L. and Wild, J. 1998. Characterisation of rice endo beta glucanase genes (*Gns2-Gns14*) defines a new subgroup within the gene family. *Gene* 223: 311-320

*Rouhier, P., Bruneteau, M. and Michel,G. 1995. Structural analysis on β-D-glucans from *Phytophthora capsici*. *J. Carbohydrate Chem.* 14: 254-274

Ruppel, G. and Almeydar, N. 1965. Susceptability of native *Piper* species to the collar rot pathogen of black pepper in Puerto Rico. *Pl. Dis. Rept.* 49: 550-551

Samaraj, J. and Jose, P.C.1966. A phytophthora wilt of pepper (*Piper nigrum* L.) *Sci. Cult.* 32: 90-92

Sambrook, J., Fritisch, E.F. and Manaiatis, T. 1989. *Molecular Cloning: A Laboratory Manual.* Second edition Cold Spring Harbor Laboratory Press, New york, USA, 1332 p.

Sarma, Y.R. and Nambiar, K.K.N. 1982. Foot rot disease of black pepper (*Piper nigrum* L.). *Proceedings of the workshop on Phytophthora disease to tropical cultivated plants* (ed. Nambiar, K.K.N.), CPCRI, Kasargod, Kerala, pp. 209-224

Sarma,Y.R., Ramachandran, N. and Anandraj, M. 1991. Black pepper disease in India. Disease of black pepper. (eds. sarma,Y.R. and Premkumar, T.). *Proceeding of the international Pepper community on Black pepper disease.* National Research Centre for Spices, Calicut, Kerala, pp. 55-101.

Schroder, M., Hahlbeock, K. and Kombrink, E. 1992. Temporal and spatial pattern of 1, 3 β-glucanase and chitinase induction in potato leaves infected by *Phytophthora infestans. Pl. J.* 2: 161-172

Shi,Y., Zhang, Y. and Shih, D.S. 2005. Isolation, characterization, and expression analysis of some beta-1, 3-glucanase genes from the strawberry plant [Online]. Available:www.ncbi.nlm.nihgov/[2005, Oct. 21]

Shinshi, H., Wenzler, H., Neuhaus, J. M., Felix, G., Hofsteenge, J and Meins, F. J. 1988. Evidence for N- and C-terminal processing of a plant defense related enzyme. Primary structure of tobacco prepro-β-1, 3- glucanase. *Proc. nat. Acad. Sci. USA. 85: 5541-5545*

Simmons, C.R. 1994. The physiology and molecular biology of plant 1, 3-β-D-glucanases and 1-3; 1-4,beta-D-glucanases. *Criti. Rev. Pl. Sci.* 13: 325-387

Simmons, C.R., Litts, J.C., Huang, N. and Rodriguez, R.L. 1992. Structure of a rice β glucanase gene regulated by ethylene, cytokinin, wounding, salicylic acid and fungal elicitors. *Pl. mol. Biol.* 18: 33-45

Simpson, G.G. and Filipowicz, W. 1996. Splicing of precursors to messenger RNA in higher plants:mechanism, regulation and sub-nuclear organization of the splicesomal machinery. *Pl. mol. Biol.* 32: 1-41

Slakeshi, N. and Fincher, G.B. 1992. Developmental regulation of (1→3, 1→4) β-glucanase gene expression in barley. *Pl. Physiol.* 99: 1226-1231

*Smith, R. F., Wiese, B. A., Wojzynski, M. K., Davison, D. B. and Worlry, K. C. 1996. BCM Search Launcher. An integrated interface to molecular biology database search and analysis services available on the world wide web. *Genome Res.* 6: 454-462

Sun, L., Gurnon, J.R., Adams, B.J., Geaver, M.V. and Etten, J.L.V. 2000. Characterization of a β-1, 3-glucanase encoded by chlorella virus PBCV-1. *Virology* 276: 27-36

Survey of Indian Agriculture. 2005. *The Hindu.* p. 75

Takeuchi, S., Yoshikawa, M., Takeba, G., Tanaka, K., Shibata, D. and Horina, O. 1990. Molecular cloning and ethylene induction of mRNA encoding a phytoalexin elicitor releasing factor, ß-1, 3-endo glucanase, in soyabean. *Pl. Physiol.* 93: 673-682

Tamas, L., Ciampoeova, M. and Luxova, M. 1998. Accumulation of pathogenesis related proteins in barley leaf intercellular spaces during leaf senescence. *Biol. Pl.* 41: 451-460

Teucker, M.L. and Milligan, S.B. 1991. Sequence analysis and comparison of avocado fruit and bean absicission cellulose. *Pl. Physiol.* 95: 928-933

Thanseem, I., Joseph, A. and Thulaseedharan, A. 2005. Induction and differential expression of beta-1, 3-glucanase mRNAs in tolerant and susceptible *Hevea* clones in response to infection by *Phytophthora meadii. Tree Physiol.* 25: 1361-1368

*Thimmapuram, J., Korban, S.S. and Kellechals, M. 1994. Isolation of apple genomic clones containing putative β-1, 3-glucanase gene (ed. Schmidt, H.). *Proceedings of the Eucarpia Fruit Breeding Section Meeting. Aug. 30 to Sept. 3, 1993.* Kluwer Academic Publisher, Netherland, pp. 339-342

Thompson J. D., Higgins D.G. and Gibson T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22: 4673-4680

Tweddell, R.J., Jabaji-hare, S.H.,and Charest, P.M. 1994. Production of chitinases and beta-1, 3-glucanases by *Stachybotrys elegans*, a mycoparasite of *Rhictonia solani. Appl. Environ. Microbiol.* 60: 489-495

*Ulmann, A., Jacob, F. and Monod, J. 1967. Characterisation by *invitro* complementation of a peptide corresponding to an operator-proximal segment of the β-galactosidase structural gene of *Eschersia coli. J. mol. Biol.* 24: 339-345

Van Kan, J. A., Joosten, M. H., Wagemakers, C. A., Van den Berg-Velthuis, G. C. and DeWit, P. J. 1992. Differential accumulation of mRNAs encoding extracellular and intracellular PR proteins in tomato induced by virulent and avirulent races of *Cladosporium fulvum*. *Pl. mol. Biol.* 20: 513-527

Van Loon, L. C., Pierpoint, W. S., Boller, T. and conejero, V. 1994. Recommendation for naming plant Pathogenesis-related proteins. *Pl. mol. Biol. Rept.* 12: 245-264

Van Loon, L.C. 1985. Pathogenesis related protein. *Pl. mol. Biol.* 4: 111-116

*Van Loon, L.C. 1997. Induced resistance in Plants and the role of pathogenesis related proteins *Eur. J. Pl. Pathol.* 103: 753-765

Varghese, J.N., Garrett, T.P.J., Colman, P.M., Chen, L., Hoj, P.B., and Fincher G.B. 1994. Three-dimensional structures of two plant beta-glucan endohydrolases with distinct substrate specificities. *Proc. nat. Acad. Sci. U.S.A.* 91: 2785-2789

Vilasini, T.N. 1982. Quick wilt disease of pepper II. The technique of screening pepper varieties against quick wilt disease caused by *Phytophthora palmivora*. M.Sc. (Ag.) thesis, Kerala Agricultural University, Thrissur, Kerala, 95 p.

Vogeli-Lange, R., Hansen-Gehri, A., Boller, T. and Meins, F. 1988. Induction of the defense related and glucanhydrolases, β-1, 3-glucanase and chitinase by tobacco mosaic virus of tobacco leaves. *Pl. Sci.* 54: 171-176

Wang, Y.X., Kausch, A.P., Chandlee, J.M., Lou, H., Ruemmele, B.A., Browining, M., Jackson, N. and Goldsmith, M.R. 2003. Co-transfer and expression of chitinase, glucanase and bar genes in creeping bent grass for conferring fungal disease resistance. *Pl. Sci.* 165: 497-506

Wilks, A.F.1989.Two putative protein tyrosine kinases identified by application of the polymerase chain reaction. *Proc. nat. Acad. Sci.USA.* 86: 1063-1067

Wu, S., Blumer, J.M., Darvill, A.G. and Albershem, P. 1996. Characterization of a Endo-ß-1, 4-glucanase gene induced by auxin in elongating pea epicotyle. *Pl. Physiol.* 110: 163-170

*Yamaguchi, T., Nakayama, K., Hayashi, T., Tanakae, Y. and Koike, S. 2002. Molecular cloning and characterization of a novel β-1, 3-glucanases gene from rice. *Bioscience* 66: 1403-1406

*Zanor, M. I., Valle, E.M. and Vallejos, R. H. 2000. Isolation and expression of a barley beta-1, 3-glucanase isoenzyme II gene. *DNA sequence* 10: 395-398

Zdobnov, E. M. and Apweiler, R. 2001. InterProScan-an intergration platform for the signature-recognition methods in Interpro. *Bioinformatics* 17: 847-848

Zeng, Y., Lan, L. Q., Luo, H., Bai, J., Yang, M. Y., Miao, C., Cai, Y.F.,Qiang, X. L. and Chen, F. 2002. RAPD Markers in diversity detection and variety identification of tibetan hulless Barley. *Pl. mol. Biol. Rept.* 20: 369-377

Zing, Yu, Xu, Y., Tang, L., Qiang, X.L. and Chen, F. 2003. Cloning and expression of a new Tibetan hullless barley beta-1, 3-glucanase gene. *Biotech. Lett.* 25: 617-622

* Originals not seen

APPENDIX I

Composition of reagents and media used for molecular biology works

1. Chilled isopropanol

   Isopropanol stored in refrigerator was used as such

2. Chloroform - isoamyl alcohol (24:1 v/v)

   To 24 parts of chloroform, 1 part of isoamyl alcohol was added and mixed properly

3. Ethanol 70 per cent and 100 per cent.

   Absolute ethanol was used as such. 70 per cent ethanol was prepared by adding 30 parts of double distilled water to 70 parts of absolute ethanol

4. Extraction buffer (4x)

   a. Sorbitol 2.5 gm
   b. Tris HCl 4.8 gm
   c. EDTA 0.74 gm

5. Dissolved in about 80 ml distilled water, adjusted the pH of buffer to 7.5 and made up to 100 ml with distilled water and then autoclaved.

6. Intercalating dye

   a. Ethidium bromide - 10 mg
   b. Distilled water - 1 ml

   It was mixed well and kept in fridge at 4°C.

7. LB-Medium (Luria Broth)

Ingredients	Concentration (g l^{-1})
Trypton	10
Yeast extract	5
NaCl	5

The above ingredients were dissolved in distilled water and pH adjusted to 7.5. The entire volume was made up to final volume of one litre. It was autoclaved and stored at room temperature.

8. LBA medium (Luria Bretani Agar)

Ingredients	Concentration ($g\ l^{-1}$)
Trypton	10
Yeast extract	5
NaCl	5
Agar	15

All the ingredients except agar was dissolved in distilled water and pH was adjusted to 7.5. The entire volume was made up to final volume of one litre. Then required quantity of agar was added to medium and melted. It was transferred to jam bottles (50 ml in each bottle) and autoclaved.

8. Lysis buffer-genomic DNA isolation
   c. IM Tris HCl (pH 8.0) 20 ml
   d. 0.25 M EDTA (pH 8.0) 20 ml
   e. 5.0 M NaCl 40 ml
   f. CTAB 2.0 g
9. Lysis buffer (Solution II)-plasmid isolation
   a. 2N NaOH
   b. 1 per cent SDS

   Dissolved CTAB in 20 ml distilled water, add rest of the chemical and volume of the solution was made up to 100 ml with distilled water and autoclaved.
10. Neutralization buffer (Solution III)
    a. 5 M potassium acetate 60 ml
    b. Glacial acetic acid 11.5 ml
    c. Distilled water 28.5 ml
11. Resuspension buffer (Solution I)
    a. 50 mM glucose
    b. 25 mM Tris
    c. 10 mM EDTA
12. Sarcosine (5%)
    a. Sarcosine 5.0 gm

Sarcosine was dissolved in 100 ml distilled water.

13. TAE buffer 50x (pH 8)
    a. Tris base - 242 gm
    b. Glacial acetic acid - 57.1 ml
    c. 0.5 M EDTA (pH 8) - 0.5 ml

    The solution was prepared, autoclaved and stored at room temperature

14. Tracking dye
    a. Glycerol - 3 ml
    b. Water - 7 ml
    c. Bromo phenol blue - 0.025 g

    Autoclaved and kept in fridge at 4°C.

15. TE Buffer (Tris 10.0 mM, EDTA 1.0 mM)
    a. Tris HCl 1.0 M (pH 8.0) 1.0 ml
    b. EDTA 0.5 M (pH 8.0) 0.2 ml
    c. Distilled water 98.8 ml

    Autoclaved and stored at room temperature

APPENDIX II

Sequence information of β-1, 3- glucanase used for primer designing.

>tobacco
MCSIQIIGAQSIGVCYGKAANNLPSDQDVINLYNANGIRKLRIYPDKNIFKA
LNGSNIEIILGVPNQDLEALANSSIANGWVQDNIRSHFPYVKFKYISIGNKVS
PTNNDQYSEFLLQAMKNVYNALAAAGLQDMIKVSTVTYSGVLANTYPPE
RSIFREEFKSFINPIIQFLARNNLPLLANVYPYFVHVSNTADVSLSYALFTQQ
GTNSAGYQNLFDAILDSMYFAVEKAGGPNVEIIVSESGWPSEGSSAATIEN
AQTYYRNLINHVKSGAGTPKKPGKTIETYLFAMFDENDKIGEITEKHFGLF
SPDQRAKYQLNFNYLPIYILR//
>prunus
MTKSNSSSVGRLLSLISIVLLLGQLVVGSLATKQHTGAPIGVCNGMVGDDL
PPQAEVVALYKTNNIPRMRLYDPNPAALEALRGSNIKLLLGVPNENLQYIA
LSQANANAWVQNNVRNYANVKFKYIAVGNEVKPSDSFAQFLVPAMRNIQ
EAISLAGLAKKIKVSTAIDTGVLGETFPPSIGSFKSEYNALLYPIIRFLVSHQS
PLLVNLYPYFAYSGNTQDIRLDYALFTAPSVVVQDGNFGYRNLFDAMLDG
VYAALEKAGGGSLKVVISETGWPSAAGTATTIDNARTFISNLIQHVKEGTP
RRPGRPIETYIFAMFDENRKTPELEKHWGLFSPTKQPKYQISFN//
>arabdiopsis
MLYLPKKLFLFFFSCIVVIVNYNNSDFVNAANSIGFVNAANSIGLNYGLLG
DNLPSPSKVITLYKSIDITKIRIFDPNTEVLNALRGHRDIAVTVGVRDQDLA
ALSASEEAVKGWFATNIEPYLSDINIAFITVGNEVIPGPIGPQVLPVMQSLTN
LVKSNLPISISTVVAMWNLEQSYPPSAGMFTSQAREQLVPVLKLLSQTNSPI
LVKIYPYFSPSSIRLDYATFNTEAIVVQDGSLGYSNMFDAIFDAFVWAMEK
EGVKDLPMVVSETGWPSAGNGNITTPDIAGTYNRNFVKHIASGKGTPKRP
NKGIDGFLFATFNENQKPVGEQNFGLYNPNDMKPIYNLF//
>rubber
MAISSSTSGTSSSLPSRTTVMLLLIFFTASLGITDAQVGVCYGMQGNNLPPV
SEVIALYKQSNIKRMRIYDPNRAVLEALRGSNIELILGVPNSDLQSLTNPSN
ANSWVQKNVRGFWSSVLFRYIAVGNEISPVNGGTAWLAQFVLPAMRNIH
DAIRSAGDQIKVSTAIDLTLVGNSYPPSAGAFRDDVRSYLDPIIGFLSSIRSPL
LANIYPYFTYAGNPRDISLPYALFTSPSVVVWDGQRGYKNLFDATLDALYS
ALERASGGSLEVVVSEWPSAGAFAATFDNGRTYLSNLIQHVKGGTPKRPN
RAIETYLFAMFDENKKQPEVEKHFGLFFPDKRPKYNLNFGAEKNWDISTE
HNATILFLKSDM//
>wheat
MASQGVASMFALALLLGAFASIPQSVESIGVCYGMSANNLPAASTVVSMF
KSNGINSMRLYAPDQAALQAVGGTGVNVVVGAPNDVLSNLAASPAAAAS
WVRSNIQAYPKVSFRYVCVGNEVAGGATQNLVPAMKNVQGALASAGLG
HIKVTTSVSQAILGVYSPPSAGSFTGEADAFMGPVVQFLARTGAPLMANIY
PYLAWAYNPSAMDMSYALFTVVQDGSYGYQNLFDTTVDAFYTAMAKHG
GSNVKLVVSESGWPSGGGTAATPANARIYNQYLINHVGRGTPRHPGAIET
YVFSMFNENQKDSGVEQNWGLFYPNMQHVYPISF//

>rice

MASQGVASMFALALLLGAFASIPQKAEAIGVCYGMSANNLPPASMYRSNG
ITSMRLYAPDQAALQSVGGTGISVVVGAPNDVLSNLAASPAAAASWVRN
NIQAYPSVSFRYVAVGNEVAGGATSSLVPAMENVRGALVSAGLGHIKVTT
SVSQALLAVYSPPSAAEFTGESQAFMAPVLSFLARTGAPLLANIYPYFSYTY
SQGSVDVSYALFTAAGTVVQDGAYGYQNLFDTTVDAFYAAMAKHGGSG
VSLVVSETGWPSAGGMSASPANARIYNQNLINHVGRGTPRHHGAIETYVF
SMFNENQKDAGVEQNWGLFYPNMQHVYPISF//

The corresponding nucleotide sequences are presented below

>tobacco
ATGGCTTTCTTGCAGCTGCCCTTGTACTTGTTGGGCTRATAATGTGCAGT
ATCCAAATCATAGGGGCACAGTCTATTGGAGTATGCTATGGAAAAGCT
GCCAACAATTTACCATCAGACCAAGATGTTATAAACCTATACAATGCTA
ATGGCATCAGAAAGTTGAGAATTTACTATCCTGATAAAAACATTTTCAA
AGCTCTCAATGGAAGTAACATTGAGATCATTCTTGGTGTCCCAAATCAA
GACCTTGAAGCCCTAGCCAATTCTTCAATAGCCAATGGTTGGGTTCAAG
ATAACATAAGAAGTCATTTCCCATATGTTAAATTCAAGTACATATCTAT
AGGAAATAAAGTATCTCCCACAAATAATGATCAATATTCAGAATTTCTT
CTTCAAGCAATGAAAAATGTGTACAATGCTTTAGCAGCAGCAGGGTTG
CAAGATATGATCAAGGTCTCAACTGTGACATATTCAGGGGTCTTAGCG
AATACCTACCCACCTGAACGTAGTATTTTTCGCGAAGAATTCAAGAGTT
TCATTAATCCGATAATCCAATTTCTAGCACGAAATAACCTTCCACTCTT
AGCCAATGTCTATCCTTATTTTGTTCACGTTTCCAACACTGCTGATGTTT
CACTTTCTTATGCATTGTTCACACAGCAAGGAACAAATTCAGCAGGGTA
TCAAAATCTTTTTGATGCTATTTTGGATTCTATGTATTTTGCTGTAGAGA
AAGCTGGAGGACCAAATGTGGAGATTATTGTATCTGAAAGTGGATGGC
CTTCTGAAGGAAGCTCTGCAGCAACTATTGAAAACGCTCAAACTTATTA
CAGAAATTTGATTAATCATGTGAAAAGCGGGGCAGGAACTCCAAAGAA
ACCTGGAAAGACTATAGAAACTTATTTGTTTGCCATGTTTGATGAAAAT
GATAAGATAGGAGAAATCACAGAGAAACACTTTGGACTGTTTTCTCCT
GATCAAAGGGCAAAATATCAACTCAATTTCAATTATTTGCCAATATATA
TATTGAGATGAGTAATAAGGACAACTGTTATGTTTTTCTCTTCAATTGA
AAATGTAACTCTGGTTTCACTTTG//
>prunus
GAATTCCAAAGCGGAATTTGATTCGGGTCCTGTCAGAGATAGTGAAGA
GAAAGATGACAGATTAAGTGAGAGGAGTGAGTGTGAGAGAAAGGTTG
GGATCTGGGGAGAGGGAAAGAGAGAGGAGAGGTAAGAGTAGAGAAA
GACATTGAGAAAATGGTGGAGGAATTATTTCAGTTTTAACCACAACTG
AGTAGTCAGGCAAGTATAGTTGTGGTTAGCAATCTAACCACCAATAAA
ACTGCGATAAATAGGCTGCCATGTATCGCATCACATGTACCCAAATAAT
GCTCACATAGAGCAAATAATCTTATAAATTACCGTAACTTAAAATATTA
TGTCTTTTCTTGGCAATATTGATGCATTAAAAAAGTTATTATAATCTCAT
TATGTTAAGGATGTTAAAGATAAATTGCAGATATAAGCTCAGTCAAGTT
GGATTAGGGGATGTGCTCCCCACTTTGCCCCGAGTTCAAATCATTTTTC
TTATAATTTAAAATAAATTAAAGTAAAATATTGTTTGTATAAAAAAAGA
CATTATTTAAATTGAGATAGAGGAATATTGTTTGTGTCATAAAGGAATA
TACTTAATACTTCCCTGACTTTTCTCCCGCCTATATAGTTGACTTGTCCT

TATTAGGTTTACTTGGACTTTCTTATTTACAATTACAATATAAGATATTT
ACATTAACACTCTTGAGGTTTTTTGTCTTTTGACAAAACTCTCCCTAAGG
TTTCAAAAATTACATGAACACCCCTTGAGGTTTTAGATTATTTTCACAA
AACCCATTTCCTTGTTTGTCCAAAAATTGATGATTTTATTAAAAACAAT
TACGGAAATGAAAAAATTAACCTCAATGATTGCATATGCAAAATCGAC
GAAATTTTCATATATATAAAATCATCAATATTTGGTCGAAAAATCTACG
AAAAGGGATTTATGTGGTTTTCTTGTCTCATAAGGTACGTACGTGAGAG
CAATGTTCGTCCCTCCTTTGCCACCTCATATCTCTCAACATCTTCTCGTC
TTGTCACGTATTTTCTGTTTGTCTCTTAAGAAACTTTGGATTAAAAGAAT
AAAAACACATTTAATTTAAGCTTATTAAATGAAAAGAAGAAAAGGACA
AGGAGAACCAGAAAACAAAACTACCCGAAGGCAGGCTTGGCCACATG
GTGAAAGTTCAACTAAGACGATAGTTATGACACATAACAAATCTTTGTT
ACAGAAATCCCACTCTAAACGCAGGGGCCTCATCCCACCAACCAAACC
AAGAAACAGCCCTGCCATGATTAGCCAAGGCCCTCCTTATAGTCTCACA
AAAGATACGAGAAAATCGAAAATGCATCAGTGAAAGACGGAATAAAC
AGACATTTGTTGTTGCAACAACGTAGACAGTTGAAATTGGACCCTCCTG
TAAAGAGATCTTGAGCAGGTGCAAATCTCTTTCTCATCTCTATAAATTA
AGGAGCAAGAGGCGGACTTGAGAGACGTACACTTAGAATATTTCTTAG
AAAATTGTATGTTTCTGACCATGACTAAATCGAATTCGTCATCAGTTGG
CAGACTCCTTTCTCTGATTTCCATAGTACTTCTACTTGGGCAGCTGGTGG
TGGGTAGCTTGGCAACAAAACAACACACAGGTATGCATATATAATTGG
CACCTCTGTCAATCTAACTGCAAATTACGACATTTTTGTGTGTGTCTATT
TTAAAGATAAGGACCAAACTGTTCCATGCAACAAACCACGCATTTCTA
ATCAATCTCACATGTAAGCAATATTAATTCCTTTTTCAATGTAGGTGCT
CCAATTGGTGTATGTAATGGAATGGTTGGCGATGACCTACCACCCCAA
GCAGAAGTTGTTGCCCTCTACAAGACAAATAACATCCCAAGAATGCGA
CTTTATGATCCAAACCCAGCCGCTCTAGAAGCCCTTCGAGGCTCCAATA
TCAAGCTCTTGCTAGGCGTACCAAATGAAAACCTTCAATACATTGCCTT
AAGCCAAGCCAACGCAAATGCATGGGTCCAAAACAATGTGAGAAACTA
TGCCAATGTGAAATTCAAGTACATTGCGGTAGGAAATGAAGTCAAGCC
TTCAGACTCCTTTGCACAGTTTCTCGTCCCAGCCATGCGAAATATTCAA
GAGGCAATTTCTCTTGCTGGTCTTGCAAAGAAAATTAAAGTTTCGACAG
CCATCGACACCGGAGTACTTGGAGAGACCTTTCCTCCTTCGATAGGCTC
ATTCAAGTCTGAATATAACGCCCTTTTATATCCCATCATCCGCTTCCTAG
TGAGCCACCAATCGCCATTGCTTGTTAACTTGTACCCTTATTTTGCTTAC
AGTGGCAACACTCAAGACATTCGTCTTGACTATGCTCTTTTCACAGCTC
CATCAGTTGTGGTACAAGATGGGAACTTTGGTTACCGAAATCTTTTCGA
TGCCATGTTAGATGGTGTTTATGCTGCTCTTGAGAAGGCTGGTGGAGGG
TCTTTGAAAGTTGTTATATCAGAGACTGGTTGGCCATCAGCTGCTGGAA
CAGCCACAACAATTGATAATGCAAGGACTTTTATATCAAATTTGATTCA
ACATGTGAAGGAAGGGACTCCAAGGAGGCCAGGAAGGCCCATAGAAA
CTTACATCTTTGCCATGTTTGATGAGAATAGAAAGACCCCAGAGCTTGA
GAAACATTGGGGGCTCTTCTCCCCAACAAAACAGCCTAAATACCAAAT
CAGTTTCAATTGATTAGCACTAAAAGGGATGTTGTGGTTGCCAATAAAA
GATTGCAAAGTTACCTGCAAAGTTATTTTTGTGTGTGTGTGTGGTCACG
AGCACCCTTGTACCAAAAAAAAAAACTCATAAAGAGCAAATAGTCTTA
ATAAGAGATTGTACGAATTATAATTGTATATGTCTTCTTTGGTAGTTTG
ACGCATTACAATTTACAGTTGTCATTGTATCTTTGGGTTTAGTCGCTACT

TGCATATGCAAATTCATTTTTAATTACTTCAATAGAAACGAACTAACTC
TTAATTTAGTTGTATTTTTTATTGGCGTGACTTGTGGACCATTGACTTTC
TTTTCTTACGCACCAATGATTCCTATTATAATTATATTTGATTTACTTTA
ATTCCTGATTTCCTATTGTAAATAGAAATAAGAATTTATTATTTACTTGC
CCATTCAGGTTTCGTTGTATTATAAATATGACCTCCTATAAGGAGAAGA
ATACACAGAAAATTCCCACAAACATATATTCTCTCATAGTTCTTATATT
TTTGCATGGTATCAGAGCGGCGATCTTGGAATTGCTTACTCTAGTTTCC
AACCCCGACACATTTTCAGATCCTGATCCAGATTCCTACTAAACTTGTA
TCCTATGCATCTTTCACTGCACAAATTATGTCTTCCCAACTAATGACCCC
GACACATGGACGTGATTGCTCATATTGTGGTGATCTAAGACACCCTCAT
GAGACTTGTTTTAAATTGCATGGCTACCCTGACTGGTGGGCCACTCTTA
AAGATCGAACAAAACCCGATACAACCCGTAATGATACTGGTTATGGAT
TCCATACTTCGGATAAGGGTGATTCCACGAGTTGGATAATTGATTCATG
TGCAACTGATCATATAACGTTTGATCTTGATGATTTTCTGAATACTATCC
AACCTCGACGAACTTGTATTGCTAACACCAATGGAGTTACTTATCATGT
GACAGAGGCTGGCACTGTTGCACTCTCTTGCTCTCTCTCACTGTCTGAT
ACTTTACTAGTTCCGTCTTTATCCAATAAATTTTTGTCAGTTAGTCAGCT
TACTAAACAATTGAATTGTTGTGTACTCATTTACTCGAG//

>arabidopsis

ATGCTCTATTTGCCTAAGAAACTCTTCTTGTTCTTCTTCTCGTGCATTGT
GGTGATTGTCAACTACAACAATAGTGACTTCGTAAACGCAGCAAATAG
CATTGGCTTCGTAAACGCAGCAAATAGCATTGGCTTGAACTACGGTCTC
CTCGGAGATAACCTCCCATCTCCGTCAAAAGTTATAACCCTTTACAAGT
CCATAGACATCACTAAAATCCGAATCTTCGACCCAAACACTGAGGTTCT
TAACGCTTTACGTGGCCATCGTGATATTGCGGTCACAGTAGGAGTTAGG
GACCAGGACTTGGCTGCTCTTTCAGCTAGCGAAGAAGCTGTTAAGGGC
TGGTTTGCGACCAACATCGAGCCTTACTTATCCGACATCAACATCGCGT
TCATTACTGTTGGTAACGAAGTCATCCCCGGACCAATCGGTCCTCAAGT
GCTTCCAGTCATGCAGTCTCTCACCAACCTCGTCAAGTCAAGGAATCTT
CCTATCTCGATAAGCACGGTGGTGGCTATGTGGAACCTCGAGCAATCAT
ACCCACCTTCCGCAGGAATGTTCACGTCTCAAGCGCGTGAACAACTTGT
CCCCGTGCTGAAACTATTATCCCAAACAAATTCGCCTATCCTCGTAAAA
ATCTACCCTTACTTCTCCTATGCGTCCGACCCATCTAGCATCCGTTTGGA
CTATGCCACCTTCAACACTGAGGCCATCGTGGTACAAGATGGATCACTG
GGCTATTCAAACATGTTTGATGCAATCTTTGATGCGTTCGTGTGGGCAA
TGGAGAAGGAAGGCGTTAAAGATTTACCAATGGTGGTGTCCGAGACCG
GATGGCCATCTGCCGGGAATGGAAACATTACCACGCCGGATATCGCGG
GTACCTATAACAGAAATTTCGTGAAGCATATAGCAAGCGGAAAAGGTA
CACCTAAAAGGCCTAACAAAGGCATCGACGGGTTTTTGTTTGCAACTTT
CAATGAAAATCAAAAGCCGGTCGGGACTGAACAAAACTTTGGGTTATA
CAATCCGAATGATATGAAGCCCATCTACAATCTATTCTAA//

>rubber

CGCCCGGGCAGGTATTTCTTCCATTAAATAAATATATTAGTTTTAATTT
GGCGATAGTTTAAATTCAAACCCCAATTGACATTAAATAAATAAATAA
GCTCCAGAAATAGTATGCTAAGGAGTCCCTATATAAAGCATAGACTTG
GTAGTAATGTAATGCATATGCATGCTCCAAATTAAGCTGCTCTCTTCTT
AGGTTCCATCCTTCTTAATGGCTATCTCCTCTTCAACTTCAGGAACTAGT

AGTTCCCTGCCCTCAAGAACTACTGTCATGCTTCTTCTGATTTTCTTTAC
AGCAAGCCTTGGTATAACAGGTCTCTCTCTCTCTCTCTCTCTCTCTCT
CTCTCTCTCCATCTATCTACTCTCATGTTAAAGTTGACGATGCTCTTTTT
TTCTCTCCTTGTTCTAAAAGGTTTCAACTAATACCTGTATTTAGGAATTC
AGATGCCCAGGTAGGTGTTTGCTATGGAATGCAAGGCAACAACCTTCC
ACCTGTTTCAGAGGTCATAGCTCTCTATAAACAATCTAACATCAAGAGA
ATGAGAATTTATGATCCAAATCGAGCAGTATTGGAAGCCCTTAGAGGC
TCAAACATTGAACTCATACTAGGTGTTCCAAACTCAGATCTCCAAAGCC
TTACCAATCCTTCCAATGCAAACTCATGGGTACAAAAAAATGTTCGTGG
CTTCTGGTCAAGTGTCCTGTTCAGATATATAGCAGTTGGCAACGAAATT
AGTCCTGTCAATGGAGGCACAGCTTGGTTGGCTCAATTTGTTTTGCCTG
CCATGAGAAATATACATGATGCTATAAGATCAGCTGGTCTTCAAGATC
AAATCAAGGTCTCCACTGCAATTGACTTGACCCTGGTAGGAAATTCCTA
CCCTCCTTCTGCAGGTGCTTTCAGGGATGATGTTAGATCATACTTGGAC
CCAATTATTGGATTTCTATCCTCTATCAGGTCACCTTTACTTGCCAATAT
TTATCCTTACTTTACTTATGCTGGTAATCCAAGGGATATTTCCCTTCCCT
ATGCTTTGTTCACTTCACCATCAGTTGTTGTGTGGGATGGTCAGCGAGG
TTATAAGAACCTTTTTGATGCAACGTTGGATGCATTGTACTCTGCTCTTG
AGAGGGCTAGTGGTGGTTCTCTGGAGGTGGTTGTTTCGGAAAGTGGCT
GGCCGTCTGCCGGAGCATTTGCTGCCACATTTGACAATGGGCGTACTTA
TCTCTCAAATTTGATCCAGCATGTTAAAGGAGGTACTCCTAAGAGGCCT
AACAGAGCTATAGAGACTTACTTATTTGCCATGTTTGATGAAAATAAGA
AGCAACCAGAGGTTGAGAAACACTTTGGACTTTTCTTTCCTGATAAACG
GCCAAAATATAATCTCAATTTTGGTGCAGAAAAGAACTGGGATATTTCT
ACTGAACACAATGCAACAATACTTTTCCTTAAGAGTGATATGTGA//
>wheat
CCACATACACACACCCACACACCCTCAACGCAGCTAGAGAGACAAAGA
GAATGGCGAGCCAAGGTGTTGCCTCCATGTTCGCTCTGGCATTGCTCCT
CGGAGCCTTCGCCTCCATCCCACAAAGCGTCGAGTCCATCGGGGTGTGC
TACGGCATGAGCGCCAACAACCTGCCGGCGGCGAGCACCGTCGTCAGC
ATGTTCAAGTCCAACGGCATCAACTCCATGCGGCTGTACGCTCCCGACC
AGGCGGCGCTGCAGGCCGTCGGCGGCACGGGCGTCAACGTCGTCGTCG
GGGCGCCTAACGACGTGCTCTCCAACCTCGCCGCCAGCCCAGCTGCGG
CCGCCTCGTGGGTCAGGAGCAACATCCAGGCCTACCCCAAGGTCTCCTT
CCGGTACGTCTGCGTCGGCAACGAGGTCGCCGGCGGCGCCACCCAGAA
CCTCGTCCCGGCCATGAAGAACGTGCAGGGCGCGCTCGCCTCCGCTGG
GCTGGGCCACATCAAGGTCACCACGTCGGTGTCGCAGGCCATTCTCGG
CGTGTACAGCCCGCCCTCCGCCGGGTCCTTCACCGGGGAGGCGGACGC
GTTCATGGGCCCCGTGGTGCAGTTCCTTGCCCGCACCGGCGCGCCGCTC
ATGGCTAACATCTACCCGTACCTGGCCTGGGCCTACAACCCGAGCGCC
ATGGACATGAGCTACGCGCTCTTCACCGCATCCGGCACCGTGGTCCAG
GACGGCTCCTACGGGTACCAGAACCTGTTCGACACCACCGTGGACGCC
TTCTACACGGCCATGGCCAAGCACGGCGGCTCCAACGTGAAGCTCGTG
GTGTCGGAGAGCGGGTGGCCCTCAGGCGGCGGCACGGCGGCGACTCCG
GCCAACGCCAGGATCTACAACCAGTACCTCATCAACCACGTCGGGCGC
GGCACCCCGCGCCACCCGGGCGCCATCGAGACCTACGTCTTCTCCATGT
TCAACGAGAACCAGAAGGACAGCGGCGTGGAGCAGAACTGGGGACTC
TTCTACCCCAACATGCAGCACGTCTACCCCATCAGCTTCTGATGAGCTA

GCTGCGTATGCGTAGCTAGTGTCCGTATGTCCGTACGTACGCGCGCGTA
CACGCGTATAAGAGCGTGTATGCGGTACGTATGTGCACATTATGTATTG
TACAGGGTTTGGGTTGGGGTGCGACCGCTGAGGCAGCTCAGATGCGTA
CGCTATAGTAGTGGTTTGCTGTAGTAGTGTATGTGTATGATTTACGATG
GAAGGGAAGCAGATGTAAACGCTCTCCCTTCGTCGATTCATCATACATT
TGATACGTACACACATGTGTGCGTAAGTAGAACTATATTGTAGGGTTCA
AAATTTCGTCAAAATTTGACGGAATTTGTCCAAAATAATATGAAATATA
TCGGCGAGACCGAAGTATTCCT//
>rice
ACTCAGAGAGGAGGCGTGTTGGTTAGCTCATTGGCAGCAGCACACACA
AACCACATCTCCTATATATAGCTCATTTTTAGCTTTTGGAATTTGAGAG
AGGTTTTGAGAGAAATGGCTAGCCAAGGTGTAGCCTCCATGTTCGCTCT
CGCATTGCTCCTCGGTGCCTTTGCCTCCATTCCTCAAAAGGCGGAGGCG
ATCGGGGTGTGCTACGGCATGAGCGCGAACAACCTGCCGCCGGCGAGC
TCGGTGGTGGGGATGTACCGCTCCAACGGCATCACGTCGATGCGGCTG
TACGCGCCGGACCAGGCGGCGCTGCAGTCGGTGGGCGGCACGGGGATC
AGCGTCGTCGTCGGCGCGCCCAACGACGTGCTCTCCAACCTCGCCGCCA
GCCCCGCCGCGGCGGCGTCGTGGGTGCGGAACAACATCCAGGCCTACC
CGTCGGTGTCGTTCCGGTACGTCGCCGTCGGGAACGAGGTCGCCGGCG
GCGCCACGTCCAGCCTGGTCCCGGCCATGGAGAACGTCCGCGGCGCGC
TGGTGTCGGCGGGGCTGGGCCACATCAAGGTGACGACGTCGGTGTCGC
AGGCGCTCCTCGCCGTGTACAGCCCGCCGTCCGCCGCGGAGTTCACCG
GCGAGTCGCAGGCGTTCATGGCGCCCGTCCTGAGCTTCCTCGCCCGCAC
CGGCGCGCCGCTGCTCGCCAACATCTACCCCTACTTCTCCTACACCTAC
AGCCAGGGCAGCGTCGACGTCTCCTACGCGCTCTTCACCGCCGCCGGC
ACCGTCGTCCAGGACGGCGCCTACGGGTACCAGAACCTGTTCGACACC
ACCGTCGACGCGTTCTACGCCGCCATGGCCAAGCACGGCGGCTCCGGC
GTCTCCCTCGTCGTCTCCGAGACAGGCTGGCCCTCCGCCGGCGGCATGT
CCGCCTCGCCGGCCAACGCCCGGATCTACAACCAGAACCTCATCAACC
ACGTCGGCCGCGGCACGCCGCGCCACCACGGCGCCATCGAGACCTACG
TCTTCTCCATGTTCAACGAGAACCAGAAGGACGCCGGCGTCGAGCAGA
ATTGGGGCCTCTTCTACCCCAACATGCAGCACGTCTACCCCATCAGCTT
CTGATGCATTCCGTACACATATACGCATACGTATGCGTATACGTGACCG
CAGGTAGTGTAGTATACACGTACACGTACCTGTACATTACAGTATTGTA
CAGAGGGCTTGGCTTGCGACCGCTGAGAGGACACGTACACCAAGTGTA
CGTCTGTCAGATGCGTACGCGATAGTATATATATTGGGTATATTATATA
TATACCGTATTATTACGGTATGTATAGAAGGGAAGCTACAGGTACAAA
ATACGGTACGCTGTGGTAGGCGAATTAGCCCCCATTTTCCCTTCCTAGA
TTACTATTACTATATAGAGAGGGTTGCAATATATATACATGTATATGTA
CGTACTTAGGAGGTGTATTTTGTACGTACACAGATTGTAATACTGTACA
TTTGTACGCCGTACGTCGAGGTATTTTGCCTCACGTAGGCGTTTGAGAA
AAAAAAAGAAAAAATTTAAAAGATTTTA//

ISOLATION AND CHARACTERISATION OF β-1, 3-GLUCANASE GENE FROM *Piper* spp.

By

MABLE ROSE GEORGE

ABSTRACT OF THE THESIS

submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture

(PLANT BIOTECHNOLOGY)

Faculty of Agriculture
Kerala Agricultural University, Thrissur

Centre for Plant Biotechnology and Molecular Biology
COLLEGE OF HORTICULTURE
VELLANIKKARA, THRISSUR - 680 656
KERALA, INDIA

2005

ABSTRACT

The study entitled isolation and characterization of β-1, 3- glucanase gene in *Piper* spp was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara from December 2003 to September 2005. β-1, 3-glucanase is reported to be involved in the defense mechanism of *Piper* against the *Phytophthora* foot rot disease. Attempts were undertaken to isolate the gene from tolerant species *P. colubrinum* and susceptible species *P. nigrum*.

The information of plant β-1, 3- glucanase gene sequence available in the public domain NCBI was collected and subjected to multiple sequence alignment to detect conserved boxes of the gene among different plant species. Based on the data, eight degenerate primers were derived and out of this, three sets of forward and reverse primers were synthesized for gene isolation. Genomic DNA isolated from *Piper* spp namely *P. nigrum* and *P. colubrinum* was subjected to PCR with the designed degenerate primers at various combinations at different annealing temperatures. The amplification with the primer combination *GluF1R2* yielded multiple bands in both the selected *Piper* species, whereas the primer combination *GluF2R1* yielded multiple bands in *P. colubrinum* only. The primer combination *GluF2R2* and *GluF3R3* yielded single intact band in both *P. nigrum* and *P. colubrinum*. The amplicons obtained were eluted and cloned in pGEM-T vector and transformed into competent cells. High level of recombination was observed on blue and white screening. Recombination of the insert was confirmed by PCR and restriction analysis of the plasmid isolated from white colonies. The cloned fragments were sequenced.

The fragments *Pnglu* from *P. nigrum* and *Pcglu* from *P. colubrinum* sequenced and subjected to BLAST search revealed, significant level of homology to β-1, 3-glucanase genes reported from other plants deposited in the public domain. Two other fragments cloned and sequenced from *P. nigrum* did not show homology to β-1, 3-glucanase genes deposited in the public domain.

The sequences of the fragment *Pnglu* and *Pcglu* were subjected to other sequence analysis utilizing bioinformatics tools including BCM Search Launcher, ORF finder, GENSCAN, Biology workbench, Conserved domain database, Interproscan and CATH. Multiple sequence alignment of nucleotide of *Pnglu* and *Pcglu* with the selected nucleotides of β-1, 3- glucanase gene sequence from different plant species indicated several conserved regions. The cloned fragment *Pnglu* and *Pcglu* had largest open reading frame of size 450 and 372 bp respectively. Internal exons of size 423 bp and 348 bp respectively were detected in the fragment *Pnglu* and *Pcglu* respectively. Restriction analysis revealed that both *Pnglu* and *Pcglu* have restriction sites for the frequent cutter *Alu*I and the rare cutter *Nae*I. The fragments had high GC content of 56.1 per cent and 56.9 per cent for *Pnglu* and *Pcglu* respectively.

The major amino acid composition deduced from *Pngl*u were alanine, leucine, arginine and proline, while in *Pcglu* major amino acids were alanine, glycine, arginine, leucine and valine. The secondary structures predicted for the polypeptides deduced from *Pnglu* and *Pcglu* had a high proportion of helices. Conserved domains detected from deduced amino acids of *Pnglu* and *Pcglu* suggested that both belong to glycosyl hydrolase family 17, which is a single domain family. Both fragments lack transmembrane regions. Domain structure comparison indicated that domains of *Pnglu* and *Pcglu* had similarity to glycosidase, which is the sequence super family of β-1, 3-glucanases. The sequence information obtained from *Pnglu* and *Pcglu* confirmed that they are genomic counter part of β -1, 3- glucanase gene from *Piper* spp and can be further utilized for the isolation of full length gene from *Piper* spp.