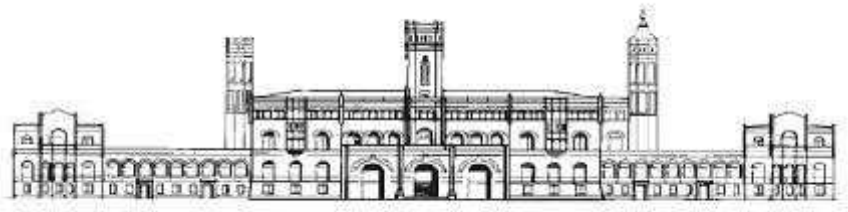

A study of pathogenesis-related (PR) proteins from pearl millet (*Pennisetum glaucum*) after infection with the downy mildew pathogen (*Sclerospora graminicola*).

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Zusammenfassung

Pathogenesis-related Proteine (PR) bilden einen Hauptbestandteil der Pflanzenabwehr gegenüber Pathogenen. Bis heute sind siebzehn PR-Proteinfamilien beschrieben worden. In der Perlhirse (*Pennisetum glaucum*), die vom Flaschen Mehltau (*Sclerospora graminicola*) befallen werden kann, wurden bisher die Phenylalanin-Ammoniak-Lyase, Peroxidase, Superoxiddismutase und Glucanase näher charakterisiert. Jedoch liegen zu Chitinasen und Thaumatin-ähnliche Proteine (TLP), die eine wichtige Rolle in der Verteidigung gegenüber Oomyceten spielen, keine Erkenntnisse aus der Perlhirse vor. In dieser Arbeit wurde die Chitinase der Perlhirse durch chromatographische Methoden aufgereinigt. Das Protein besitzt ein apparentes Moleklargewicht von 24 kDa auf einer SDS PAGE und zeigte eine chitinolytische Aktivität im „In Gel" Assay. Außerdem konnte das Protein durch eine immunologische Kreuzreaktion mit dem Antichitinase Antikörper aus Tabak identifiziert werden. Darüberhinaus fand eine Identifizierung des Proteins durch *de novo* Sequenzierung von tryptischen Fragmenten mittels ESI Q-ToF Massenspektrometrie statt. Die Homologiesuche der abgeleiteten Aminosäurensequenz ergab, dass das Protein zur Gruppe I der Chitinasen (Lysozym-Superfamilie) gehört und somit zu den PR-3 Proteinen. Zusätzlich wurden Primer von einer Mais-Chitinase für die Amplifikation des Volllängengens abgeleitet. Mit ihnen konnte ein 482 bp Fragment der Perlhirse amplifiziert werden, welches Homologien zu einer Mais Chitinase (NCBI Zugriffsnummer gi: 195627425) der Familie 19 der Glycosyl-Hydrolasen (GH 19) aufweist. Die Nukleotidsequenz der Perlhirsen Chitinase wurde unter der NCBI Zugriffsnummer gi: 296011300 hinterlegt und beinhaltet zwischen den Positionen 241 und 348 ein Intron von 107 Basen. Das zweite wichtige Protein, das untersucht wurde, ist das Thaumatin-ähnliche PRotein (TLP) der Perlhirse, dass zur Familie der PR-5 Proteine gehört. Das TLP (23 kDa) der Perlhirse wurde partiell durch Chromatographie aufgereinigt und zeigte eine immunologische Kreuzreaktion mit einem Anti-Thaumatin Antikörper der Douglasie. Durch *de novo* Sequenzierung von tryptischen Fragmenten mittels ESI Q-ToF Massenspetrometrie des TLPs wurden partielle Aminosäuresequenzen erzeugt, die für die Ableitung von Primer genutzt wurden. Mit diesen konnte ein 422 bp Fragment des TLP Gens amplifiziert werden. Die Homologiesuche der abgeleiteten Aminosäuresequenz ergab eine große Übereinstimmung zu einem hypothetischen Protein mit 231 Aminosäure und Ähnlichkeiten zu einem Thaumatin-ähnlichen Proteinen aus *Sorghum bicolor* (NCBI Zugriffsnummer gi:242038635). Darüber hinaus zeigte das TLP *in vitro* eine Curdlan-bindende (Wasser-unlösliches Glucan) Aktivität, die essentiell für das Binden an die mykotische Membran ist. Durch diesen Kontakt wird die Pilzmembran permeabel und durch das anschließende Ausströmen von Ionen aus der Pilzzelle deren Zelltod eingeleitet. Der positive Nachweis von *S. graminicola* Sporangien mit Alcain Blau, einem Kompetitor für die TLP-Bindestelle an der Zellwand des Pilzes, zeigte die Existenz einer solchen Bindungsstelle beim Falschen Mehltau auf. Die Hemmung der Beweglichkeit von *S. graminicola* Zoosporen durch ein Glucan-bindendes Protein zeigte den toxischen Effekt des TLPs auf den Falschen Mehltau. Die Permeabilisierung der pilzlichen Membran durch das TLP kann mit Sytox Grün, einem Nukleinsäurefarbstoff, nachgewiesen werden. Dieser Nachweis konnte erstmalig durch Messung der Sytox Fluoreszenz für das TLP aus der Perlhirse bestätigt werden. Zusätzlich wurden verschiedene Primer basierte Techniken (Genome walking) für die Amplifizierung der Volllängenklone der Chitinase und des TLPs aus Perlhirse durchgeführt. Das Multiple Sequenzalignment der Chitinase und des TLPs aus der monocotylen Perlhirse ergab eine phylogentische Verwandschaft zu anderen Pflanzenspezies und zeigte eine Divergenz zu den bisher bekannten monocotylen Chitinasen und TLPs. Interessanterweise sind die Chitinase und das TLP der Perlhirse in einer eigenen Untergruppe einzuordnen.

Schlüsselworte: Chitinase, Falscher Mehltau (*Sclerospora graminicola*) Perlhirse (*Pennisetum glaucum*).

Summary

Pathogenesis-related (PR) proteins constitute a major defense mechanism of plants against pathogens. Seventeen PR protein families have been classified till date. Some of the defense related proteins studied in pearl millet (*Pennisetum glaucum*) downy mildew pathogen (*Sclerospora graminicola*) interaction include phenyl alanine ammonia lyase, peroxidase, super oxide dismutase, and glucanase are characterized; however, chitinase and thaumatin like proteins (TLPs) from pearl millet which play in general an important role in host defense against oomycete pathogen are not characterized. Therefore this doctoral study concentrated on the characterization of pearl millet chitinase and TLP.

In this study pearl millet chitinase was purified by chromatography. The purified protein had an apparent molecular weight of 24 kDa on SDS PAGE and showed a chitinolytic activity in an in gel assay. The protein also showed immunological cross reaction with an anti-chitinase antibody from tobacco. Moreover, the protein was identified by *de novo* sequencing of trypsin digested peptide fragments by ESI Q-TOF. Homology search of the deduced amino acid sequence revealed that the purified protein belongs to class I chitinase (lysozyme super family) belonging to the PR-3 family of pathogenesis related proteins. Additionally, primers were designed for the amplification of the full length gene considering the deduced amino acid sequence and a maize chitinase gene. The 482 bp (partial length) pearl millet chitinase gene shared homology with many plant chitinases belonging to family 19 of glycosyl hydrolases (GH 19) classification system. The sequence was submitted to NCBI (NCBI accession number: gi|296011300). It was also observed that the pearl millet *chitinase* gene is interrupted by a 107 bp intron at the position 241 bp to 348 bp. The obtained nucleotide sequence shared homology to the chitinase from Maize (NCBI Accession number: gi|195627425). The second important PR protein studied was the pearl millet thaumatin like protein belonging to PR-5 family. TLP from pearl millet was partially purified by chromatography. The 23 kDa pearl millet TLP showed immunological cross reaction with anti-TLP antibody from Douglas fir. The protein was characterized by *de novo* sequencing of trypsin digested peptide fragments by ESI Q-TOF. Based on the deduced partial amino acid sequence primers were designed and a 422 bp (partial length) pearl millet tlp gene was amplified. The obtained nucleotide sequence was translated and the partial amino acid sequence showed homology to the hypothetical protein (231 aa) from *Sorghum bicolor* (similar to thaumatin like protein NCBI Accession number: gi|242038635). Moreover, the protein also exhibited *in vitro* curdlan (water insoluble glucan) binding activity, which is essential before TLP acts on fungal membrane and hydrolyzes the fungal plasma membrane leading to ion leakage and death of invading fungal pathogen. Positive staining of *S. graminicola* sporangium with alcian blue – a competitor of TLP for binding sites on cell wall of the fungus showed the presence of TLP binding sites on cell wall of downy mildew. Inhibition of *S. graminicola* zoospore motility by the glucan bound protein showed toxic effect of TLP on downy mildew pathogen. Fungal membrane permeabilization by TLP was investigated with Sytox green, a nucleic acid dye that enters the cells with compromised plasma membrane. Fluorescence of Sytox green stained sporangia after treatment with curdlan bound protein proved membrane permeabilization by pearl millet TLP. Different primer based genome walking techniques were employed to amplify the full length gene of pearl millet chitinase and TLP. Multiple sequence analyses of both chitinase and TLP revealed their phylogenetic relationship of pearl millet chitinases and TLPs with that of other plant species. Interestingly both pearl millet chitinase and TLP showed divergence from other monocotyledonous chitinases and TLPs and it was placed in separate subgroup.

Key words: Chitinase, Downy mildew pathogen (*Sclerospora graminicola*), Pearl millet (*Pennisetum glaucum*).

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List of abbreviations

APS	Ammonium persulphate
ATP	Adenosine triphosphate
Bp/bp	Base pair
BCIP	5-Bromo-4-chloro-3-indolyl-phosphate (X-phosphate) 4-toluidine salt
BSA	Bovine serum albumin
CBB	Coomassie brilliant blue
cDNA	Complementary deoxyribonucleic acid
CTAB	Hexadecyltrimethylammonium bromide
cv.	Cultivar
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotriphosphate
DPEC	Diphenyleneiodonium chloride
ESI-Q-TOF	Electron spray ionisation quadrupole time of flight
HEPES	4-(2-hydroxyethyl)-1-piperazine ethansulfonate
IPTG	Isopropyl-ß-D-thiogalacto pyranosid
kDa	KiloDalton
LB medium	Lauria Bertani medium
MCS	Multiple cloning site
MOPS	3-(N-morpholino) propane sulfonic acid
mRNA	Messenger RNA
NBT	Nitro blue -tetrazolium
NC	Nitrocellulose
PAGE	Polyacrylamide gel electrophoresis
PCI	Phenol chloroform isoamylalcohol
PCR	Polymerase chain reaction
PEG	Polyethylenglycol
PMSF	Phenylmethylsulfonylfluorid
PR protein	Pathogenesis-related protein
PVDF	Polyvinylidenefluoride
PVP	Polyvinylpyrrolidone
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rotations per minute
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulfate
TAE	Tris-acetate-EDTA buffer
Taq	DNA polymerase from *Thermus aquaticus*
TEMED	N,N,N',N'- tetramethylethylenediamine
UV	Ultra violet
X-Gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

1. Introduction.

Pearl millet [(*Pennisetum glaucum* L.) R. Br.] belongs to *Poaceae* (alt. *Gramineae*). It is also known with alternative names like *Pennisetum typhoides* (Burm.f.) Stapf and Hubbard, *P. americanum* (L.) Leeke and *P. spicatum* Roem and Schult. Chromosome number of pearl millet is n=7, haploid genome size of pearl millet is 2450 Mbp (ICRISAT).

1.1 Cultivation.

P. glaucum is mostly grown as a grain crop, being the staple food in those parts of tropical Africa and India. The crop comes to harvest in approximately three months from the day of sowing depending on the cultivar. India is the largest producer of this crop, both in terms of area (9.1 million hectares) and production (7.3 million tons) (Khairwal *et al.,* 2004). The crop is well adapted to production systems characterized by drought, low soil fertility, and high temperature. It performs well in soils with high salinity or low pH. Because of its tolerance to difficult growing conditions, it is grown in areas where other cereal crops, such as maize or wheat, would not survive. *P. glaucum* is susceptible to water logging. Although this species tolerates poor, infertile soils better than most other crop species, it is more productive on fertile soils. The ideal pH range is 5.5-7.0, but the crop can be cultivated in extremely acidic and alkaline soils with pH ranging from 4.5-8.3 and in soils containing high exchangeable aluminium. By interfering Pi absorption, root elongation and metabolism Al^{3+} inhibits crop growth and yield (Tang *et al.,* 2002). Pearl millet requires an annual rainfall of 125-750 mm, though for forage production a minimum of 500 mm annual rainfall is essential.

1.2 Economic importance.

Millet ranks sixth in importance in terms of contribution to global cereal supply, after wheat, rice, maize, barley and sorghum. It is also used for grazing, green chop and silage, and, with appropriate management, for hay. Whole grains are fed to poultry and livestock, and the straw is used for bedding, thatching, fencing and fuel. Pearl millet was domesticated as a food crop in the tropical region of West Africa at least 4000 years ago. Its use as a food grain has grown over the centuries, with an estimated 64 million acres of pearl millet grown in Africa and India (this acreage is equivalent to the total U.S. corn crop). Ninety four percent of the world's millet production is in developing countries. Pearl millet is the staple food for 90 million people of the semiarid tropics, annually grown on more than 29 million hectares in the semi-arid tropical regions of Asia, Africa and Latin America. The crop is used for a variety of food products, and is even made into a type of beer (ICRISAT).

1.3 Important diseases of pearl millet.

Some of the more important diseases are downy mildew caused by *Sclerospora graminicola*, smut (*Moeszimyces* (*Tolyposporium*) *penicillariae*), ergot (*Claviceps fusiformis*), rust (*Puccinia substriata*) and pyricularia leaf spot (*Pyricularia grisea*). Downy mildew caused by *S. graminicola* [(Sacc). Schroet] leads upto 80% yield loss as the grains are replaced by leaf-like structures in the earhead (Howarth and Yadav, 2002). It is every pearl millet farmer's nightmare; more so in India where plant breeders have introduced genetically uniform single-

cross hybrids that make the crop especially vulnerable to epidemics of this potentially devastating disease. In India, the monetary loss due to a single epidemic of downy mildew was estimated to be € 8.77 million (Hash *et al.,* 2003).

1.4 Downy mildew disease

Downy mildew disease caused by biotrophic oomycetous fungus *Sclerospora graminicola* [(Sacc). Schroet] is the major threat to pearl millet cultivation.

Synonym *Sclerophthora macrospora*

1.4.1 Systematic position.

Kingdom:	Chromalveolata
Phylum:	Heterokontophyta
Class:	Oomycetes
Order:	Sclerosporales
Species:	***Sclerospora graminicola***

1.4.2 Pathogen Characteristics.

Asexual sporangia are produced during the night under moderate temperatures and high humidity. Optimum sporangium production occurs at 20 °C. Sporulation does not occur below 70 percent relative humidity. Sporangiophores have an average length of 268µm. Sporangia are borne on short sterigmata, elliptical in shape of varied size (12-21 x 14-31µm) with distinctive papillate operculum at apex. Oospores are pale brown and 22 to 35µm in diameter. Sporangia germinate to liberate 1 to 12 zoospores, which encyst and germinate by germ tube. Sporangia generally do not remain viable very long after daybreak. Sexual oospores are thick-walled, spherical, brownish yellow, and 22 to 35 µm in diameter. Oospores are formed in colonized plant tissue and can survive from 8 months to 13 years (ICRISAT).

1.4.3 Host range.

S. graminicola exhibits a wide host range infecting a variety of crops like maize, sorghum, *Echinochloa crus-galli, Panicum miliaceum, Pennisetum leonis, P. spicatum, Setaria italica, S. lutescens, S. verticillata, S. viridis, S. magna, Euchlaena maxicana,* and *Agrostis alba.* However, cross-inoculation studies to different hosts have usually been unsuccessful when attempted. The existence of variability in *S. graminicola* has been reported and different host specific pathotypes have been identified (Thakur and Rao 1997; Thakur 1999; Thakur *et al.,* 2003).

1.4.4 Disease symptoms.

Disease symptoms often vary depending on the growth stage and severity of systemic infection. Symptoms begin as stunted growth of the infected seedlings (Fig 1a) and chlorosis at the base; successively higher leaves show progressively greater chlorosis (Fig 1c). Healthy plant with normal growth is shown in the Fig 1b. All the photos were taken at downy mildew sick

plot maintained by AICPMIP (All India Coordinated Pearl Millet Improvement Programme) at University of Mysore campus.

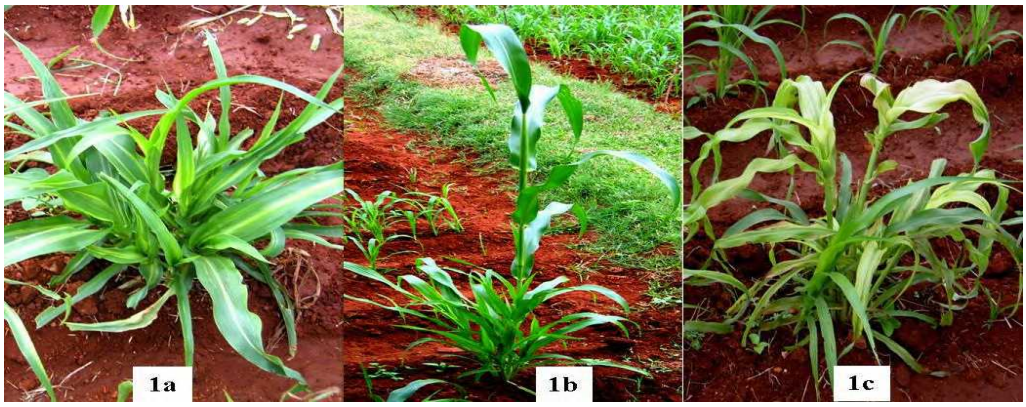


Fig 1a) Infected plant of pearl millet showing stunted growth. 1b) Healthy plant showing normal growth 1c) Infected plant showing chlorosis (all the three plants are 40 day old).
Photographed from downy mildew sick plot maintained by Downy mildew research laboratory, University of Mysore, Mysore.

Infected chlorotic leaf areas can support abundant white asexual sporulation on the lower leaf surface. Basal portion of lower surface of leaf showing sporulation is called half leaf symptom (Fig 2).

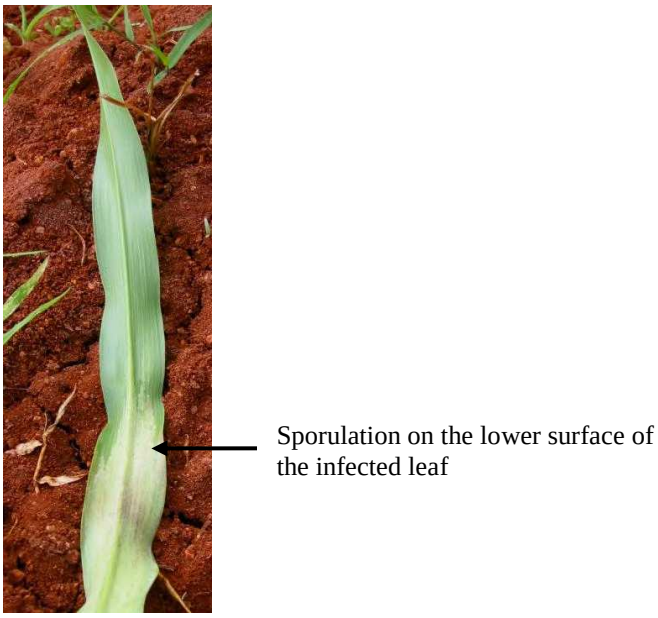


Fig 2. Infected pearl millet leaf showing downy sporulation.

Photographed from downy mildew sick plot maintained by Downy mildew research laboratory, University of Mysore, Mysore.

Severely infected plants are generally stunted and do not produce panicles. Green ear symptoms result from transformation of floral parts into leafy structures (Fig 3).

Fig 3. Earhead of pearl millet.

A: Infected earhead showing malformed floral buds, B: Green ear with all the flower buds transformed into leafy structure, C: Healthy earhead. Photographed from downy mildew sick plot maintained by Downy mildew research laboratory, University of Mysore, Mysore.

1.5 Spread of disease.

Sclerospora graminicola reproduces both sexually by producing oospores and asexually by producing sporangia that liberate zoospores at maturity. Oospores are thick-walled structures that can survive for several years on leaf debris and in soil and also contaminate the seed lots becoming externally seed borne which acts as primary sources of inoculum in the field along with infested field soil and leaf debris. Once the seedlings are infected, sporulation occurs on the lower surface of the foliage, which serves as a source of secondary inoculum for the spread of disease within and between fields.

1.6 Disease Management.

Systemic fungicide Metalaxyl is used to manage downy mildew disease of pearl millet. Seed treatment with Metalaxyl at the rate of 2 g a.i/kg seed controls the disease excellently for about the first 35 days after sowing. Other fungicides used in seed treatment are Maxim (Fludioxonil and Mancozeb), Ridomil, Apron and Subdue (a.i. Mefenonxam which is the active isomer in Metalaxyl). Foliar application of these fungicides at the rate of 125 mg active ingredient per liter arrests further development of the disease in systemically infected plants. If sprayed before floral initiation, disease-free heads are produced. However, fungicide application as foliar spray is not recommended for pearl millet as the crop is also used as a forage crop (ICRISAT).

1.7 Host-pathogen interaction - general overview.

Plants possess both preformed and inducible mechanisms to resist pathogen invasion. Extant morphological barriers, secondary metabolites (phytoanticipins), and antimicrobial proteins must be avoided or overcome by the pathogens in order to invade a plant. Plants possess a basal resistance against their pathogens, which is overcome, manipulated, or suppressed by these pathogens to allow successful infection and tissue colonization (Cui *et al.,* 2005, Nomura *et al.,* 2005, Schulze-Lefert 2004, Zimmerli, 2004). Once the contact with host is established, elicitors produced and released by the pathogen induce further defenses, comprising the reinforcement of cell walls, the production of phytoalexins, and the synthesis of defense-related proteins (Slusarenko *et al.,* 2000). Plant microarray data showed that in both compatible and incompatible plant-pathogen interactions, hundreds of genes are up and down regulated (Tao *et al*., 2003). Studies on *Arabidopsis* mutants with enhanced disease susceptibility (EDS) showed that susceptibility and resistance is determined by the speed and magnitude with which defense mechanisms are activated expressed and by their effectiveness against individual pathogens (Rogers and Ausubel, 1997). Most PRs and related proteins are induced through the action of the signaling compounds salicylic acid, jasmonic acid, or ethylene, and possess antimicrobial activities *in vitro* through hydrolytic activities on cell walls, contact toxicity, and perhaps an involvement in defense signaling. The evolutionary conservation of similar defense-related proteins in monocots and dicots, but also their divergent occurrence in other conditions, suggest that these proteins serve essential functions in plant life, whether in defense or not (Van Loon *et al.,* 2006).

1.8 Role of Pathogenesis-Related Proteins in plant defense.

Protective plant proteins specifically induced in pathological or related situations are called pathogenesis-related proteins" (PR proteins). PR proteins are part of an immune surveillance mechanism that protects the plant primarily against invasion by microorganisms that are generally perceived as nonpathogenic. These inducible defense-related proteins have been detected in many plant species upon infection with oomycetes, fungi, bacteria, or viruses, or insect attack. The expression of some PR genes is constitutive, developmentally regulated, and tissue- and organ-specific. Although the nomenclature of PR proteins was described as early as 1994 (Van Loon, 1994), classification of PR proteins beyond tobacco and tomato has been hampered by the poise of data on properties of these proteins in various species to the mRNA and cDNA level. Originally PR-proteins were classified into 14 families based on their enzymatic properties. The numbering was done in the order of their invention. The list was extended by addition of new PR proteins as they were discovered and at present there are 17 families of PR proteins classified based on the sequence similarities, serologic or immunologic relationships, and enzymatic properties. Related proteins occurring in the absence of pathogen interaction were to be referred to as Pathogenesis related-like proteins (PRLs). Now the term is not encouraged because even the healthy plants possess these proteins and their quantity is observed to increase after pathogen infection or due to some physiological stress (Van Loon, 1994). Classification of PR proteins according to Van Loon and coworkers (2006) is summerised in table 1.

Table 1. Recognised families of pathogenesis-related proteins.

Family	Type member	Properties	Gene name	Reference
PR-1	Tobacco PR-1a	Unknown	*Ypr1*	Antoniw *et al*., 1980
PR-2	Tobacco PR-2	β-1,3-glucanase	*Ypr2*, [*Gns2* (‘*Glb*’)]	Antoniw *et al*., 1980
PR-3	Tobacco	Chitinase I, II, IV, V, VI, VII	*Ypr3*, *Chia*	Van Loon, 1982
PR-4	Tobacco	Chitinase I, II	*Ypr4*, *Chid*	Van Loon, 1982
PR-5	Tobacco	Thaumatin-like protein	*Ypr5*	Van Loon, 1982
PR-6	Tomato	Inhibitor I Proteinase-inhibitor	*Ypr6*, *Pis* (‘*Pin*’)	Green and Ryan, 1972
PR-7	Tomato	Endoproteinase	*Ypr7*	Vera and Conejero, 1988
PR-8	Cucumber	Chitinase III	*Ypr8*, *Chib*	Métraux *et al*., 1988
PR-9	Tobacco	Lignin-forming peroxidase	*Ypr9, Prx*	Lagrimini *et al*., 1987
PR-10	Parsley	Ribonuclease-like protein	*Ypr10*	Somssich *et al*., 1986
PR-11	Tobacco	Chitinase type I	*Ypr11*, *Chic*	Melchers *et al*., 1994
PR-12	Radish	Defensin	*Ypr12*	Terras *et al*., 1992
PR-13	Arabidopsis	Thionin	*Ypr13*, *Thi*	Epple *et al*., 1995
PR-14	Barley	Lipid-transfer protein	*Ypr14*, *Ltp*	García-Olmeda *et al*., 1995
PR-15	Barley	Oxalate oxidase	*Ypr15*	Zhang *et al*., 1995
PR-16	Barley	Oxalate-oxidase-like protein	*Ypr16*	Wei *et al*., 1998
PR-17	Tobacco	Unknown	*Ypr17*	Okushima *et al*., 2000

1.9 Chitinase.

Chitinases (EC 3.2.1.14) catalyse the hydrolytic cleavage of the β-1,4-glycoside bond present in biopolymers of *N*-acetylglucosamine, mainly in chitin. Many purified plant endochitinases also show some degree of lysozyme (EC 3.2.1.17) activity and can hydrolyse β-1,4-linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine residues in peptidoglucan (Boller, 1988, Majeau *et al*., 1990).

1.9.1 Classification of chitinases.

Based on their amino acid sequence similarity chitinases have been classified into four major families encoded by different classes of genes named chiA, chiB, chiC and chiD genes

(Neuhaus *et al.*, 1996; Neuhaus, 1999). Three classes of plant chitinases have been proposed based on the primary structure (Shinshi *et al.,* 1990). Classes I and IV are characterized by the presence of an N-terminal, cysteine rich, usually referred to as hevein-like domain or chitin-binding domain (CBD), which is important for binding chitin but not for catalytic activity (Iseli *et al.*, 1996). When present, the CBD is separated from the catalytic domain by a hinge region, variable in length and amino-acid sequence. Class II chitinases lack the N terminal cysteine-rich domain but have a high amino acid sequence identity to the main structure of class I chitinases. Most class I chitinases have molecular masses of around 32 kDa and are defined into two subclasses class Ia and Ib. Class Ia involves basic chitinases possessing a leucine- or valine rich carboxy-terminal signal peptide that is essential for targeting into the plant cell vacuole (Neuhaus *et al.*, 1991), whereas class Ib chitinases are acidic, lack the signal peptide and are therefore, extracellular (Flach *et al.*, 1992). Class II chitinases, mainly found in dicolyledons, have molecular masses of 27 to 28 kDa. Their amino acid sequences are very similar to class I chitinases, but lack the CBD and the carboxy-terminal vacuolar targeting signal indicating that they are secreted to the apoplast (Shinshi *et al.*, 1990). Class III and class V chitinases are bifunctional enzymes of about 28 to 30 kDa displaying chitinase/lysozyme activity. They are mainly plant and fungal in origin (Heitz *et al*., 1994, Hamel *et al*., 1997) and do not share amino acid sequence homology to any other class. Class IV and VII enzymes are similar to class I, but are smaller in size due to deletions; but, class IV and class I chitinases are serologically distinguishable (Mikkelsen *et a/.,* 1992). Class V is principally comprised of bacterial chitinases; however, two class V chitinases, resembling bacterial chitinases, have been isolated from tobacco (Melchers *et al.*, 1994); class V chitinases have a C-terminal extension for vacuolar targeting and may contain a chitin-binding domain as well (Heitz *et al*., 1994; Ponstein *et al*., 1994). Structure of different classes of chitinases is schematically represented in the Fig 4. Classification of chitinases encoded by different gene families is based on primary structure. Class I and IV structurally similar but serologically distinguishable. Class V chitinases are mainly bacterial chitinases (also found in tobacco plant). Class VI chitinases possess a carboxy terminal extension for vacuolar targeting as well as chitin binding domain.

Schematic representation classification of chitinases.

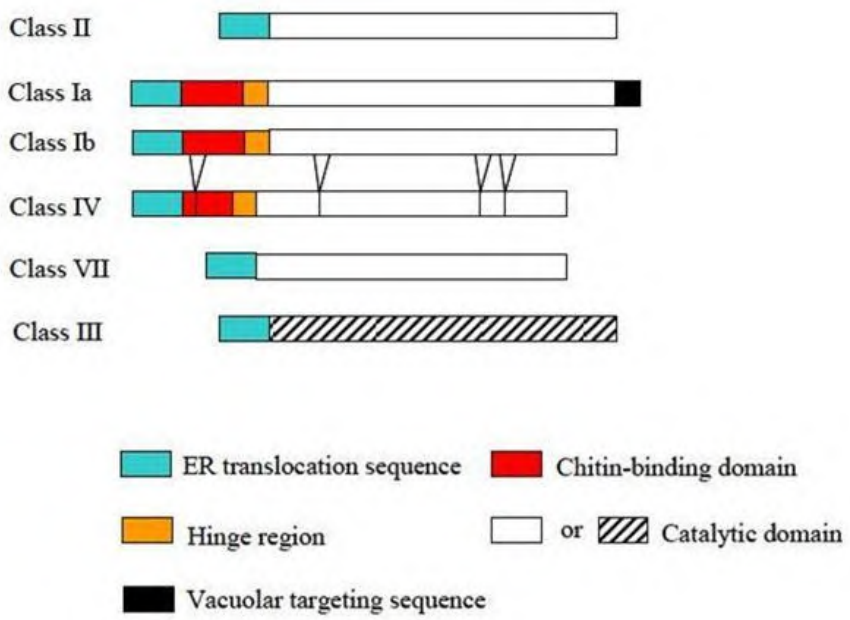


Fig 4. Schematic representation classification of chitinases (Classification is according to Collinge, 1993; Schematic representation: Agdour, 2007).

In glycosyl hydrolase classification system, which is based on amino acid sequence similarity of the catalytic domains, chitinases have been placed in families 18 and 19 (Henrissat, 1991). Family 18 chitinases are found in bacteria, fungi, yeast, viruses, plants and animals whereas family 19 members are almost exclusively present in plants. Family 18 members hydrolyze GlcNAc-GlcNAc or GlcNAc-GlcN linkages whereas family 19 chitinases do so with GlcNAc-GlcNAc or GlcN-GlcNAc linkages (Ohno *et al*., 1996). Family 18 chitinases are likely to function according to a substrate-assisted catalysis model (Brameld *et al*., 1998), whereas family 19 chitinases use a general acid-and-base mechanism (Garcia-Casado *et al*., 1998; Hart *et al*., 1995). In all plants analyzed to date, chitinases of both families are present (Graham and Sticklen, 1994). They are organized in five different classes numbered from I to V, according to their sequences and structure (Neuhaus *et al*., 1996); Chitinases of class I, II, IV, VI and VII belong to the PR-3 family. Class III chitinase belongs to PR-8 family, while class V belongs to PR-11. Seed chitinases belong to classes I, II, IV and VII as well as to the more divergent class III (Kasprzewska, 2003).

1.9.2 Role of chitinase in defense response.

Chitinases can degrade microbial cell wall components and, thereby, might contribute to the expression of resistance (Broekaert *et al*., 2000, Grover and Gowthaman 2003). Additionally chitinase and glucanase-like proteins have both enzymatic and antifreeze activities (Atici and Nalbantog, 2003 and Griffith and Yaish, 2004). At freezing temperatures, these antifreeze

proteins alter ice crystal shape inside plant tissues and reduce freezing injury by slowing the growth and recrystallization of ice (Griffith 2005). Both glucanases and chitinases could also act on endogenous plant substrates and, thereby, aid in the generation of signal molecules that may function as endogenous elicitors of further defensive mechanisms (Van Loon *et al.,* 2006).

1.10 Thaumatin like protein-TLP (PR-5).

PR proteins of the family PR-5, structurally related to the sweet tasting protein thaumatin from the West African shrub *Thaumatococcus danielli* (Cornelissen, 1986) are among the families of antimicrobial proteins associated with plant defense (Veronese *et al.,* 2003). The family includes low molecular weight proteins (15-30 kDa) acidic or basic in nature, resistant to heat and acidic treatments. They are relatively resistant to proteolytic degradation and can accumulate extra-cellularly, in cell vacuoles of various organs. These proteins are also called as Permatins because of their ability to cause transmembrane pores in the plasma membrane of the fungi. These transmembrane pores cause ion leakage and kill fungus (Roberts and Selitrennikoff, 1990).

1.10.1 Classification of thaumatin like (PR-5) proteins.

The system of nomenclature is somewhat arbitrary because Osmotin like proteins (OLP) and Thaumatin like proteins have similar levels of sequence similarity with the TLP from *T. danielli*. Similarly, there appears to be no obvious reason why isoelectric points of a family of proteins should be related to function. Thus, both TLPs and OLPs can be referred to as PR-5 proteins in general (Osmond *et al.,* 2001). PR-5 proteins with acidic isoelectric points are often referred to as thaumatin-like proteins (TLPs) because of their sequence similarity with the intensely sweet protein, thaumatin, from the West African shrub *Thaumatococcus danielli* (Cornelissen, 1986). Basic PR-5 proteins are referred to as osmotin-like proteins (OLPs) because of their similarity to osmotin, a salt stress induced protein from tobacco (Singh *et al.,* 1987). In this study, the term TLP will be used to indicate pearl millet PR-5 protein, in accordance with the previous studies from Radhajeyalakshmi and coworkers (2003) on existence of Thaumatin-like Proteins (TLPs) in seeds of cereals where the pearl millet PR-5 protein is addressed as TLP.

1.10.2 Role of TLPs in defense response

TLPs are inducible or constitutive and sometimes both (Dudler *et al.,* 1994). TLPs have also been used as marker of SAR in dicots (Yamakawa *et al*., 1998) as well as in monocots (Morris, 1998). Various TLPs were shown to be antifungal *in vitro* as well as in transgenic plants (Hu and Reddy 1997, Liu *et al*., 1994, Yun *et al*., 1997). Constitutive cereal seed permatins was observed to be antifungal on several types of fungi (Yun *et al*., 1997). Over expression of PR-5 genes from rice has been demonstrated to reduce infection of rice by *R. solani* (Grover and Gowthaman, 2003), of wheat by *Fusarium graminearum* (Chen *et al*., 1999), of tobacco by *A. alternata* (Velazhahan and Muthukrishnan, 2003/4), and of carrot by *A. dauci*, *Alternaria petroselini*, *A. radicina*, *B. cinerea*, *R. solani*, and *S. sclerotiorum* (Punja, 2005). Osmotin from tomato protected transgenic orange plants against *Phytophthora citrophthora* (Fagoaga *et al*., 2001).

1.10.3 Mode of action of TLPs

Zeamatin, as well as chemically induced extracellular PR- 5-type proteins from barley and pea leaves, showed binding to polymeric β-1,3-glucans, whereas tobacco osmotin did not (Trudel *et al.,* 1998). Osmotin, but not zeamatin, exhibited endo-β-1,3-glucanase activity on these substrates. Analysis of other thaumatin-like proteins confirmed a general lack of correlation between antifungal activity, β-1,3-glucan binding, and β-1,3-glucan hydrolysis (Grenier *et al.,* 1999, Menu-Bouaouiche *et al.,* 2003). Thus, the main antifungal action of PR-5-type proteins must reside in a different property. Osmotin induces apoptosis in the yeast *Saccharomyces cerevisiae*, apparently by binding to phosphomannans in the cell wall (Ibeas *et al.,* 2000), which facilitates access to a 7-transmembrane-domain receptor-like protein in the plasma membrane that regulates lipid and phosphate metabolism and is homologous to a mammalian receptor for the hormone adiponectin. Like adiponectin, osmotin activates AMP kinase in murine myocytes via adiponectin receptors, suggesting that osmotin action is receptor-mediated (Narasimhan *et al.,* 2005). Over expression of the stress-related yeast PIR2 cell wall glycoprotein protected *S. cerevisiae* from the toxic action of osmotin (Yun *et al.,* 1997), and similar result was obtained for *Fusarium oxysporum* f.sp. *nicotianae*, allowing increased disease severity and fungal growth in tobacco seedlings (Narasimhan *et al.,* 2003). Thus, osmotin seems to contribute to basal resistance of tobacco against *F. oxysporum* f.sp. *nicotianae*. Other PR-5 proteins are much less active against yeast cells than osmotin but active against other fungal species (Yun *et al.,* 1997). It seems that cell wall binding facilitates the action of osmotin and contributes to their fungal target specificity. Four PR-5 proteins from *Arabidopsis* and seven from *Oryza sativa* have extensions that end in a hydrophobic stretch that could serve as a membrane anchor (Van Loon *et al.,* 2006) suggestive of a (temporary) attachment of these proteins to a membrane. Others contain a potential transmembrane segment followed by a kinase domain, suggestive of a function in extracellular sensing of perhaps β-1,3-glucan fragments and signal transduction. Three of these receptor-like kinases are present in *Arabidopsis thaliana* and two in rice. One of the PR-5-like receptor kinases from *A. thaliana* is expressed constitutively at low levels in leaves and siliques and at higher levels in flower stems and roots, and has been suggested to recognize the same targets as the related PR-5 proteins (Wang *et al.,* 1996). Moreover, Salzman and coworkers (2004) demonstrated that inorganic cations mediate antifungal activity of plant PR-5 protein; understanding of the exact mechanism of action of PR-5 proteins however, needs further studies.

1.11 Aim of the Research

Study of pathogenesis related proteins is very important for complete understanding of the host defense mechanism. PR proteins from pearl millet are not well characterized and the host defense mechanism needs a deeper investigation in order to develop strategies to combat downy mildew disease of pearl millet. Occurrence of different pathotypes of *Sclerospora graminicola*, resistance breakdown of many cultivars over a very short period is a big challenge for pearl millet downy mildew researchers. Moreover obligate nature of downy mildew pathogen, light and temperature sensitivity of the zoospores, great variation among 13 different races of the pathogen originating from semi-arid regions of Africa and India makes the study more complicated than any other crop species.

Considerable amount of research is carried out at Downy mildew laboratory, Mysore, India on induction of host defense mechanism using various biotic and abiotic elicitors. Defense related enzymes like peroxidase, glucanase, proton ATPase, super oxide dismutase, thionins, and phenylalanine ammonia lyase are well characterized in pearl millet. Great amount of research is being carried out at ICRISAT in Africa and India on marker assisted screening of resistance genes. However, a clear insight to defense mechanism needs further study.

In this doctoral study, the main aim was to purify pearl millet chitinase and thaumatin like protein (TLP), for further characterization and study of mechanism of action against *Sclerospora graminicola.* Chitinase and TLP are known to have synergistic action against oomycete pathogen attack. Though the mode of action of chitinase and TLP are well studied in other organisms, the mode of action of these proteins in pearl millet is not well understood. The purified proteins should further *de novo* sequenced to get the partial amino acid sequences for the amplification of the full length genes. Since neither amino acid nor nucleotide sequence of pearl millet chitinase and tlp are available in the database this partial amino acid sequence was very important to conduct molecular studies.

Next effort was isolation of pearl millet chi and tlp genes. Because of the lack of nucleotide sequence data of pearl millet chi and tlp genes, earlier studies have used barley, rice or wheat gene fragments as probes for northern and southern blot analysis. Therefore, it was important to isolate these genes from pearl millet and carry out sequence evaluation of pearl millet chitinase and TLP using different bioinformatics tools.

The next important task was to isolate the full-length genes coding pearl millet chitinase and TLP. For this different genome, walking techniques to amplify the unknown regions of the gene flanking the known sequence had to be followed. However, large genome size and non availability of the nucleotide sequences posed a great challenge in genome walking.

Since the knowledge of molecular data of pathogenesis-related proteins from pearl millet is currently limited, this doctoral thesis will build the basis for further characterization for ongoing studies in the plant pathogen defense system of pearl millet against the fungal pathogen *Sclerospora graminicola*.

2 Materials and Methods.

Part A-Preliminary studies.

2.1 Infection of pearl millet seedlings with downy mildew pathogen.

2.1.1 Plant material.

Pearl millet (*Pennisetum glaucum*) seeds used in the study were downy mildew resistant cultivar IP18292 (less than 5% disease incidence) and downy mildew susceptible cultivar 7042S (more than 85% disease incidence). They were obtained from the 'All India Coordinated Pearl Millet Improvement Project' (AICPMIP), Jodhpur, Rajasthan, India and the International Crop Research Institute in Semi Arid Tropics (ICRISAT), India. Pearl millet seedlings were grown under green house condition with a high relative humidity of > 95% (RH) at 22-30°C with 12 h alternating light and dark period.

2.1.2 Maintenance of inoculum.

Soil from the sick plot maintained at the University of Mysore campus, (India) serves as the source of primary infection (oospore). The sick plot soil contains oospores from the infected plant debris. When a susceptible cultivar is grown on this *S. graminicola* infested soil under high relative humidity of >95% (RH) at 22-30°C, the seedlings get downy mildew infected. In this manner phytopathogenic oomycete *S. graminicola*, was maintained on pearl millet cv. 7042S under field conditions. Zoospores from the infected plants were used to infect susceptible seedlings. Under greenhouse conditions, the susceptible seedlings are grown in the green house and sprayed with zoospore suspension (40,000/mL) from two leaf stage for three consecutive days (Safeeulla, 1976).

2.1.3 Inoculum preparation.

The phytopathogenic oomycete *S. graminicola* maintained on pearl millet cv. 7042S, under greenhouse conditions with 12 hrs of alternating light and dark period was used for all inoculation experiments. Leaves of pearl millet showing profuse sporulation of *S. graminicola* on the abaxial side were collected in the evening from plants maintained under greenhouse conditions (25–30°C, >95% RH). Collected leaves were thoroughly washed under running tap water to remove the previous crop of sporangia. The leaves were then blotted dry, cut into small pieces, and incubated overnight in a moist chamber for sporulation at 22°C (Safeeulla, 1976).

2.1.4 Harvest of zoospores and inoculation.

After 12 hrs fresh crop of sporangia was harvested into sterile deionised water by washing the lower surface of the leaf. For use as inoculum, the zoospore concentration was adjusted to 4×104/ mL with a haemocytometer. The seedlings were sprayed with this zoospore

suspension (approximately 50 µL/seedling) and incubated in dark to facilitate infection process (Jones *et. al*, 2001).

2.1.5 Sample harvest.

The seedlings are harvested for the study by removing them from the inoculum and blot drying. The seedlings were frozen by dipping in liquid nitrogen and maintained at -70°C until further use.

Part B-Analytical methods.

2.2 Immunological detection of TLP from pearl millet.

2.2.1 Extraction of total soluble protein.

Frozen pearl millet seedlings were homogenized by crushing with ice-cold 50 mM sodium acetate buffer, pH 5.0, containing 3 mM phenylmethanesulfonyl fluoride (PMSF) until homogeneity. The homogenate was centrifuged at 10,000 x g (Sorvall centrifuge GS3 rotor) for 30 min at 4°C to precipitate the cell debris. The supernatant obtained is the total soluble protein fraction.

2.2.2 Determination of protein concentration by Bradford method.

Calibration curve was prepared by using a standard protein sample BSA (Bovine Serum Albumin, Sigma). Afterwards 10 µL from protein sample was added to 80 µL deionised water, and 100 µL deionised water was used as blank. Nine hundred µl of Bradford reagent was added to each sample and as well as the blank, mixed well and after 5 min the absorbance was measured by spectrophotometer at 595 nm (Bradford, 1976). The corresponding concentrations were calculated with the aid of calibration curve.

Bradford reagent.

96 % Ethanol	50 mL
85 % Ortho-Phosphoric acid (H3PO4)	100 mL
Coomassie Brilliant Blue G-250 (CBB)	100 mg

Volume is made up to 1 liter with deionised water.
After dissolving Coomassie Brilliant Blue (G250) the solution was filtrated and stored at 4°C.

2.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)-Laemmli method.

2.3.1 Gel preparation.

Separating gel was prepared by mixing all the components mentioned below and poured into the gel cassette; mini gels of the size 9 x 11 cm and 0.75 mm thickness were casted. Polymerization was facilitated by covering separating gels with iso-butanol. After polymerization the iso-butanol was removed, and the gel was washed with deionised water to

remove traces of iso-butanol. Gel cassette was fixed into electrophoresis cell and 4 % stacking gel solution was and poured above separating gel, and comb (with 10 teeth) was inserted into the stacking gel. After polymerization of the stacking gel, 1X Laemmli running buffer was poured into the Electrophoresis apparatus and the comb was removed carefully. The wells were washed with running buffer before loading the samples (Laemmli, 1970). 600, Pharmacia, Sweden). Composition of separating gel (12%), stacking gel (4%) and Laemmli running buffer is given below.

Separating gel.	**(12% Acrylamide)**
Components	12% gel
H_2O	10.2 mL
1.5 M Tris-HCl, pH 8.8	7.5 mL
20% (w/v) SDS	0.15 mL
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	12.0 mL
10% (w/v) ammonium persulfate (APS)*	0.15 mL
TEMED	0.02 mL

Stacking Gel Solution.	**(4% Acrylamide)**
Deionised H_2O	3.075 mL
0.5 M Tris-HCl, pH 6.8	1.25 mL
20% (w/v) SDS	0.025 mL
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	0.67 mL
10% (w/v) ammonium persulfate (APS)*	0.025 mL
TEMED	0.005 mL

* freshly prepared

1X Laemmli Running Buffer.	
Tris-HCl	25 mM
Glycine	200 mM
SDS	0.1% (w/v)

5 X SDS-Laemmli loading buffer.	
Tris-HCl (pH 6.8)	0.2 M
SDS	10 % (w/v)
Beta-mercapto-ethanol	10 mM
Glycerol	20 % (v/v)
Bromophenol blue	0.05% (w/v)

2.3.2 Sample preparation for SDS-PAGE and electrophoresis.

Protein sample was mixed with 5 X SDS-Sample buffer (Laemmli loading buffer containing SDS in a 1.5 mL micro centrifuge tube by vortexing. The sample is denatured by incubating in boiling water bath for 10 min and snap cooled by putting on ice. The sample was centrifuged for 10 sec at 5000 x g (Eppendorf centrifuge, rotor F45-24-11) and loaded onto the gel. The gel was run at 50V (15-20 mA current) when the samples are in the stacking gel and at

75V (20-25 mA current) once the samples move to the separating gel using electrophoresis power supply (EPS).

2.3.3 Staining of protein gel.

2.3.3 i) Staining of protein gel with Coomassie Brilliant Blue.

The gel was stained with Coomassie Brilliant Blue according to the method of Maldener, 2004. The staining solution contained isopropanol (25%), acetic acid (10%) and Coomassie Brilliant Blue-R250 (0.05%). The gel was destained with 10% acetic acid.

2.3.3 ii) Staining of protein gel with Silver nitrate.

For detection of low amounts of proteins (1–5 μg) after gel electrophoresis, silver staining method is followed. To achieve the best results, that are high sensitivity and low background, it is very important to follow closely the incubation time of all steps as given in the protocol and all the reagents were freshly prepared.

Fixing: The gel was removed from the cassette after electrophoresis and fixed by incubating in the fixing solution for 1 hour or overnight on a shaker. The fixing solution contained 50% ethanol, 12% acetic acid and 0.05%.

Washing: After fixation the fixative is discarded and the gel was washed 3 times for 15 min each with 20% ethanol.

Thiosulphate treatment: After the washing step, gel was incubated in 0.02% sodium thiosulphate for exactly 1 min with continuous shaking.

Staining: After treating the gel with sodium thiosulphate it was washed thrice with deionised water (1 min each) and was stained by incubating in 0.2% silver nitrate solution containing 0.076% formalin for 45 min on a shaker.

Developing: The gel was washed thrice with deionised water and developed by incubating in 6% sodium carbonate solution containing 0.0004% sodium thiosulphate and 0.05% formalin. The reaction was arrested after the brown bands appeared against pale background by adding 1% acetic acid.

Drying: After staining the gel, it was scanned for documentation purpose and dehydrated by dipping in 20% ethanol and dried on a gel drier, sealed between two transparent plastic sheets.

2.3.4 Electrophoretic transfer of protein-wet blot method.

After electrophoresis was finished, gel was removed from the chamber, washed with transfer buffer and soaked in it for 15 min to remove the electrophoresis salts and detergents. PVDF membrane was activated by dipping in methanol and equilibrated with the transfer buffer for 15 min before assembly for electro transfer. The gel was assembled for electro transfer in the following order. Care is taken so that no air bubble was trapped between the layers. Composition of electro transfer buffer is given below.

Electro transfer Buffer.

Na bicarbonate ($NaHCO_3$)	10 mM
Na carbonate ($NaCO_3$)	3 mM
Methanol	20 % (v/v)

Sandwich for electro transfer of proteins.

The gel and membrane were arranged in the following order to transfer protein from gel onto the membrane.

Cathode, negative electrode

1 Sponge sheet
1 layers of Whatman filter paper
Electrophoresed Gel
Nitrocellulose membrane
2 layers of Whatman filter paper
1 Sponge sheet

Anode, positive electrode

Direction of protein transfer is from cathode to anode. Transfer was carried for 90 min at 400V. The gel was run in duplicates; once Electro transfer was complete one of the gels was stained with Coomassie Brilliant Blue for comparison. Membrane was stained with 0.1% (w/v in deionised water) Ponceau red to confirm the transfer of protein onto the membrane. Protein appears as pale red band on membrane, which can be distained by washing repeatedly with distilled water.

2.4 Immunological detection of proteins.

Proteins were transferred onto the membrane and subsequently blotted.

2.4.1 Blocking.

The membrane was blocked using blocking buffer (1 x PBS containing 5% defatted skimmed milk powder) for 1h at 4ºC. Membrane was then removed from the blocking buffer and washed with phosphate buffer saline (Composition of PBS and PBST (pH 7.4) are given below).

Components	**mM**	**g/L**
Sodium chloride (NaCl)	7.40	8.00
Potassium chloride (KCl)	2.70	0.20
Disodium phosphate (Na_2HPO_4)	10.00	1.44
Sodium dihydrogen phosphate (NaH_2PO_4)	1.76	0.24

1 x PBST for washing steps was prepared by adding 0.01% Tween 20 to 1 x PBS.

2.4.2 Incubation antibodies.

Incubation with the primary antibody: Membrane was incubated 1 x PBS containing the primary antibody overnight at 4ºC with gentle shaking. Incubation with the secondary antibody: Membrane was removed from primary antibody and washed with 1 x PBST thrice (10 min each), incubated with secondary antibody conjugated with alkaline phosphatase for 90 min at 37ºC.

2.4.3 Development of immuno blot.

An ideal system for staining of immunoblots with alkaline phosphatase conjugated secondary antibody is the use of combination of NBT (Nitro Blue Tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt). Together, they yield an intense, black-purple precipitate that provides much greater sensitivity than either substrate alone. This reaction proceeds at a steady rate, allowing accurate control of its relative sensitivity. NBT/BCIP characteristically produces sharp band resolution with little background staining of the membrane. NBT is a tetrazolium salt; upon reduction, it yields NBT-formazan, a highly colored, water-insoluble product and hydrolysis of BCIP by alkaline phosphatase results in a blue-purple precipitate that can be deposited on nitrocellulose membrane. Membrane was washed with PBST and equilibrated in the developing (TMN) buffer for 10 min. For 10 mL of TMN buffer 33 µl BCIP and 33 µl of NBT were added and the blot was incubated in this developing solution until the colour developed on shaker at room temperature. The reaction was arrested when purple coloured bands appear on the membrane (2-10 min) by washing with water. Composition of TMN developing buffer, BCIP and NBT stock solutions is given below.

TMN Buffer

Tris/HCl	100 mM
$MgCl_2$	5 mM
NaCl	100 mM

pH was adjusted to 9.5 using 1M HCl.

BCIP: BCIP stock solution was prepared by dissolving 50 mg 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt in 1 mL in Dimethyl Formamide (DMF), and stored at -20°C.

NBT: NBT stock solution was prepared by dissolving 50 mg Nitro Blue Tetrazolium (NBT) in 1 mL deionised water and stored at -20°C.

2.4.4 Immunoblotting of the purified protein.

Proteins were separated on 12% Laemmli gel as explained in section 2.3 of materials and methods. The protein was electro blotted onto PVDF membrane as mentioned in section 2.3.4 of material and methods. Immunoblotting was carried out according to section 2.4 of materials and methods. The primary anti-chitinase antibody as well as anti-TLP antibody was raised in rabbit. Therefore secondary anti rabbit antibody raised in goat tagged with alkaline phosphatase (Sigma) was used for immuno blotting of proteins. The immuno blot was developed with BCIP-NBT as explained in 2.2.6 of materials and methods.

Part C - Biochemical methods.

2.5 Substrate binding assay.

2.5.1 Substrate Preparation.

One gram of commercially available curdlan (water insoluble ß-1-3, glucan, substrate for TLP) was suspended in 10 mL water and boiled for 30 min to induce gel formation (Saito et.al, 1968). The gel was homogenized by grinding and washed extensively with water.

2.5.2 Binding of TLP to curdlan.

For substrate binding assay involving PAGE analysis, total protein extract was incubated with 5% (w/v) of gelled curdlan equilibrated with 50 mM sodium acetate buffer (pH 5.0), in a micro centrifuge tube in 0.1 mL of 50 mM sodium acetate buffer, pH 5.0, with continuous vortexing at 22°C for 15 min (Trudel *et al.*, 1998).

2.5.3 Elution of the curdlan bound protein.

Bound proteins were recovered from the insoluble pellet by centrifugation at 13,000 x g (Eppendorf centrifuge rotor F45-24-11) for 5 min at 22°C after the pellet was washed twice with 10 mm sodium acetate buffer (pH 5.0), by boiling in the Laemmli sample loading buffer. Eluted bound protein was analyzed on 12% (w/v) denaturing SDS polyacrylamide gel (Laemmli, 1970). Protein bands were visualized by Coomassie staining as mentioned under section 2.3.3 of materials and methods. Bound Protein was released from curdlan by incubating the washed pellet for 15 min at 22°C in 0.1 mL of 0.1 M HCl (pH 2.2). Immediately after centrifugation at 15,000 x g (Eppendorf centrifuge, rotor F45-24-11) for 5 min at 22°C, the supernatant containing the released proteins was neutralized with equal amount of 0.1 M NaOH (Trudel *et al.*, 1998). the recovered protein was equilibrated with 50 mM sodium acetate buffer (pH 5.0) by washing the sample with the same buffer using a Vivaspin protein concentrator tubes with a molecular cut off of 5 kDa at 8000 x g (Sorvall centrifuge GS3 rotor). Though the method resulted in partial purification of the protein, only a weak protein band was seen when the bound protein fraction was separated on SDS-PAGE due to the loss of bound protein during washing steps.

2.5.4 Immunoblotting of the curdlan bound protein with anti-TLP antibody.

The curdlan bound protein was eluted from the substrate by boiling with SDS loading buffer. The sample was loaded onto 12% polyacrylamide gel containing SDS. Proteins were resolved by SDS-PAGE as detailed under section 2.3 of materials and methods. Proteins separated by electrophoresis were transferred onto PVDF membrane as explained in section 2.3.4 of materials and methods. Immunoblotting was carried out using anti-osmotin antibody from tobacco raised in rabbit as elaborated in section 2.4 of materials and methods and the gel was prepared in duplicates; one set of samples were stained by Coomassie Brilliant Blue and the other one was used for immuno blot analysis.

2.6 Action of TLP on downy mildew pathogen of pearl millet.

2.6.1 Inhibition of zoospores with curlan bound protein.

Curdlan bound proteins were eluted neutralized and desalted by dialysis. Living zoospores of *S. graminicola* suspended in ice cold sterile deionised water (4 X 104 zoospores/mL) was treated with curdlan bound protein (10 μg/mL). The zoospores were observed under bright field microscope at 20 X magnification for their mobility before and after treatment with curdlan bound protein.

2.6.2 Microscopic observation of membrane permeabilization using Sytox green dye.

This experiment was carried out according to the method of Thevissen and coworkers (1999). The method was used to study membrane permeabilization induced by plant defensins in *Neurospora crassa*. To investigate membrane permeabilization, a high-affinity nucleic acid stain that fluoresces upon nucleic acid binding and for which no endogenous channels exist - Sytox Green (Molecular Probes Eugene, Oreg) was chosen. Sporangial suspension harvested in ice cold deionised water was resuspended in 100 mM HEPES-KOH (pH 6.5) with 5 mM MgCl2 and 0.2 mM Sytox Green, this served as control. Ninety micro liter of this zoospore suspension (4 X 104 zoospores/mL) was mixed with 10 μl of curdlan bound protein, which contains partially purified TLP (10 μg/mL) and incubated in a micro centrifuge tube in dark for 30 min on ice. Fluorescence was observed with Leitz Diaplan fluorescent microscope using Leitz I2/3 excitation filter: BP450-490, dichroic mirror: RKP510 emission filter: LP515. (Excitation wavelength, 450 to 490 nm; Emission wavelength 520 nm) under 200X (20x10) magnification.

2.7 Purification of pearl millet chitinase.

Purification of pearl millet chitinase by chromatographic method was optimized. The steps followed are mentioned below.

2.7.1 Propagation of pearl millet seedlings.

Seeds were surface sterilized by soaking in 0.1% (v/v) of sodium hypochlorite for 5 min. Seeds were then thoroughly washed with water to remove traces of hypochlorite. The seeds were spread on a sterile wet blotter sheet. The seedlings are grown at 25°C for 2 days.

2.7.2 Treatment of pearl millet seedlings with silver nitrate solution for PR protein over expression.

Pearl millet seedlings were induced to over express PR proteins by treating one week old seedlings with 1mM aqueous silver nitrate solution. Seedlings were sprayed once in a day with 1mL of 1 mM silver nitrate solution for five consecutive days (Conejero & Granell, 1986 and Rahimi *et al.,* 1993). Seedlings sprayed with deionised water served as control. The treated seedlings were harvested on the sixth day and snap frozen by dipping in liquid nitrogen and stored at - 70°C.

2.7.3 Extraction of total soluble protein from pearl millet seedlings.

Total soluble protein from silver nitrate treated pearl millet seedlings was extracted as explained in under section 2.2.1. Total protein extract was extracted from untreated seedlings. Both the samples were analysed on SDS-PAGE for comparison. As the silver nitrate treated seedlings showed over expression of proteins, they were used for purification purpose. Different concentrations of silver nitrate were tested for the induction of proteins in pearl millet seedlings. A concentration of 1 mM was found to be effective and the higher concentration of silver nitrate had toxic effect on seedlings leading to death of the seedlings.

2.7.4 Ammonium sulphate fractionation of proteins.

Ammonium sulphate fractionation of proteins works on the principle of existence of hydrophobic and hydrophilic amino acids in protein molecules. After protein folding in aqueous solution, hydrophobic amino acids usually form protected hydrophobic areas while hydrophilic amino acids interact with the molecules of solvation and allow proteins to form hydrogen bonds with the surrounding water molecules. If enough of the protein surface is hydrophilic, the protein can be dissolved in water. When the salt concentration is increased, some water molecules are attracted by the salt ions, which decreases the number of water molecules available to interact with the charged part of the protein. As a result of the increased demand for solvent molecules, the protein-protein interactions are stronger than the solvent-solute interactions; the protein molecules coagulate by forming hydrophobic interactions with each other. This process is known as salting out. Total soluble protein fraction from silver nitrate treated pearl millet seedlings was fractionated at 0-30%, 30-60% and 60-100% ammonium sulphate saturation (Table 2).

Table 2. Ammonium sulphate fractionation of total soluble protein fraction from pearl millet.

Protein fraction	Percentage saturation	Volume of the fraction	Amount of $(NH_4)_2SO_4$ added	Final concentration of $(NH_4)_2SO_4$
1	0-30%	122 mL	20g	164g/L
2	30-60%	128 mL	23g	181g/L
3	60-100%	133 mL	37g	279g/L

2.7.5 Separation of proteins by ion exchange chromatography.

Separation by ion exchange chromatography depends upon the reversible adsorption of charged solute molecules to immobilized ion exchangers of opposite charge. The type of charged groups determines the nature and strength of ion exchanger; their total number and availability determines the capacity. An ideal pH range depends on the p*I* of the proteins. Hence, proteins would bind to positively charged molecules of the resin. Change in the buffer pH condition

changes the charges on the protein molecule dependent on its pI. Among the most commonly used charged molecules are CM (carboxymethyl) and DEAE (Diethylaminoethyl); these charged molecules are coupled to inactive materials like cellulose, sepharose, sephacel or sephadex; sepharose is preferred here because the column would not shrink at higher sodium chloride concentrations unlike sephadex.

2.7.6 Chromatography using DEAE cellulose column.

DEAE -Diethylaminoethyl (-O-CH_2-CH_2-NH(CH_2CH_3)$_2$ cellulose is a weak anion exchanger. DEAE-cellulose contains an ionizable tertiary amine group therefore positively charged at neutral pH and DEAE-cellulose is an anion exchanger. The counter ion for DEAE-cellulose is normally Cl^-. Ligand begins to lose its charge above pH 9; therefore pH range while working with DEAE-cellulose is pH 5 - 9.

In this case DEAE cellulose column was equilibrated with 10 mM Tris-HCl buffer (pH 8.0) and packed into a 20 cm column of 1.25 cm radius with 10 cm bed; bed volume of the column was 49 cm3 and the void volume was 16 cm3. Ammonium sulphate precipitated protein at 30-60% saturated is also dialysed against the 10 mM Tris-HCl buffer (pH 8.0) before loading into the column. The sample is centrifuges at 4°C for 15 min at 15,000 x g (Sorvall centrifuge GS3 rotor) to settle down the impurities and particles that would otherwise block the column. Flow rate of 0.4 mL/ min was adjusted and unbound proteins were eluted with 2 bed volumes of 10 mM Tris-HCl buffer (pH 8.0). Bound proteins were eluted using a 0-1000 mM linear NaCl gradient using gradient mixer. The column material was regenerated and stored in 20% ethanol at 4 °C after use.

2.7.7 Chromatography using hydroxyapatite column.

Hydroxyapatite-HA (Bio-Gel, HTP DNA-grade; Bio-Rad) was used to fractionate the proteins as DEAE-cellulose did not result in good separation. Hydroxyapatite chromatography is a method of purifying proteins that utilizes an insoluble hydroxylated calcium phosphate [$Ca_{10}(PO_4)_6(OH)_2$], which forms both the matrix and ligand. Functional groups consist of pairs of positively charged calcium ions (C-sites) and clusters of negatively charged phosphate groups (P-sites). The interactions between hydroxyapatite and proteins are complex and multi-mode. An acidic protein usually binds to C-sites via a coordination bond to carboxyl group, while a basic protein binds to P-sites through charge interaction with the amine group as shown in Fig 5 (Shepard, 2000). The higher the phosphate concentration, the less strong is adsorption.

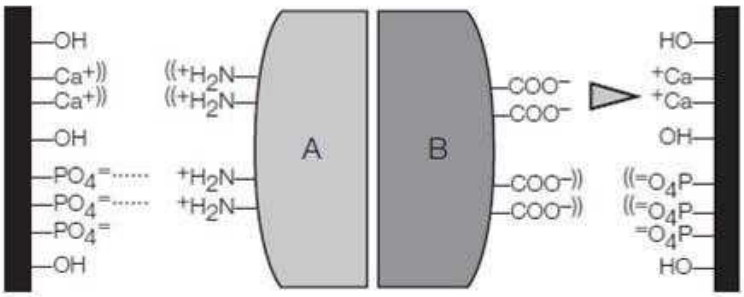


Fig 5. Schematic representation of protein binding to hydroxyapatite.
A is a basic protein and B is an acidic protein. Double parentheses indicate repulsion. Dotted lines indicate ionic bonds. Triangular linkages indicate coordination bonds. Source: Shepard, 2000.

When hydrated, hydroxyapatite occupies approximately 2-3 mL per dry gram. All buffers were degassed prior to the addition of the dry hydroxyapatite. Buffer pH was 7.0

In this case gel was soaked in 10 mM sodium phosphate buffer (pH 7.0) overnight for swelling; the fine particles forming cloudy upper level on top of the settled bed were decanted and the swollen column material was degassed before packing into the column of 25 cm length and 1.25 cm radius. Height of the bed was 10 cm. (The void volume of hydroxyapatite is about 75% of the bed volume). The column was washed 3 bed volumes of elution buffer (10 mM Na phosphate buffer, pH 7.0) before loading the sample. Two mg of protein per mL of bed volume was loaded. Flow rate was adjusted to 0.4 mL/ min. Fraction size was 2 mL. Adsorbed proteins from the column were eluted by washing with 1 M sodium phosphate buffer (pH 7.0).

2.7.8. Ion exchange chromatography using CM Sephadex.

Carboxymethyl (CM) -O-CH_2-COO-) Cation exchange resin with bound positive counter ions, at neutral pH this group is ionized as -$CH_2OCH_2COO^-$ so that CM-cellulose is negatively charged, therefore it is a cation exchanger; for CM-cellulose the counter ion is usually Na^+ CM-cellulose begins to lose its charge below about pH 5, therefore starting pH range when using CM-cellulose is about pH 5 - 9.

In this case CM Sephadex was swollen in 10 mM sodium acetate (pH 5.5) overnight and washed three times with the same buffer and degassed before packing into the column of 25 cm length and 1.25 cm radius. Height of the bed was 15 cm. Flow rate was adjusted to 0.4 mL/min. After elution of unbound proteins the column was washed with three bed volume of elution buffer. Bound proteins were eluted with a linear NaCl gradient of 0-400 mM.

2.7.9 Substrate affinity chromatography using colloidal chitin.

2.7.9 i) Preparation of colloidal chitin.

Chitinase was purified by an affinity procedure employing chitin, which was prepared according to the method used for preparation chitin (for actenomycetes culture medium) of Hsu and Lockwood (1975) from powdered lobster shells. Forty gram crab shell was ground into paste in 400 mL of concentrated HCl for 50 min under fume hood. The chitin was precipitated as a colloidal suspension by adding it slowly to 2 liters of water on ice. The suspension was collected by filtration with suction on a coarse filter paper and then washed by suspending it in about 5 liters of tap water and refiltering. The washing was repeated at least three times or until the pH of the suspension was about 3.5. This colloidal chitin was dried in over at 100°C. The powder was resuspended in deionised water or required buffer autoclaved and stored at room temperature.

2.7.9 ii) Substrate affinity chromatography.

Twenty mL of total protein extract was mixed with 180 mL of 10 mM Na phosphate buffer (pH 7.0) and the mixture was passed through colloidal chitin (15.0 cm height and 1.25 cm radius) previously equilibrated with the same buffer; unbound proteins were discarded and the adsorbed proteins were eluted with 10 mM acetic acid. The eluted protein fraction was dialysed against10 mM sodium phosphate buffer (pH 7.0) and concentrated using Vivaspin sample concentrator tubes. The samples were analysed using SDS-PAGE as explained in section 2.3 of materials and methods.

2.8 Identification of the purified protein.

Sample preparation for *de novo* sequencing by MS (In-gel trypsin digestion of the protein band).

Separation of purified protein on 12% SDS-PAGE was carried out as explained under section 2.3 of materials and methods. The gel was stained with Coomassie Brilliant Blue solution as described under section 2.3.3.1 and destaining as explained under section 2.3.4 of materials and methods.

2.8.1 Washing and complete destaining of the excised band.

The target bands were excised from Coomassie-stained gel using a scalpel. Excised bands were put in micro centrifuge tubes and washed two times for 15 min with double volume of deionized water/acetonitrile 1:1 (v/v). Washing solution was discarded and bands were incubated for 5 min in one volume acetonitrile at RT. Acetonitrile solution was replaced by one volume of 100 mM NH_4 HCO_3 and incubated for 5 min at RT. After addition of one volume acetonitrile to the same tube, they were incubated again for 15 min at RT with shaking. The solution was decanted and the gel pieces were dried by SpeedVac. The gel pieces shrink and form a ball when completely dry.

The dried gel pieces were gradually quenched by adding 10 µL (10 ng/µL in 50 mM ammonium bicarbonate) sequencing grade trypsin (Serva Electrophoresis GmbH) and incubated in ice for 10 min. Quenching step was repeated several times until saturation of gel pieces with trypsin solution. The excess of trypsin solution was discarded and gel pieces were soaked in excess of 25 mM ammonium bicarbonate solution. Samples were incubated overnight at 37°C.

2.8.2 Recovery of trypsin digested peptide fragments from the gel.

After overnight digestion, samples were shortly centrifuged and sonicated for 2 min; supernatant was collected in a new micro centrifuge tube. Gel pieces were incubated in one volume of 25 mM ammonium bicarbonate with shaking for 20 min. To this one volume acetonitrile was added and incubated for 15 min under shaking. The mixture was sonicated for 2 min; the supernatant was collected in the same reaction tube. Gel pieces were incubated two times for 15 min in 5% (v/v) formic acid/ acetonitrile (1:1) and supernatant was collected in the same tube, final sample volume was reduced to approximately 10 µl by SpeedVac and stored at -20°C.

2.8.3 *De novo* sequencing of peptide fragments by ESI Q-TOF.

De novo sequencing was done in positive ion mode on a quadrapole time-of-flight hybrid mass spectrometer (Q-TOF) Micromass, Waters, Manchester, United Kingdom). Amino acid sequences were identified by homology search by using the program Peptide Search (EMBL, Heidelberg, Germany).

2.8.4 In-gel assay to detect pearl millet chitinase activity.

Chitinase activity was determined by in-gel assays. For this, separating gel was prepared with 1% glycol chitin which is the substrate for chitinase. 3 % Stacking gel was poured on top of it. Thirty µg of total soluble protein fraction and 10 µg of the 24 kDa purified protein was mixed well with loading buffer by vortexing and loaded onto the gel and electrophoretically separated on 12% polyacrylamide gel under denaturing conditions. Remaining steps for PAGE were followed as mentioned under section 2.3 of materials and methods. The proteins in the gel were renatured after electrophoresis by incubating in renaturation buffer at 37°C overnight (Potvin *et al.*, 1998, Audy *et al.*, 1989) with gentle shaking.

Renaturation buffer.

HEPES free acid	5.96 g
pH 7.0 adjusted with	3 M KOH
Triton X-100	5.00 mL
Final volume	500.00 mL

The gel was washed twice with 100 mM Tris HCl and stained with 100 mL of 500 mM Tris-HCl, pH 9.0 containing 3-4 grains of fluorescent brightener (Fluorescent brilliant 28, Sigma # F343) for 5 min. The gel was rinsed twice with deionised water and observed under UV

illuminator. Glycol chitin, (substrate for chitinase) in the gel hydrolyzed by chitinase band appeared as dark blue (slightly diffused) band against pale blue background of the gel.

2.9 FPLC (Fast Protein Liquid Chromatography).

This chromatographic method involves separation of proteins by the principle of size exclusion. Here gel matrix with specific pore size serves as stationary phase; the small protein molecules can enter the gel beads and are eluted slowly from the column. The larger protein molecules cannot enter the gel beads because of their bigger size and hence pass through the space between gel particles and therefore move quickly through the column. Retention time of the protein molecule therefore is determined by its size; from retention time of the protein molecule, size can be estimated. Gel filtration chromatography (GFC) involves the use of aqueous mobile phase, whereas gel permeation chromatography (GPC) involves a non aqueous mobile phase (example: organic solvents).

In the present study Superose-12 column (Pharmacia, particle size 10 ± 2 μm) was used. The standard protein solutions used were of 1 mg/mL concentration; volume of the standards loaded to the column was 500 μL. Flow rate was adjusted to 0.5 mL/min. Different protein standards used to calibrate the FPLC column with their corresponding molecular weights and retention time are listed in the table 3.

Table 3. Standards used to calibrate Superose-12 FPLC column; proteins were detected using a UV detector attached to FLC at 280 nm. *Elution volume represented in mL.

Serial No.	Standard	Size in kDa	Retention time	Elution volume *	Retention factor
1	Bluedextran	2000.0	17 min	8.5	1.00
2	Ferritin	400.0	21 min	10.5	0.81
3	Catalase	240.0	23 min	11.5	0.74
4	BSA	66.0	25 min	12.5	0.68
5	Ovalbumin	45.0	27 min	13.5	0.63
6	Carboanhydrase	30.0	31 min	15.5	0.55
7	Myoglobin	17.0	32 min	16.0	0.53
8	Cytochrome C	12.4	29 min	14.5	0.59

Bluedextran is used to determine the dead volume of the column. (Dead volume of the column is taken as the retention volume of a solute of very large molecular weight, having a molecular size much greater than the pore size of the packing so that it is completely excluded from the pores; and which is not retained by the stationary phase).

Part D - Molecular biology methods.

2.10 RNA Isolation and quantification.

2.10.1 Isolation of the total RNA from pearl millet seedlings.

For isolation of total RNA from pearl millet seedlings approximately 0.3 g of frozen leaves was crushed well in liquid nitrogen in a pre cooled mortar and pestle. The powder obtained was mixed with 750 µL of pre warmed (50°C) lysis buffer and 750 µL of PCI solution and shaken for 20 min. The mixture was centrifuged for 20 min at 13000 x g (Eppendorf centrifuge rotor F45-24-11). The supernatant was collected and mixed well with 750 µL of PCI solution and again centrifuged for 20 min at 13000 x g. Supernatant was collected and mixed with 0.75% volume of 8 M Lithium chloride solution and stored overnight at 4°C to allow precipitation. The mixture was centrifuged for 20 min at 13000 x g (Eppendorf centrifuge rotor F45-24-11); After removal of the supernatant, pellet was resuspended in 300 µL deionised DEPC water, 50 µL 3M sodium acetate (pH 5.2) and 500 µL of cold 96% ethanol (-20°C), and stored for 1 h at –20 °C. After centrifugation for 20 min at 13000 x g (Eppendorf centrifuge rotor F45-24-11), the pellet was washed with 500 µL of cold 70% ethanol (-20°C) and centrifuged again for 20 min. Supernatant was carefully removed and pellet was dried by SpeedVac. Eventually the pellet was dissolved in 1 mL deionised DEPC water, divided into 50 µL aliquots and stored at –80°C until further use. All the centrifugation steps are carried out at 4°C and care is taken to avoid RNase contamination and degradation of extracted RNA. All the tips and micro centrifuge tubes used were washed with DEPC water and autoclaved.

Composition of lysis buffer, PCI solution, 8 M Lithium chloride solution, 10 x MOPS and preparation of DEPC water are given below.

Lysis buffer.

NaCl	600 mM
EDTA	20 mM
SDS	4% (v/w)
Tris-HCl (pH 8.0)	100 mM

Lithium Chloride (8M).

Eight molar lithium chloride solution was prepared by dissolving 33.912 g lithium chloride in 100 mL deionised water. The solution was sterilized by autoclaving and stored at 4°C.

PCI (25:24).

Phenol	25 mL
Chloroform	24 mL

PCl solution was saturated with autoclaved 100 mM sodium acetate (pH 4.5) and stored at 4°C in dark.

MOPS (3-Morpholino-1-propansulphonic acid) 10 x.

MOPS	200 mM
Na acetate	50 mM
EDTA	100 mM

The pH is adjusted to 7.0, and the solution is sterilized by autoclaving storage at 4°C.

Working solution.

10 x MOPS solution was diluted to 1 x with deionised water and used for running RNA gel electrophoresis.

DEPC water.

0.1% DEPC was added to deionised water and shaken overnight at 37°C and sterilized by autoclaving. DEPC decomposes into carbon dioxide and ethanol.

2.10.2 Determination of quantity and purity of RNA.

Isolated RNA was diluted 1:100 and the absorption was measured at 260 nm and 280 nm. RNA concentration was determined by the following formula.

$$\frac{E260 \times 40 \times \text{dilution factor}}{1000} = \mu g \text{ RNA} / \mu L$$

The quotients E260/E280 and E260/E230 give information about contamination with proteins and polysaccharides respectively. A quotient between 1.8 and 2 shows sufficient purity.

2.10.3 Electrophoresis of RNA.

2.10.3 i) Preparation of agarose gel.

One gram molecular biology grade agarose was melted in 72 mL deionised water and then cooled to 60°C. To this melted agarose 10 mL of 10 x MOPS buffer and 18 mL 37% formaldehyde was added under the fume hood; gel was poured to the cassette under the fume hood and allowed to solidify (Bio-Rad Midi gel - 10 x 10 cm or maxi gel - 15 x 10 cm or 15 x 15 cm depending on the number of samples). Percentage of agarose was varied according to the requirement. Composition of 1% agarose gel for electrophoresis of RNA is given below.

Agarose gel for electrophoresis of RNA.

Molecular biology grade Agarose	1 g
Deionised water	72 mL
10X MOPS buffer	10 mL
37% formaldehyde (12.3 M)*	18 mL
Final quantity of the gel	100 mL

*added after cooling the gel to 60°C.

2.10.3 ii) RNA Sample preparation and electrophoresis.

Two micro liters of RNA sample, was taken and mixed with 18 µL Northern-Mix. The mixture was mixed by vortexing and incubated at 65°C for 15 min. Samples were chilled on ice, centrifuged and loaded to the gel.

Northern Mix.

Solution 1.

Formaldehyde	6.5%
Formamide	50% in 1x MOPS (pH 7.0)

Solution 2.

Bromophenol blue	0.25%,
Xylene Cyanol	0.25%
EDTA (pH 8.0)	1 mM in 50% Glycerol

Northern Mix was prepared by mixing 5 mL Solution 1 + 1 mL Solution 2. To this mixture 40 µL Ethidium bromide (5 mg/mL) was added and stored at -20°C.

Samples were electrophoretically separated at 75 V for midi gel and 125 V for maxi gel for 30 min, 1x MOPS with 0.01% Ethidium bromide served as running buffer. RNA was visualized under UV light.

2.11 DNA Isolation and quantification.

2.11.1 Isolation of genomic DNA from pearl millet seedlings.

Around 2 g of leaf materials from pearl millet were harvested and ground in a pre cooled mortar with liquid nitrogen. The fine powder was transferred equally into two 50 mL tubes. Preheated 1.5 mL CTAB ($C_{16}H_{33}$)N(CH_3)$_3$Br, cetyltrimethylammonium bromide) buffer were added to each tube mixed and incubated for 30 min at 65°C. To this 1.5 mL of PCI mix was added and gently mixed to avoid genomic DNA breaking. Samples were centrifuged for 10 min at 10000 x g (Sorvall centrifuge GS3 rotor). After centrifugation, the upper aqueous phase containing DNA was transferred into a new tube and the lower organic phase was discarded; this step was repeated twice obtain a clear separation of aqueous layer. To this aqueous fraction, 2/3 volume of a precooled (-20°C) isopropanol was added and gently mixed. The samples were incubated overnight at 4°C to precipitate DNA (or for 60-90 min at -80°C). Then the samples were centrifuged for 10 min at 13000 x g at 4°C. Supernatant was discarded and pellet was washed by 1 mL washing buffer. Afterwards the washing buffer was carefully decanted and the pellet was resuspended in 500 µL 10 mM Tris HCl buffer (pH 8.0) supplemented with DNase free RNase A (final concentration 10 µg/mL). After incubation for 30 min at 37°C, 500 µL of 7.5 M ammonium acetate and 1.5 mL 96% alcohol were added and gently mixed. The samples were centrifuged for 10 min at 13000 x g (Sorvall centrifuge GS3 rotor) and subsequently the

supernatant was completely removed; pellet was resuspended in a suitable volume of sterile deionised water. DNA sample is divided into smaller aliquots of 100 µL and stored at -20°C.

CTAB Buffer.

CTAB*	3.0% (w/v)
NaCl	1400 mM
ß-Mercaptoethanol*	0.2% (v/v)
EDTA	20 mM
Tris-HCl (pH 8.0)	100 mM
PVP- 40 (Poly vinyl pirrolidone)	1.0% (w/v)

* CTAB and ß-Mercapto-ethanol were added after autoclaving

Phenol chloroform iso amyl alcohol Mix (25:24:1).

Phenol	25 mL
Chloroform	24 mL
Isoamylalcohol	1 mL

Wash buffer (WB).

Ethanol	76%
Ammonium acetate	10 mM

Tris-HCl buffer for DNA storage.

Tris-HCl, (pH 8.0)	10 mM

EDTA is omitted as it interferes in PCR reaction.

2.11.2 Determination of quantity and purity of DNA.

Isolated genomic DNA was diluted 1:100 and the absorption was measured 260 nm and 280 nm. DNA concentration was determined by the following formula.

$$\frac{E260 \times 50 \times \text{dilution factor}}{1000} = \mu g \text{ DNA} / \mu L$$

The quotients E260/E280 and E260/E230 give information about contamination with proteins and polysaccharides respectively. A quotient between 1.8 and 2 shows sufficient purity.

2.11.3 Electrophoresis of DNA.

2.11.3 i) Preparation of agarose gel.

Agarose gel is prepared by melting molecular biology grade agarose in 1x TAE buffer and when the melted agarose was cooled, 0.01% EtBr was added to it and the gel is poured into the gel cassette and allowed to solidify.

TAE buffer (50 X) Stock solution.

Tris HCl	40 mM
Na acetate	10 mM
EDTA	1 mM

The pH was adjusted to 7.8 using acetic acid

Working solution: TAE buffer was diluted to 1 x concentration. To this 0.01% EtBr was added.

2.11.3 ii) Sample preparation and electrophoresis of DNA.

DNA sample was mixed with appropriate amount of 6 x DNA loading buffer on a parafilm strip and loaded onto agarose gel. DNA samples were electrophoretically separated at 75 V for midi gel and 125 V for maxi gel for 45 min, 1x TAE with 0.01% ethidium bromide served as running buffer. DNA was visualized under UV light.

6 x DNA loading buffer.

Tris-HCl (pH 7.6)	10 mM
Bromophenol blue*	0.03%
Xylene cyanol	0.03%
Glycerol	60%
EDTA	60 mM

*Orange G was used instead on Bromophenol blue and xylene cyanol and this loading dye was used for Electrophoretic separation of lower molecular weight PCR products.

2.12 Polymerase chain reaction (PCR).

2.12.1 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).

RT-PCR was performed by using peqGold M-MuLV Reverse Transcriptase RNase H- (Peqlab-Biotechnologie GmbH, Germany). cDNA synthesis was carried out in Thermocycler (PTC 200, Biozym, Oldendorf, Germany). Mix 1 and mix 2 were prepared as outlined below.

Mix 1

RNA	0.5 – 1.0 μg
Oligo (dT) 23 primer	1.0 μL
dNTP Mix (10 mM each)	1.0 μL
Deionised sterile water to	10.0 μL
Final volume	10 μL

This mix was incubated initially at 70 °C for 10 min and snap chilled on ice.

Mix 2

10x buffer for M-MuLV-RT	2 μL

RNase inhibitor (20u/µl)	1 µL
M-MuLV reverse transcriptase	1 µL
Deionised sterile water	6 µL

The first strand of cDNA was built by adding mix 1 to mix 2 and incubating the mixture at 50°C for 50 min in Thermocycler (PTC 200, Biozym, Oldendorf, Germany). After completion of cDNA synthesis, the reaction mixture was transferred onto ice.

2.12.2 PCR using genomic DNA as template.

PCR was carried out using pearl millet genomic DNA as template. Reaction mixture was prepared as per specifications for dream *Taq* polymerase (Fermentas #EP0701) which is mentioned below.

PCR with dream *Taq* polymerase.

10 x DreamTaq™ Buffer	5 µL
dNTP Mix (10 mM)	1 µL
Forward primer (100 pM)	1 µL
Reverse primer(100 pM)	1 µL
Template (genomic DNA)*	100 ng
DreamTaq™ DNA polymerase (5u/ µL)	0.2 µL
Deionised sterile water to	50 µL
Total volume	50 µL

*For RT PCR 500 ng of cDNA was used as template.

When PCR additives like DMSO/DMF, $MgCl_2$, and betaine were used, final volume was made up to 50 µL using sterile deionised water accordingly. Composition of 10 x Dream Taq buffer is given below.

Composition of 10X Dream Taq buffer.

Tris-HCl (pH 8.8)	750 mM
Ammonium sulphate $(NH_4)_2SO_4$	200 mM
Tween 20	0.1% (v/v)
$MgCl_2$	20 mM

2.12.3 PCR reaction conditions.

Thermocycler was programmed as outlined below.

1. Initial denaturation @ 98°C (when genomic DNA was used as template).
   Initial denaturation @ 95°C for 1 min (when cDNA was used as template) 1cycle.
2. Denaturation @ 95°C for 30 sec.
3. Annealing depending on the primers used.
4. Extension @72°C for 30 sec.
5. 30 cycles of step 2 – 4.
6. Final Extension @ 72°C for 3 min.

7. Maintained @ 4°C thereafter.

For temperature gradient PCR, annealing temperature gradient was set between 45-65°C. Stepdown PCR was carried out with initial 10 cycles at 65ºC annealing temperature, followed by 20 step down cycles with every cycle lower from the previous cycle by one degree Celsius annealing temperature.

Positive control was ~ 350 bp (*nonspecific lipid transfer protein- nsltp*) product amplified by the primers Remo forward primer 5′-GGGCTGGTCCTCGAATTCAC-3′ Remo reverse primer 5′-GTGTATGAGTAATGGATCG-3′ using 100 ng of genomic DNA from *Malus domestic* cv. Remo as template.

2.13 Purification of PCR products.

2.13.1 Purification of PCR products using E.Z.N.A. cycle pure kit.

PCR products were purified before cloning to remove any contaminants that might otherwise affect the sequencing reaction. E.Z.N.A. Cycle-Pure Kit (Peqlab Biotechnology, GmbH) was used to purify the PCR products. To the total volume of PCR products 4-5 volumes of CP buffer were added. Subsequently, 750 µL of the mixture was loaded to the DNA binding column fitted to a collecting tube and centrifuged at RT for 1 min at 16000 x g (Eppendorf centrifuge rotor F45-24-11). DNA-wash buffer (with 1.5 volumes of ethanol) 750 µL was added to the column and was centrifuged for 1 min at 16000 x g (Eppendorf centrifuge rotor F45-24-11). Washing step was repeated to remove traces of impurities. In all the centrifugation steps the solution collected in the collecting tubes was discarded. The column was dried by centrifuging for 1 min. The dried column was placed on a sterile micro centrifuge tube and 50 µL of sterilized deionized water was added directly on to the membrane of the column, incubated at RT for 2-3 min and centrifuged for 1 min at 16000 x g (Eppendorf centrifuge rotor F45-24-11) to elute DNA bound to the column.

2.13.2 Purification DNA fragments by phenol chloroform method.

The DNA fragments were purified by phenol chloroform method for southern blot analysis and genome walking experiments. For this 95 µL phenol 80% equilibrated to pH 8.0 was added and mixed by vortexing at low speed for 5-6 sec. Tube was spun briefly to separate the aqueous and organic phases. The aqueous phase was transferred into a fresh tube and the organic phase was discarded. To the same tube 95 µL of chloroform was added, tube was shortly vortexed and briefly spun. Again the upper layer was transferred into fresh tube. For DNA precipitation two volumes (190 µL) of ice cold 95% ethanol and 1/10 volume (9.5 µL) of 3 M sodium acetate buffer (pH 5.2) were added and vortexed at low speed for 5-6 sec. Centrifugation was done at 16000 x g (Eppendorf centrifuge rotor F45-24-11) for 10 min to precipitate DNA. Subsequently, supernatant was removed and pellet was washed with 100 µL of ice cold 70% ethanol and centrifuged for 5 min at 16000 x g (Eppendorf centrifuge rotor F45-24-11). The supernatant was decanted and pellet was air dried and dissolved in 20 µL deionised nuclease free water and stored at -20°C.

2.13.3 Purification of PCR products with QIAEX II Gel Extraction kit (Qiagen #20021).

The standard QIAEX II Gel Extraction protocol is used to extract DNA from 0.3–2% low-melt agarose gels in TAE buffer. QX1 in the QIAEX II Gel Extraction Kit is used for solubilization of agarose gel slices and binding of DNA to the QIAEX II silica particles. Buffer QX1 contains a pH indicator, allowing easy determination of the optimal pH for DNA binding. DNA adsorption requires a pH 7.5, and the pH indicator appears yellow in this range. If the pH is >7.5, the binding mixture turns orange or violet. This means that the pH of the sample exceeds the buffering capacity of Buffer QX1 and DNA adsorption will be inefficient. In this case, the pH of the binding mixture can easily be corrected by addition of a small volume of 3 M sodium acetate, pH 5.0, before proceeding with the protocol.

A typical agarose gel slice was solubilized by adding 3 volumes of Buffer QX1 to 1 volume of gel (e.g., 300 µL of Buffer QX1 was added to 100 mg gel slice) and incubated at 50°C for 10 minutes. The high concentration of a chaotropic salt in Buffer QX1 disrupts hydrogen bonding between sugars in the agarose polymer, allowing solubilization of the gel slice. In addition, the high salt concentration dissociates DNA binding proteins from the DNA fragments. DNA molecules bind to the QIAEX II particles during the adsorption step, and all non nucleic acid impurities such as agarose, proteins, ethidium bromide, and salts remain in the supernatant. A high salt wash with Buffer QX1 removes residual agarose, QIAEX II pellet containing bound DNA was washed twice with 400 µL ethanol-containing Buffer PE efficiently remove salt contaminants. All traces of supernatant were carefully removed at each step to eliminate impurities and reduce buffer carryover. Washed QIAEX II particles carrying adsorbed DNA were pelleted and air-dried at room temperature for 10–15 minutes. Drying the pellet is necessary to remove all traces of residual ethanol which may interfere with subsequent enzymatic reactions. The pellet was then resuspended in 20 µL deionised water by vortexing to elute the bound DNA and centrifuged at 10000 x g for 10 min at room temperature. The supernatant containing the purified DNA was collected in a fresh micro centrifuge tube and stored at -20°C. Elution step was repeated again with 10 µL of deionised water to elute traces of bound DNA. The purified DNA can also be eluted in TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

2.14 Preparation of competent *E. coli* cells and transformation.

2.14.1 Preparation of electro competent *E. coli* cells.

For the preparation of ultra competent cells for heat shock transformation glycerin stock culture of required *Escherichia coli* strain (for example, XL-Blue1, NM522 or DH5α) was cultured overnight in 5 mL LB medium at 37°C. Afterwards 250 mL of LB medium was inoculated with 500 µL of this overnight culture. The culture flask is incubated at 18 °C on shaker at 155 rpm. The OD of the culture was measured at every 1 hour interval, and when the OD_{600} reaches 0.55-0.6, culture flask was transferred to an ice bath and incubated for 10 minutes. Here onwards all the steps were carried out at 4ºC and the temperature was maintained by keeping the cell suspension of ice throughout the preparation process. Then the suspension was centrifuged for 10 min at 4000 x g (Sorvall GSA rotor) at 4ºC in autoclaved centrifugation bottles of 500 mL

capacity. The supernatant was discarded and the remaining supernatant was drained by inverting the centrifuge tube on tissue paper. After centrifugation for 10 min at 4000 x g, the pellet was resuspended in 5 mL distilled water, and subsequently with 250 ml with distilled water. Centrifugation was done again for 20 min at 4000 x g, and the pellet was resuspended in 250 mL distilled water (this step was repeated once again). Pellet was resuspended in 25 mL 15% glycerol and centrifuged for 30 min at 4000 x g. Finally the pellet was resuspended in 1.5 volume of 15% glycerol, mixed gently and divided into 100 µL aliquots; snap frozen by dipping in liquid nitrogen and stored at – 70°C.

2.14.2 Electro transformation of *E. coli* cells.

For transformation of *E. coli* cells by electroporation, 100 µL of electro competent cells were thawed on ice and mixed with 3 µL ligate in a precooled electroporation cuvette. Electroporation was done in BTX cell at 1.25 kV. After electroporation, 900 µL LB medium without antibiotic was added to cell suspension and incubated for 90 min; eventually, bacterial cells were cultivated on LBA plates containing appropriate (100 µg/mL Amp) antibiotic for selection. For blue-white selection LBA plates containing ampicillin, IPTG (0.100 µM) and X-gal (20-40 µg/mL) were used.

2.14.3 Preparation of heat shock ultra-competent cells.

For the preparation of ultra competent cells for heat shock transformation glycerin stock culture of required *Escherichia coli* strain (for example, XL-Blue1, NM522 or DH5α) was cultured overnight in 5 mL LB medium at 37°C. Next morning 250 mL of LB medium was inoculated with 500µL of this overnight culture. The culture flask is incubated at 18 °C on shaker at 155 rpm. The OD of the culture was measured at every 1 hour interval, and when the OD_{600} reaches 0.55-0.6, culture flask was transferred to an ice bath and incubated for 10 minutes. Here onwards all the steps were carried out at 4ºC and the temperature was maintained by keeping the cell suspension of ice throughout the preparation process. Then the suspension was centrifuged for 10 min at 4000 x g (Sorvall GSA rotor) at 4ºC in autoclaved centrifugation bottles of 500 mL capacity. The supernatant was discarded and the remaining supernatant was drained by inverting the centrifuge tube on tissue paper. The pellet was resuspended in 19.2 mL ice cold Inoue transformation buffer and incubated for 10 min on ice. The suspension was centrifuged for 10 min at 4000 x g at 4ºC. The supernatant was discarded and pellet was resuspended in 2.4 mL filter sterilized, ice cold Inoue buffer. This suspension contains ultra competent cells which can be transformed by heat shock. To this cell suspension, 84 µL DMSO was carefully pipetted in the middle, mixed by gently tilting and swirling. As DMSO is cytotoxic aliquoting was done quickly. The ultra competent cells were divided into small aliquots of 100 µL each and pipetted out into ice cold micro centrifuge tubes with ice-cold tips. The aliquots were snap-frozen by dipping in liquid nitrogen and stored at – 70ºC. Composition of PIPES and Inoe buffer are given below.

PIPES (piperazine-1,2-bis[2-ethanesulfonic acid])

PIPES	15.10 g
Deionised water	80.00 mL

pH was adjusted to 6.7 using 5 M KOH

Volume was made up to 100 mL using deionised water.
PIPES solution was filter sterilized using disposable pre-rinsed 0.45-µm Nalgene filter and stored at -20°C in aliquots.

Inoue transformation buffer

Reagent	**Amount per liter**	**Final concentration**
$MnCl_2.4H_2O$	10.88 g	55 mM
$CaCl_2.2H_2O$	2.20 g	15 mM
Potassium chloride	18.65 g	250 mM
PIPES (0.5 M, pH 6.7)	10.00 mL	10 mM
Final volume	1 liter	

The solution was filter sterilized using pre rinsed 0.45-µm Nalgene filter and stored at -20°C.

2.14.4 Heat shock transformation of *E. coli* cells.

The ultra competent cells were thawed on ice bath till they are crunchy and not liquid. When they turn just crunchy 10 ng of plasmid or 2 µL of ligation mixture was added, mixed and incubated on ice for 10 min. For heat shock transformation the competent cells with ligate were incubated on a water bath at exactly 42ºC for 45-55°C (accurate temperature and time is important). The cells were removed from water bath after incubation and put back into ice bath and to the same micro centrifuge tube 950 µL of SOC medium maintained at RT was added. The cells were allowed to multiply at 37ºC for 60-90 min on a shaker. The lid of micro centrifugation tube was punctured with to facilitate aeration. Once the transformants had recovered from heat shock and multiplied on antibiotic free broth, they were (50 µL, 100 µL, and the rest of the cells) were plated out on separate plates with selective medium; incubated at 37ºC overnight.

Composition of SOC medium

Bacto-tryptone	(2% w/v)	20 g
Bacto-yeast extract	(0.5% w/v)	5 g
NaCl (10 mM)		0.584 g
KCl (2.5 mM)		0.186 g
$MgCl_2$ (10 mM)		0.952 g
Glucose (20 mM)		3.603 g
pH was adjusted to 7.0 with		10 M NaOH

Final volume was made up to 1000 mL using deionised water; Broth was sterilized by autoclaving, 20 mL of filter sterilized (pre-rinsed Nalgene filter, 0.45 µm pore size) 1 M glucose was added to the broth before use.

Preparation of LB Agar-Amp^{100} plates with X-gal, IPTG.

Thirty grams of Luria Broth Agar was suspended in 900 mL of deionised water and the pH was adjusted to 7.2, final volume was made up to 1L and the medium was sterilized by autoclaving. The medium was cooled to 50°C; 1 mL of Amp stock solution (100 mg/mL), 1 mL of X-gal stock solution (20 mg/mL) and 1 ml of IPTG stock solution (0.1 M) were added, mixed thoroughly; poured onto petriplates and allowed to solidify. The LB agar plates were stored at 4°C for not more than 7 days. These plates were used to screen the colonies by blue white selection. Only Amp^{100} plates were used to screen the Amp resistant transformants.

X-gal stock solution (20 mg/mL)

X-gal stock solution was prepared by dissolving 200 mg 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (Sigma # B4252) in 10 mL molecular biology grade dimethyl Formamide (DMF), the solution was filter sterilized using pre rinsed 0.45-µm Nalgene filter and stored at -20°C.

IPTG Stock solution (0.1 M)

IPTG stock solution was prepared by dissolving 0.238 g Isopropyl beta-D-thiogalactopyranoside (Sigma # I5502) in 10 mL of deionised water (formula weight is 238.3g/mol), The solution was filter sterilized using pre rinsed 0.45-µm Nalgene filter and stored at -20°C.

Ampicillin Stock solution

Ampicillin stock solution was prepared by dissolving 100 mg of Ampicillin (sodium salt) in 1mL of deionised water. The solution was filter sterilized using pre rinsed 0.45-µm Nalgene filter and stored at -20°C.

2.14 Cloning of PCR products into cloning vector.

2.14.1 TA Cloning.

Inserts generated by PCR using *Taq* polymerase have poly A overhangs The PCR fragments were purified by gel extraction and immediately cloned into pGEM-T Easy vector from Promega (# A3600) that facilitates TA cloning. Three µl of the purified PCR product was mixed with 1 µL pGEMT Easy vector and 1 µl of T4-DNA ligase in presence of 10 µL of 2 X ligation buffer. The ligation assay was incubated for 30 min at room temperature. From this ligation 3 µL were used for transformation. To calculate the appropriate amount of PCR product (insert) to include in the ligation reaction, the following equation was used.

(ng of vector X kb size of insert / kb size of vector) X (3:1)* = ng of insert to be added
*3:1 being the Insert : Vector ratio

2.14.2 Blunt end cloning.

Amplicons generated by PCR using enzymes with proof reading activity like *Pfu*, KAPA HiFi polymerase (but not *Taq* polymerase) do not have overhangs and are thus blunt. To clone these blunt end PCR products generated by KAPA HiFi polymerase from Peqlab (KK 2101, CloneJet blunt end cloning vector from Fermentas (# K1231) was used. The vector was mixed with purified PCR product at 1:3 ratio. One μL T4 ligase, Ligation buffer at a final concentration of 1 X, 0.5 mM ATP and 1 mM DTT, 3% PEG 4000 were added to the reaction tube. The reaction volume was adjusted to 20 μl by sterile deionised water. Reaction tubes were incubated overnight at 16°C.

2.15 Screening the transformed colonies.

2.15.1 Blue white selection.

The blue-white screen is a molecular technique that allows for the detection of successful ligations in vector-based gene cloning. DNA of interest is ligated into a vector. The vector was then used to transform competent *E. coli* cell. The transformants cells were grown in the presence of X-gal and IPTG. If the ligation was successful, the bacterial colony was white; otherwise the colony was blue. This technique allows quick and easy detection of successful ligation. Several white and as well as blue colonies were picked and sub cultured overnight in LB broth in the presence of 100 μg/mL ampicillin. Afterwards, plasmid was isolated from these cultures by HB-lysis or using GeneJET plasmid Miniprep kit.

2.15.2 Antibiotic selection.

Bacterial transformation is the process by which bacterial cells take up naked DNA molecules. If the foreign DNA has an origin of replication recognized by the host cell DNA polymerases, the bacteria will replicate the foreign DNA along with their own DNA. When transformation is coupled with antibiotic selection techniques, bacteria can be induced to uptake certain DNA molecules, and those bacteria can be selected for that incorporation. Plasmids used for the cloning and manipulation of DNA have been engineered to harbor the genes for antibiotic resistance. Here the bacterial transformation was plated onto media containing ampicillin, only bacteria which possess the plasmid DNA and thus have the ability to metabolize ampicillin and form colonies were grown on the ampicillin agar plates. In this way, bacterial cells containing plasmid DNA were selected.

2.16 Isolation of plasmid DNA from *E coli* cells

2.16.1 Isolation of plasmid DNA by HB-lysis

For isolation of the plasmid, 1.5 mL of overnight culture was centrifuged for 2 min at 16000 x g (Eppendorf centrifuge rotor F45-24-11). Pellet was resuspended in 300 μL buffer 1, and another 300 μL buffer 2 was added. The mixture was incubated for 5 min at room temperature. After mixing with an additional 300 μL buffer 3, mixture was incubated for 10 min

in ice. Afterwards mixture was centrifuged for 10 min at 16000 x g (Eppendorf centrifuge rotor F45-24-11) at RT. The supernatant was transferred into a fresh micro centrifuge tube and recentrifuged for 10 min at 16000 x g (Eppendorf centrifuge rotor F45-24-11). Supernatant was pipetted out and mixed with 800 µL were mixed with ice cold 700 µL 2-propanol and centrifuged for 30 min at 16000 x g at 4°C. Pellet was incubated with 500 µL cold 70% ethanol for 2 min subsequently centrifugation was done for 10 min at 16000 x g (Eppendorf centrifuge rotor F45-24-11) at RT. After air drying the pellet it was resuspended in 50 µL deionised water. Plasmid DNA was dissolved at 50°C on a heating block and stored at – 20°C. Composition of buffer 1, 2 and 3 are given below.

Buffer 1:

Tris-HCl (pH 8.0)	50 mM
EDTA	10 mM
RNase A*	40 µg/ml

Buffer 2:

Sodium hydroxide (NaOH)	200 mM
SDS	1%

Buffer 3:

Potassium acetate	2.55 M

The pH was adjusted to 4.8 using acetic acid.

* All the three buffers were autoclaved. RNase was added after autoclaving for buffer 1 and stored at 4°C. Buffer 2 and 3 were stored at RT.

RNase A Stock solution:

10 mg/ml of DNase free RNase A dissolved in deionised water and stored at -20°C.

2.16.2 Plasmid preparation using GeneJET Plasmid Miniprep Kit (Fermentas # K0502).

The GeneJET Plasmid Miniprep Kit (Fermentas # K0502) is a simple, rapid and cost-effective system for the isolation of high quality plasmid DNA from recombinant *E. coli* cultures. The kit utilizes an exclusive silica-based membrane technology in the form of a convenient spin column.

Principle: A bacterial culture is harvested and lysed. The lysate is then cleared by centrifugation and applied on the silica column to selectively bind DNA molecules at a high salt concentration. The adsorbed DNA is washed to remove contaminants, and the pure plasmid DNA is eluted in a small volume of elution buffer or water.

Steps involved

Bacterial culture was centrifuged in a micro centrifuge tube at 4000 x g (Eppendorf centrifuge rotor F45-24-11) and then resuspend in 250 µL of the resuspension solution. to this 250 µL of the Lysis Solution was added and mix thoroughly by inverting the tube 4-6 times

until the solution becomes viscous and slightly clear (not by vortexing as it would shear chromosomal DNA and incubated for not more than 5 min (to avoid denaturation of super coiled plasmid DNA). To the same mixture 350 µL of the neutralization solution was added and mixed thoroughly by inverting the tube 4-6 times. The neutralized bacterial lysate is cloudy and viscous; it was centrifuged for 5 min to pellet cell debris and chromosomal DNA. The supernatant was transferred to the GeneJET spin column by decanting (care was taken to avoid transferring the white precipitate). The spin column was inserted into a collection tube and centrifuged for 1 min. The flow-through was discarded and column was placed back into the same collection tube. Spin column was washed with 500 µL of the wash solution (diluted with ethanol prior to first use as) and centrifuged for 30-60 sec. The flow-through was discarded and spin column was washed again with the same amount of wash solution. The flow-through was discarded and spin column was dried by centrifugation for an additional 1 min to remove residual wash solution. This step is essential to avoid residual ethanol in plasmid preps. GeneJET™ spin column was transferred into a fresh 1.5 mL micro centrifuge tube. Twenty five µL of the elution buffer was added to the center of GeneJET™ spin column membrane to elute the plasmid DNA (care was taken not to contact the membrane with the pipette tip). Spin column was incubated for 2 min at room temperature and centrifuged for 2 min. Elution was repeated with another 20 µl of elution buffer. The eluted plasmid DNA in the micro centrifuge tube was stored at -20°C. The purified DNA is ready for immediate use in all molecular biology procedures such as fast and conventional digestion with restriction enzymes, transformation and automated sequencing.

2.17 Sequencing.

Plasmids containing inserts were digested by using restriction enzymes from multiple cloning site (MCS) of the vector. After selection of the right plasmid, the plasmid preparation was sent to a company for sequencing (Sequence Lab, Göttingen, Germany).

2.18 Northern blot analysis.

The northern blot is a technique used in molecular biology research to study gene expression by detection of isolated mRNA in a sample. With northern blotting it is possible to observe cellular control over structure and function by determining the particular gene expression levels during differentiation, morphogenesis, as well as abnormal or diseased conditions. Northern blotting involves the use of electrophoresis to separate RNA samples by size and detection with a hybridization probe complementary to part of or the entire target sequence. The term 'northern blot' actually refers specifically to the capillary transfer of RNA from the electrophoresis gel to the blotting membrane; however the entire process is commonly referred to as northern blotting. The northern blot technique was developed in 1977 by James Alwine and coworkers at Stanford University.

2.18.1 Capillary blotting of RNA from the gel onto nylon membrane.

Capillary blotting technique was used to transfer RNA from agarose gels onto nylon membrane. For this, agarose gel was equilibrated with 2 x SSC for 5 min. Capillary blotting was carried out using a blotting apparatus containing 10 x SSC and a big Whatman sheet with both

ends of it dipping in 10 x SSC buffer. The gel was placed on three layers of Whatman sheets, exactly cut to the size of the gel and soaked in 2 X SSC, The membrane was laid on top of the agarose gel, followed by three thick Whatman sheets soaked with 2 x SSC and a stack of filter tissues. The Whatman sheets below the gel and on the membrane were exactly the same size of the gel. Weight was put on top of the blotting construction to ensure even capillary force on the gel which would allow uniform and complete transfer of RNA onto the membrane; transfer was carried out overnight. The following day transferred RNA was fixed onto the membrane by baking the blot in a hot air over at 80°C for 60 min. Blots were stored in dark at room temperature for later use in a clean sealed bag. Composition of all the buffers used is given below.

20 x SSC

Na citrate (pH 7.0)	300 mM
NaCl	3000 mM

50 x Denhardt's solution

PVP 10	1% (w/v)
BSA (filter sterilized)	1% (w/v)
Ficoll 400	1% (w/v)

20 x SSPE

NaCl	3600 mM
Na dihydrogen phosphate (NaH_2PO_4)	200 mM
EDTA (pH 7.4)	20 mM

Prehybridization solution (100 mL)

50 x Denhardt's solution	10 mL
20 x SSPE	25 mL
10% SDS	2 mL (w/v)
Salmon sperm DNA (10 mg/mL)	2 mL

TE Buffer

Tris-HCl (pH 7.5)	10 mM
EDTA	1 mM

TNE buffer

Tris-HCl (pH 7.5)	10 mM
NaCl	100 mM
EDTA	1 mM

Hybridization solution

One hundred µL radioactive ^{32}P labeled DNA probe was mixed with 5 mL prehybridization solution, incubated in boiling water bath for 10 min and cooled on ice. This hybridization was used for overnight hybridization of the blots.

Wash solution (to wash the blot after hybridization)

2 x SSC containing 0.1% SDS preheated to 60 °C was used to wash the blots; finally the blots were rinsed with 2 x SSC.

2.18.2 Prehybridization.

The blots were incubated in prehybridization solution for 2 hrs in a rotating oven at 60 °C.

2.18.3 Radioactive labeling of probe.

The preparation of radioactively labeled probes for hybridization of Southern blots was performed using the Prime-It II Random Primer Labeling Kit (Stratagene). 10 µl of random oligonucleotide primer was added to probe DNA solution containing 25-30 ng of probe DNA in 24 µl. The reaction was heated for 5 min at 95°C and placed back on ice for 5 min. This allows annealing of random primer to the probe DNA. To this primer annealed probe 10 µL of 5 x dCTP primer buffer, 5 µL of α-32P labeled dCTP, and 1 µL of 5 U/µL Exo(-)Klenow enzyme were added, mixed thoroughly, and incubated for 20 min at 37°C. The reaction is stopped by adding 2 µL of stop solution. The reaction volume was made up to 100 µL using TNE buffer. The micro centrifuge tube was given a short spin to bring its contents to the bottom of the tube.

2.18.4 Purification of the labeled probe.

Radiolabeled probe was purified to remove free ^{32}P before use with 1 mL Sephadex G-50 spin columns with a filter in the bottom (35 µm for pore size) equilibrated with TE (Tris HCl 100 mM, 10 mM EDTA, pH 8.0) buffer. The column was centrifuged at 1500 x g for 5 min in 2 ml micro centrifuge tube and the flow through containing free ^{32}P was discarded. The column was washed with 100 µL TNE buffer (composition given below) to elute the radiolabeled probe and centrifuged at 1500 x g for 5 min. Before hybridization, the probe was denatured by boiling in hot water bath for 5 min at 95°C; and it was used immediately for hybridization.

2.18.5 Hybridization of RNA.

The blotted membrane containing fixed RNA molecules were hybridized by incubating the membrane in hybridization solution containing the probe overnight in a rotating oven. During this process, the labeled probes complementary bind to any complementary nucleotide sequence on the gel.

2.18.6 Washing of blots.

Blots were washed after hybridization to remove excess of adhering radiolabeled probe. This step was carried out at 60°C in a rotating oven. The blots were incubated in preheated (to 60°C) 2 x SSC containing 0.1% SDS and finally rinsed with 2 x SSC.

2.18.7 Visualization of radioactive hybridization.

The blots were sealed between thin transparent plastic sheets and kept facing the autoradiography film overnight and scanned using phosphor image (Raytest autoradiography equipment) the next day.

2.18.8 Stripping.

To remove bound probes from the membrane, blots were washed three times in 0.4 M NaOH for 30 min at 45°C. The membrane was neutralized two times for 15 min at 45°C with 0.1x SSC containing 0.1% SDS and hybridized with another probe. Finally the membrane was rinsed with 1x SSC buffer. The blot is used for radioactive labeling with another probe of interest. The same blot can be stripped and reused 2-3 times.

2.19 Southern blot analysis

Southern blot is a method routinely used in molecular biology to determine the number of copies of a gene in the genome. Southern blotting combines transfer of electrophoretically separated DNA fragments to a filter membrane and subsequent fragment detection by hybridization with labeled probe. The method is named after its inventor, the British biologist Edwin Southern.

2.19.1 Restriction digestion of genomic DNA.

Approximately 10 µg pearl millet genomic DNA extracted by CTAB method was digested with restriction enzymes in Final volume 100 µL for 2 h at 37°C. Afterwards digestion assay was vortexed at low speed, centrifuge for few seconds and incubated overnight at 37°C. Restriction enzyme was inactivated by incubating at 65°C for 15 min.

2.19.2 Purification of restriction digested DNA.

Restriction digested DNA was purified by phenol chloroform precipitation as explained in section 2.13.2 of materials and methods.

2.19.3 Electrophoresis of restriction digested DNA.

Ten µL restriction digested DNA was run on 0.8% agarose gel overnight at 40V, 15 mA current, 1 kb plus ladder was used as reference.

2.19.4 Preparation of the gel for blotting.

Electrophoretically separated DNA was depurinated by incubating in 0.25 M HCl; HCl provokes partial depurination of DNA which in turn leads to strand cleavage, this allows better transfer of large DNA restriction fragments. The DNA was then denatured by washing the agarose gel two times for 20 min with denaturation solution with gentle agitation. The gel was washed with distilled water thrice for 5 min and neutralized by washing two times for 20 min

with the neutralization solution and finally washed thrice for 5 min with distilled water. Composition of denaturation buffer and neutralization buffer are given below.

0.25 M HCl for depurination

HCl (12.1 M)	10.30 mL
Deionised water	489.70 mL
Final volume	500.00 mL

Denaturation buffer

NaCl	1500 mM
NaOH	500 mM

Neutralization buffer

Tris-HCl (pH 8.0)	1000 mM
NaCl	2000 mM

2.19.5 Capillary blotting of DNA from the gel onto the nylon membrane.

Capillary blotting technique was used to transfer DNA from agarose gels to the nylon membrane. For this agarose gel was soaked for 20 min in denaturing buffer after finishing electrophoresis of restriction digested DNA fragments. The gel was then washed thrice with deionised water with gentle shaking and neutralized with neutralization buffer, by incubating in it for 20 min with gentle shaking. The gel was then washed thrice with deionised water to remove the traces of neutralization solution. Then the gel was equilibrated with 2 x SSC for 5 min. Capillary blotting was carried out as explained under section 2.18.1 of materials and methods. The following day transferred DNA was fixed onto the membrane by baking it in a hot air over at 80°C for 60 min. Blots were stored in dark at room temperature for later use in a clean sealed bag.

2.19.6 Prehybridization.

Prehybridization was carried out as mentioned in section 2.18.2 of materials and methods.

2.19.7 Hybridization

Hybridization was carried out as mentioned in section 2.18.5 of materials and methods hybridization was carried out overnight.

2.19.8 Washing

Washing was carried out as mentioned in section 2.18.6 of materials and methods.

2.19.9 Visualization of radioactive hybridization

The blots were sealed between thin transparent plastic sheets and kept facing the autoradiography film overnight and scanned using phosphor image (Raytest autoradiography equipment) the next day.

2.19.10 Stripping

To remove bound probes from the membrane, blots were stripped off as explained under section 2.18.8 of materials and methods. The blot is used for radioactive labeling with another probe of interest. The same blot can be stripped and reused 2-3 times.

2.20 Amplification of unknown flanking regions using different PCR based Genome walking techniques

2.20.1 Inverse PCR to amplify unknown flanking regions

Inverse PCR is a simple method for the rapid in vitro amplification of DNA sequences that flank a region of known sequence (Ochman *et al.,*. 1998 and http://www.51protocol.com/ PCR /base/20070307/5473.html). The method uses the polymerase chain reaction (PCR), but it has the primers oriented in the reverse direction of the usual orientation. The template for the reverse primers is a restriction fragment that has been self ligated to form a circle. This procedure of inverse PCR (IPCR) has many applications in molecular genetics, for example the amplification and identification of sequences flanking transposable elements. Typical PCR amplifications utilize oligonucleotide primers that hybridize to opposite strands. The primers are oriented such that extension proceeds inwards across the region between the two primers. Since the product of DNA synthesis of one primer serves as the template for the other primer, the PCR procedure of repeated cycles of DNA denaturation, annealing of primers, and extension by DNA polymerase results in an exponential increase in the number of copies of the region bounded by the primers (Saiki *et al.* 1985; Scharf, Horn and Erlich 1986; Faloona and Mullis 1987). However, using the conventional PCR procedure, DNA sequences that lie immediately outside the primers are apparently inaccessible because oligonucleotide that prime DNA synthesis in to flanking regions, rather than included regions, allow only a linear increase in the number of copies. The linear increase occurs because, for each primer, there is no priming of DNA synthesis in the reverse direction. Inverse PCR is schematically represented in Fig 6.

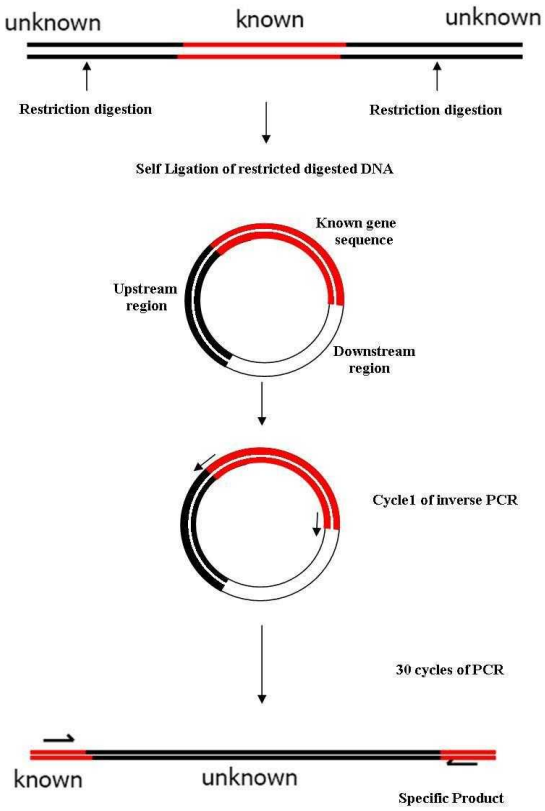


Fig 6. Schematic representation of inverse PCR.

The known sequence is depicted in orange. Upstream region is coloured black and downstream region is in while. Inverse PCR involves primers walking away from each other, direction of DNA synthesis is shown by the arrows. www.51protocol.com

Steps involved

Genomic DNA was partially cut into smaller fragments by blunt end cutter not cutting the known portion of the gene sequence. After restriction enzyme digestion, the DNA fragments produced by the restriction enzyme are diluted and ligated under conditions that favor the formation of monomeric circles. The resulting intramolecular ligation products are then used as substrates for enzymatic amplification by PCR using oligonucleotide primers homologous to the ends of the known sequence, but facing opposite orientations. The primary product of the resulting amplification is a linear double-stranded molecule including segments situated both 5' and 3' of the known sequence. The junction between the original upstream and downstream regions, otherwise ambiguous, can be identified as the restriction site of the restriction enzyme that was used to produce the linear fragments prior to ligation. By selecting a restriction enzyme that cleaves inside a known core sequence, the IPCR procedure will produce products containing only the upstream or only the downstream flanking regions.

2.20.1 Restriction digestion of genomic DNA.

To construct genomic DNA libraries which serve as template for PCRs, 2.5 µg pearl millet genomic DNA was cut with 8 µL of 6 bp blunt end non-cutters in presence of the suitable reaction at an end concentration of 1 x in a micro centrifuge tube. The total reaction volume was adjusted to 100 µL using deionised water. The tubes were incubated at 37°C. After 2 h incubation, the contents of the micro centrifuge tube were mixed by vortexing at low speed for 5-6 sec. Again the samples were incubated at 37°C overnight (16-18 h). To check the complete digestion of genomic DNA 5 µL of the digestion mixture run on a 0.5 % agarose gel and the pattern was visualised in the presence of ethidium bromide under UV light. Restriction enzymes were inactivated by incubating the reaction mixture in a hot water bath at 70°C for 10 min and subsequently cooled on ice.

Six base pair blunt end cutters which do not cut 483 bp pearl millet chi used to create genomic DNA libraries were *Sma*I (Buffer Tango, # BY5) at 30°C), *Stu*I (Blue buffer, # BB5 at 37°C), *Nru*I (*Bsp*68I) (Orange buffer, # BO5 at 37°C) and *Eco*32I (*Eco*RV) (Red Buffer, # BR5 at 37°C).

Six base pair blunt end cutters which do not cut 422 bp pearl millet *tlp* used to create genomic DNA libraries were *Sma*I (Buffer Tango, # BY5 at 30°C), *Stu*I (Blue buffer, # BB5 at 37°C), *Nru*I (*Bsp*68I) (Orange buffer # BO5 at 37°C) and *Nae*I (Buffer Tango at 37°C). Composition of various restriction enzyme buffers used is given below

1X Buffer Tango (yellow # BY5) composition

Tris-acetate (pH 7.9)	33 mM
Mg-acetate	10 mM
K-acetate	66 mM
BSA	0.1 mg/mL

1X Buffer B (Blue # BB5) composition

Tris-HCl (pH 7.5 at 37°C)	10 mM
$MgCl_2$	10 mM
BSA	0.1 mg/mL

1X Buffer O (Orange# BO5) composition

Tris-HCl (pH 7.5)	50 mM
$MgCl_2$	10 mM
NaCl	100 mM
BSA	0.1 mg/mL

1X Buffer R (Red # BR5) composition

Tris HCl (pH 8.5)	10 mM
$MgCl_2$	10 mM
KCl	100 mM
BSA	0.1 mg/mL

2.20.2 Purification of restriction digested genomic DNA.

To each digestion assay was purified by phenol chloroform method explained under section 2.13.2 of materials and methods. DNA pellet was dissolved in 20 µL 10 mM TE buffer pH 7.5. The sample was vortexed at low speed for 5-6 sec and 1 µL from the digested DNA was run on 0.5 % agarose gel to determine the approximate quantity of purified DNA.

2.20.3 Dephosphorylation of the restriction digested genomic DNA.

A very common reason for failed ligation is carryover of the phosphatase in to the ligation reaction. Thus the minimal amount of enzyme should be used during dephosphorylation. A number of enzymes are available for dephosphorylating the cut ends - calf intestinal phosphatase (CIP), shrimp alkaline phosphatase (SAP), bovine alkaline phosphatase (BAP) are a few examples. At end of the reaction, the enzymes are deactivated by heating at 70°C for 10 minutes. In this case, SAP was used. Purified, digested DNA was incubated with 1 U SAP / µg DNA in final 1x SAP buffer in 30 µL volume at 37°C for 15 min. Phosphatase was heat inactivated by incubating at 65°C for 15 min. DNA was purified by phenol/chloroform extraction as explained under section 2.13.2 of materials and methods.

2.20.4 Self ligation of the restriction digested DNA fragments

In order to generate template for inverse PCR the restriction digested and purified genomic DNA was self ligated to for a circular DNA. For this ligation was carried out overnight at 15°C overnight in the presence of T4 DNA Ligase (Fermentas #EL0016). The ligated DNA was purified using E.Z.N.A. Cycle-Pure Kit (Peqlab # D6492-01) as explained under section 2.13.1 of materials and methods.

2.20.5 Primer designing and PCR

Inverse PCR requires oligonucleotide primers homologous to the ends of the known sequence, but facing opposite orientations. The primary product of the resulting amplification is a linear double-stranded molecule including segments situated both 5' and 3' of the known sequence. The primers used in inverse PCR to clone unknown upstream and downstream regions flanking the known gene fragments of pearl millet *chi* and *tlp* are given in table 4 and 5 below.

Table 4. Primers designed to amplify unknown flanking regions of pearl millet *tlp* by inverse PCR.

	Primer	Sequence
1	PR3_72_C	5'-gttgaagaacgcgtcggtga-3'
2	PR3_76_C	5'-tgccgttgaagaacgcgtcg-3'
3	PR3_375	5'-agaacaacgcctactgctcg -3'
4	PR3_ 447	5'-cgcgtcagatctcgtggaac-3'

Table 5. Primers designed to amplify unknown flanking regions of pearl millet *tlp* by inverse PCR.

	Primer	Sequence
1	PR5_59 Rev	5'-CGACTTCTTCGACATCTCGCT-3'
2	PR5_267 Fwd	5'-CGACTACTCGAGGTACTTCAAGGG-3'
3	PR5_256 Fwd	5'-GACTGCGGCGGCGTGCT-3'
4	PR5_208 Fwd	5'-GTGTTCAAGACGGACCAGTA-3'
5	PR5_271 Fwd	5′-CTGCAGTGCACTGGGTA-3′

PCR reaction mixture

Ligated DNA (0.1 μg/μL)	1 μL
10 x PCR buffer	5 μL
25 mM $MgCl_2$	4 μL
10 mM dNTP's	1 μL
Primer 1 (10 μM)	1 μL
Primer 2 (10 μM)	1 μL
Taq DNA Polymerase (5u/ μL)	0.2 μL
Final reaction volume made up with deionised water	50 μL

PCR master mix was prepared as explained under section 2.12.2 of materials and methods. As the gene to be amplified is GC rich 3% DMSO was used and then the final volume of the reaction was made up to 50 μL with deionised water. Contents of the tubes were mixed by vortexing without introducing bubbles and given a short before thermo cycling.

PCR conditions.

Denaturation at 96°C for 1.5 min, primer annealing at 48°Cfor 1 min, and extension by *Taq* polymerase at 70°C for 4.0 min 30 cycles of amplification was carried out using thermo cycler PTC 200 (Biozym, Oldendorf, Germany). Ten μl of the PCR products were analyzed on 1.5% agarose gel.

2.21.1 Genome walking with universal genome walker kit from Clontech (# 638904).

Genome walking is a simple method for amplifying unknown sequences flanking known gene sequence. The method was developed by Siebert and coworkers in 1995; genome walker universal kit from Clontech (#638904) is designed using this principle by Gwyneth Ingram and Karine Coenen. The method involves construction of genomic DNA libraries, ligation of the restriction digested DNA fragments with Genome walker adaptors (GWAs), designing of gene specific primers and primary and nested PCRs using adaptor primers (APs) and gene specific primers (GSPs). These gene specific primers were designed using the known gene sequence. Two gene specific primers, GSP 1 and GSP 2 were used to perform primary and nested PCR along with adaptor primers 1 and 2 respectively. The method is schematically represented in Fig 7.

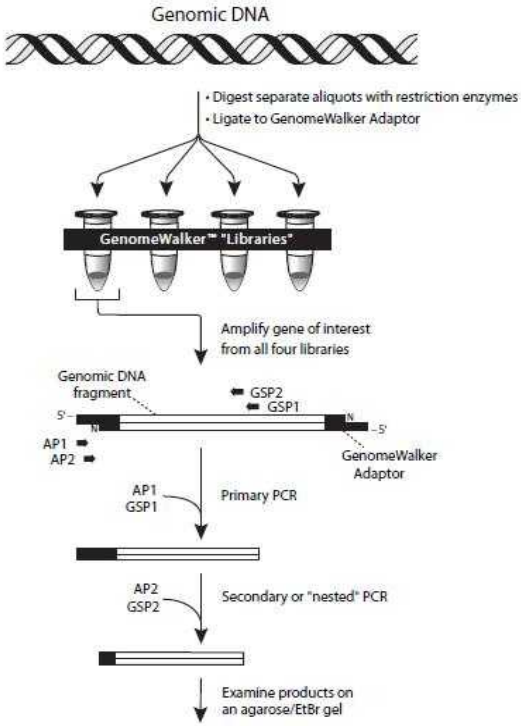


Fig 7. Schematic representation of genome walking using universal genome walker kit
AP: Adaptor primers. GSP: Gene-specific primers. Source: Clontech user manual for the kit.

2.21.2 DNA extraction by CTAB method.

To amplify upstream region of pearl millet *chitinase* gene, genomic DNA from pearl millet seedlings was extracted by CAB method (Murray and Thompson., 1980) as explained under section 2.11.1 of materials and methods.

2.21.3 Restriction digestion of genomic DNA.

To construct genomic DNA libraries which serve as template for PCRs, 2.5 µg pearl millet genomic DNA was cut with 8 µL of blunt end cutters (*Dra*I, *Eco*RV, *Pvu*II and *Stu*I) separately, in presence of the suitable reaction at an end concentration of 1X (*Eco*RV (R buffer - Red buffer), *Dra*I (Tango buffer), *Pvu*II (G buffer - Green buffer) and *Stu*I (B buffer - Blue buffer) in a micro centrifuge tube. The total reaction volume was adjusted to 100 µL using deionised water. The tubes were incubated at 37°C. After 2 h incubation, the contents of the micro centrifuge tube were mixed by vortexing at low speed for 5-6 sec. Again the samples were incubated at 37°C overnight (16-18 h). To check the complete digestion of genomic DNA 5 µL of the digestion mixture run on a 0.5% agarose gel and the pattern was visualised in the presence of ethidium bromide under UV light.

2.21.4 Purification of restriction digested genomic DNA.

To each digestion assay was purified by phenol chloroform method explained under section 2.13.2 of materials and methods. DNA pellet was dissolved in 20 µL of 10 mM TE buffer pH 7.5. The sample was vortexed at low speed for 5-6 sec and 1 µL from the digested DNA was run on 0.5% agarose gel to determine the approximate quantity of purified DNA.

2.21.5 Dephosphorylation of the restriction digested genomic DNA.

Dephosphorylation of the restriction digested purified genomic DNA was carried out as explained under section 2.13.2 of materials and methods and dephosphorylated DNA was again purified by phenol chloroform extraction as explained under section 2.13.2 of materials and methods.

2.21.6 Ligation of restriction digested genomic DNA to Genome Walker adaptor.

To construct each library, 4 µL of digested purified DNA were mixed with 1.9 µL Genome Walker adaptor (25 µM), 1.6 µL 10 x ligation buffer and 0.5 µL T4 DNA ligase (6 units/µL). Mixture was incubated at 16°C overnight. The reaction was stopped by incubation at 70 °C for 5 min.

2.21.7 Primer designing.

Genome walker adaptor primers.

PCR based DNA walking in genomic libraries requires set of walker adaptors and adaptor primers. The nucleotide sequence of these genome walker adaptors (GWA) and adaptor primers (AP) are depicted in the Fig 8. The 5' termini of long oligonucleotide fragment of GWA and 3'-OH group of small oligonucleotide fragment of GWA is blocked by a primary amino group to prevent an extension reaction by DNA polymerase.

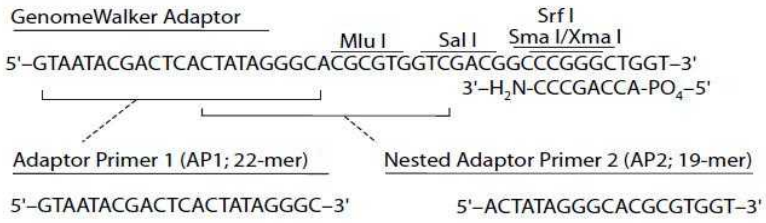


Fig 8. Structure of Adaptors primers from universal Genome Walker Kit (Clontech).

To amplify the upstream region as well as the downstream region, gene specific primers were designed using 'Primer' software. All the primers are compiled in table 6 and 7.

Table 6. Adaptor primers and gene specific primers to amplify unknown flanking regions of pearl millet chitinase gene (*chi*).

Primer	Primer Sequence	Purpose
Genome walker Adaptor	5'-GTAATACGACTCACTATAGGGCACG CGTGGTCGACGGCCCGGGCTGGT-3' -3'CCCGACCA-5	Walker adaptor
Adaptor Primer 1	5'-GTAATACGACTCACTATAGGGC-3'	Adaptor primer 1
Adaptor Primer 2	5'-ACTATAGGGCACGCGTGGT-3'	Adaptor primer 2
1a) PR3_129 GSP1	5'-GCTCCGCGTGTAGAAGTTCC-3'	Upstream *chi*
1b) PR3_72 GSP2	5'-GTTGAAGAACGCGTCGGTGA-3'	Upstream *chi*
2a) PR3_129 GSP1	5'-GCTCCGCGTGTAGAAGTTCC-3'	Downstream *chi*
2b) PR3_76 GSP2	5'-TGCCGTTGAAGAACGCGTCG-3'	Upstream *chi*
3a) PR3_230 GSP1	5'-GTGCGAACGTGGCTAATGTG-3'	Downstream *chi*
3b) PR3_447 GSP2	5'-CGCTGCAGATCTCGTGGAAC-3'	Downstream *chi*
4a) PR3_230 GSP1	5'-GCTCCGCGTGTAGAAGTTCC-3'	Downstream *chi*
4b) PR3_375 GSP2	5'-AGAACAACGCCTACTGCTCG-3'	Downstream *chi*

Table 7. Adaptor primers and gene specific primers to amplify unknown flanking regions of pearl millet thaumatin like protein gene (*tlp*).

Primer	Primer Sequence	Purpose
1a) PR5_594 GSP1	5'-GCACTGCCCCTTGAAGTA-3'	Upstream *tlp*
1b) PR5_59 GSP2	5'-CGACTTCTTCGACATCTCGCT-3'	Upstream *tlp*
2a) PR5_271 GSP1	5'-CTGCAGTGCACTGGGTA-3'	Downstream *tlp*
2b) PR5_208 GSP2	5'-GTGTTCAAGACGGACCAGTA-3'	Downstream *tlp*
3a) PR5_271 GSP1	5'-CTGCAGTGCACTGGGTA-3'	Downstream *tlp*
3b) PR5_312 GSP2	5'-GGAGTACGCGCTGAAGCAGT-3'	Downstream *tlp*

2.21.8 Primary PCR.

2.21.8 i) Primary PCR.

Master mix for primary PCR was prepared as follows

Deionized water to	50 µL
10 x PCR Reaction Buffer	5 µL
dNTP Mix (10 mM for each)	1 µL
$Mg(OAc)_2$ (25 mM)	2.2 µL
DMSO	2.5 µL
Adaptor primer 1 (AP1) (10 µM)	1 µL

Gene specific primer 1 (GSP1-10 pM)	1 µL
Template DNA library (0.1 µg/ µL)	1 µL
Advantage Genomic Polymerase Mix (50X)	1 µL
Final reaction volume	50 µL

10X *Taq* Buffer (with KCl and 15 mM $MgCl_2$)

Tris-HCl (pH 8.8 at 25°C)	100 mM
KCl	500 mM
Nonidet P40	0.8% (v/v)
$MgCl_2$	15 mM

As the template is GC rich DMSO was used at a final concentration of 5% DMSO in primary and secondary PCR. DMSO was added after adding rest of the components to the Master Mix. Contents of the reaction tubes were mixed carefully by vortexing without introducing bubbles and given a short spin. The two-step PCR reaction was carried out in thermo cycler PTC 200 (Biozym, Oldendorf, Germany) as follows according to manufacturer's specification:
7 cycles of 94°C 25 sec and 72°C 3 min
32 cycles of 94°C 25 sec and 67°C 3 min

The extremely short incubation time at 94°C may be necessary to preserve the integrity of the larger genomic DNA templates required for long distance PCR in the Genome walker protocol. To analyze the primary PCR products, 10 µl were run on 1.5 % agarose gel.

2.21.8 ii) Secondary (nested) PCR.

The secondary PCR master mix was prepared as follow:

Deionized water to	50 µL
10 x PCR Reaction buffer	5 µL
dNTP mix (10 mM for each)	1 µL
Mg $(OAc)_2$ (25 mM)	2.2 µL
Adaptor primer 2 (AP2) (10 µM)	1 µL
Gene specific primer 2 (GSP2-10 pmol)	1 µL
DNA Template 1 µl of 1:100 diluted primary PCR product	1 µL
Advantage Genomic Polymerase Mix (50X)	1 µL
Final reaction volume	50 µL

Contents of the tubes were mixed by vortexing without introducing bubbles and given a short spin before thermo cycling. The thermo cycler was programmed as in the primary PCR. Ten µl of the PCR products were analyzed on 1.5% agarose gel.

2.21.9 Cloning of PCR production and sequencing.

The obtained fragments by secondary PCR were purified cut from the gel and purified using gel extraction kit (QiaEx II # 20021) from Qiagen as explained under section 2.13.3 of materials and methods. KAPA Robust DNA polymerase (Peqlab) generates blunt end products as it has proof reading activity. Purified PCR products with poly A tail were cloned to PGEM-T easy cloning vector from Promega as explained under section 2.14.1of materials and methods; Blunt end products were cloned to CloneJet, blunt end cloning vector (Fermentas # K1231) as explained under section 2.14.2 of materials and methods. The ligation assay was incubated overnight at 15°C. Three µl of this ligated CloneJet plasmid was used to transform XL-Blue1 strain of *E. coli*; subsequently transformants grown on ampicillin plates were based on ampicillin resistance.

2.21.10 Plasmid isolation from the transformant colonies.

The transformants were grown on ampicillin (100 µg/mL) containing LB broth; plasmid was isolated using plasmid mini prep kit (GeneJet Plasmid mini prep kit Fermentas # K0502) as explained under section 2.16.2 of materials and methods. Isolated plasmid sample was analysed on 1% agarose gel.

2.22 Straight walk: A ligation-mediated method of genome walking (Tsuchiya *et al.,* 2009).

An important step in plant genome research is the isolation of unknown genomic regions flanking known sequences. Several polymerase chain reaction (PCR) (Abd-Elsalam, 2003)-based techniques have been developed for this purpose. Although these methods have been successfully used for plants with relatively small genomes (e.g., *Arabidopsis thaliana* and *Oryza sativa*), they often fail for plants with large genomes because the genome size has a negative effect on PCR amplification (Garner, 2002). The problem of PCR-based genome walking in plants with large genomes is likely caused by the extremely low ratio of the number of target DNA molecules to other genomic sequences in the reaction mixes. For this reason, the amplification of the desired product can completely fail or be obscured due to the excessive accumulation of nonspecific products. Therefore, a genome walking method that allows both high sensitivity and high specificity is needed to obtain satisfactory results in plants with large genomes. This is an improved ligation-mediated PCR (LM–PCR) procedure that is especially effective for plants with large genomes. An outline of the LM–PCR strategy used in this study as illustrated in (Tsuchiya *et al.,* 2009) the Fig 9.

Schematic representation of Straight Walk.

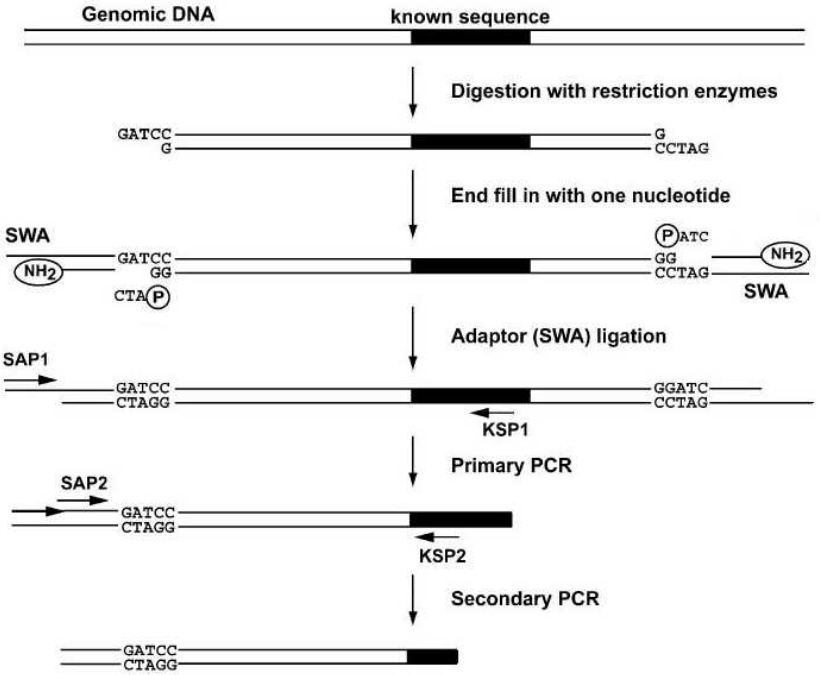


Fig 9. Schematic representation of Straight Walk: SWA adaptor composed of SWA-F and SWA-R1/R2; NH2, primary amino group to block the extension reaction of polymerase; P, phosphorylated 50 end; SAP1 and SAP2, adaptor primers specific for SWA; KSP1 and KSP2, primers annealing to the known sequence. The known sequence is shaded. Source: Tsuchiya *et al.*, 2009

2.22.1 Primer designing.

For straight walking to amplify unknown genomic regions flanking known sequences of *chi* and *tlp* gene from pearl millet, adaptor primers were designed as explained by Tsuchiya and coworkers (2009). These adaptor primers include straight walk adaptors (SWA - forward and reverse), Specific adaptor primers SAP - 1 and 2) which work regardless of the genome used (Tsuchiya *et al.,* 2009).

Table 8. Straight Walk adaptor (SWA) primers and specific primers for the amplification of unknown flanking regions of chitinase (*chi*) gene.

Primer	Primer Sequence	Purpose
SWA-F	5'-CGCAGGCTGGCAGTCTCTTTA GGGTTACACGATTGCTT-3'	SW fwd primer
SWA-R1	5'-ATCAAGCAATCGTGT-3'	SW rev primer 1
SWA-R2	5'-TAGAAGCAATCGTGT-3'	SW rev primer 2
SAP1	5'-CGCAGGCTGGCAGTCTCTTTAG-3'	Sp adaptor primer 1
SAP2	5'-ATGCGGCCGCTCTCTTTAGGGTT ACACGATTGCTT-3'	Sp adaptor primer 2
1a) PR3_129 KSP1	5'-GCTCCGCGTGTAGAAGTTCC-3'	Upstream *chi*
1b) PR3_72 KSP2	5'-GTTGAAGAACGCGTCGGTGA-3'	Upstream *chi*
2a) PR3_129 KSP1	5'-GCTCCGCGTGTAGAAGTTCC-3'	Downstream *chi*
2b) PR3_76 KSP2	5'-TGCCGTTGAAGAACGCGTCG-3'	Upstream *chi*
3a) PR3_230 KSP1	5'-GTGCGAACGTGGCTAATGTG-3'	Downstream *chi*
3b) PR3_447 KSP2	5'-CGCTGCAGATCTCGTGGAAC-3'	Downstream *chi*
4a) PR3_230 KSP1	5'-GCTCCGCGTGTAGAAGTTCC-3'	Downstream *chi*
4b) PR3_375 KSP2	5'-AGAACAACGCCTACTGCTCG-3'	Downstream *chi*

- The 5' termini of SWA-R1 and SWA-R2 are phosphorylated for ligation, and 3'-OH is blocked by a primary amino group to prevent an extension reaction by DNA polymerase.
- SWA is composed of a combination of SWA-F and either SWA-R1 or SWA-R2. The 5'-end sequences of SWA-R1 and SWA-R2 are compatible with the restriction enzyme sites of *Bam*HI, *Bgl*II, *Bcl*I, or *Sau*3AI and *Spe*I, *Nhe*I, or *Xba*I, respectively.
- SAP2 has a NotI restriction enzyme site to ease cloning.

Table 9. Adaptor primers and gene specific primers to amplify unknown flanking regions of pearl millet thaumatin like protein gene (*tlp*).

Primer	Primer Sequence	Purpose
1a) PR5_594 KSP1	5′-GCACTGCCCCTTGAAGTA-3′	Upstream *tlp*
1b) PR5_59 KSP2	5'-CGACTTCTTCGACATCTCGCT-3'	Upstream *tlp*
2a) PR5_271 KSP1	5′-CTGCAGTGCACTGGGTA-3′	Downstream *tlp*
2b) PR5_208 KSP2	5'-GTGTTCAAGACGGACCAGTA-3'	Downstream *tlp*
3a) PR5_271 KSP1	5'-CTGCAGTGCACTGGGTA-3′	Downstream *tlp*
3b) PR5_312 KSP2	5'-GGAGTACGCGCTGAAGCAGT-3'	Downstream *tlp*

Known sequence-specific primers (KSPs) were designed to amplify upstream unknown genomic DNA sequences of pearl millet *chitinase* and *tlp*. For each gene two KSPs were designed using the nucleotide sequence of the known gene fragment to perform nested PCR. The primers are compiled in the table 8 and 9 above.

2.22.2 DNA extraction and restriction digestion of genomic DNA.

Intact genomic DNA was extracted from pearl millet using cetyltrimethylammonium bromide (CTAB)-based method by Murray and Thompson (1980). Five hundred ng of genomic DNA was digested separately with 10 units of *BamH*I, *Bgl*II, *Bcl*I, *Sau*3AI, *Spe*I, *Nhe*I, and *Xba*I. All enzymatic reactions were carried out in a total volume of 10 µL at 37°C except for BclI which was incubated at 55°C. The four base 5' overhangs were partially filled by adding 1µL of 1 mM deoxyguanosine triphosphate (dGTP) for the *Bam*HI, *Bgl*II, *Bcl*I, and *Sau*3AI digestions. For the *Spe*I-, *Nhe*I-, and *Xba*I-digested DNAs 1µL of 1 mM deoxycytidine triphosphate (dCTP) was added. The end fill reactions were performed with 1 unit of Klenow enzyme (3' → 5' exonuclease activity, Klenow Fragment, Fermentas # EP0051) at 25°C for 15 min in a total volume of 12 µL. After the end fill in reaction, the Klenow enzyme was heat-inactivated by 75°C for 20 min. The partially end-filled DNA fragments generated by *Bam*HI, *Bgl*II, *Bcl*I, or *Sau*3AI were ligated to the adaptor composed of 1 µL of 100 µM SWA-F and 1 µL of 100 µM SWA-R1 The DNA fragments generated by *Spe*I, *Nhe*I, and *Xba*I were ligated to the adaptor composed of 1 µL of 100 µM SWA-F and 1 µL of 100 µM SWA-R2 by adding 10 µL of Ligation High Kit solution (Toyobo). The reaction mixtures were incubated at 16°C for 15 h.

2.22.3 PCR with constructed libraries.

2.22.3 i) Primary PCR.

In primary PCR, each adaptor-ligated DNA was diluted with deionised water to a total of 100 µL, and 1 µL of this dilution was used as a PCR template.

Composition of primary PCR reaction mixture.

10 x PCR buffer	2.5 µL
dNTP Mix (10 mM each)	1 µL
$MgSO_4$(25 mM)	1 µL
KAPA2G Robust DNA polymerase (Peqlab)	1 unit
*Ksp*1 (100 pM)	10 µL
SAP1 (100 pM)	10 µL
DNA template (0.1 µg/ µL)	1 µL
Total reaction volume	25 µL

Contents of the tubes were mixed by vortexing without introducing bubbles and given a short spin before thermo cycling. The thermo cycler was programmed as mentioned below.

PCR conditions.

Hot-start denaturation (initial) at 98°C for 20 sec, 35 cycles of denaturation at 94°C for 30 sec, primer annealing at 65°C for 30 sec, and extension at 68°C for 5 min. The PCR reaction was carried out in thermo cycler PTC 200 (Biozym, Oldendorf, Germany).

2.22.3 ii) Secondary PCR.

Product of primary PCR was diluted at 1:100 dilution and 1 µL was used as template for secondary PCR. Master mix was prepared similar to that of primary PCR; the primer pair KSP2 and SAP2 was used. Contents of the tubes were mixed by vortexing without introducing bubbles and given a short spin before thermo cycling.

PCR conditions.

The PCR conditions were the same as for the first PCR except that only 30 thermal cycles were performed. After the secondary PCR amplification, 10 µL of the PCR products were analyzed on 1.5% agarose gel.

2.22.4 Cloning of PCR products and sequencing.

The obtained fragments by secondary PCR were purified cut from the gel and purified using gel extraction kit (Qiaex II # 20021) from Qiagen as explained under section 2.13.3 of materials and methods. KAPA2G Robust DNA polymerase (Peqlab # KK5004) generates blunt end products as it has proof reading activity. The purified PCR product was cloned to blunt end cloning vector CloneJet (Fermentas # K1231). The ligation assay was incubated overnight at 15°C. Three µL of this ligated CloneJet plasmid was used to transform XL-Blue 1 strain of *E. coli* as explained under section 2.14 of materials and methods; subsequently transformants screened as explained under section 2.15 of materials and methods.

3 Results.

This doctoral research work involves study of pearl millet-downy mildew pathogen interaction. The study concentrates on two important pathogenesis-related proteins, namely chitinase and thaumatin-like protein from pearl millet. These proteins are known to act synergistically in plant's defense mechanism against phytopathogenic oomycetes. Preliminary studies include detection of these two important PR proteins in pearl millet. Chromatographic techniques were developed to purify 24 kDa pearl millet chitinase to complete homogeneity was optimized. The purified protein was identified immunologically and by *de novo* sequencing as chitinase. Thaumatin-like protein was also purified partially by chromatographic techniques. Molecular biological studies involved isolation and sequencing of pearl millet chi and tlp genes. Partial length fragment of housekeeping gene actin was also isolated and sequenced. Different genome walking techniques were followed to isolate the full length chi and tlp gene. Results of all the experiments carried out during the doctoral study are summerised below.

Part A- Preliminary studies.

3.1 Infection of pearl millet seedlings.

Pearl millet seeds were germinated and infection with downy mildew spores was investigated. Therefore surface sterilized pearl millet seeds grown on moist blotter for 2 days were inoculated with *S. graminicola* zoospore suspension ($4x10^4$ spores/mL) as explained under section 2.1.4 of materials and methods. The seedlings were incubated in dark at 22°C to facilitate infection process as the pathogen is light sensitive (Fig 10).

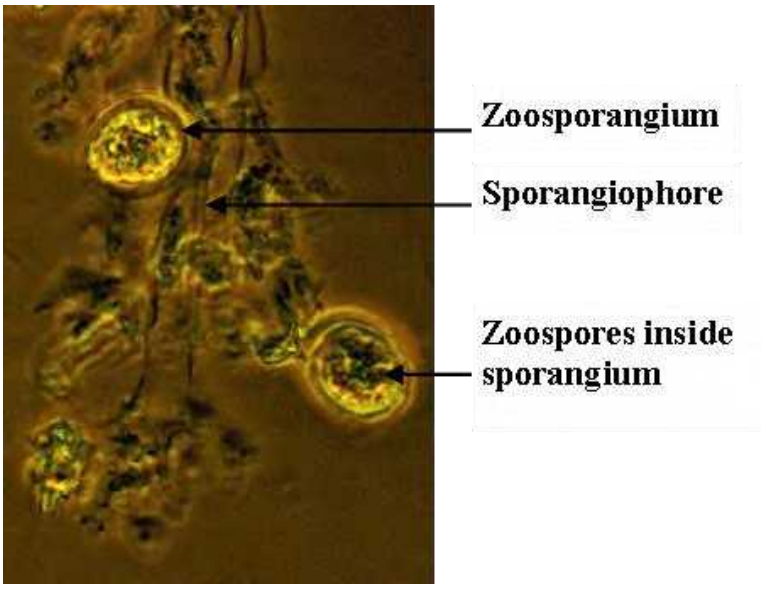


Fig. 10. Zoosporangia of *Sclerospora graminicola* as viewed under compound microscope. Bright field image captured at 200 x magnification.

Part B - Preliminary studies (analytical).

3.2 Detection thaumatin-like protein (TLP) in pearl millet.

Studies involved detection of thaumatin like protein in pearl millet. The total soluble protein was isolated from 2 day old pearl millet seedlings inoculated with *S. graminicola* (section 2.2.1 of materials and methods) and separated on SDS-PAGE (section 2.3 of materials and methods) and immuno blotted against anti TLP antibody from Douglas fir raised in rabbit (section 2.4 of materials and methods). Immuno blot analysis using anti TLP antibody revealed cross reaction of a 23 kDa protein indicating the presence of TLP in pearl millet with apparent molecular weight of 23 kDa; the results of the same are summerised below.

The pearl millet total protein was extracted with 50 mM sodium acetate buffer containing 3 mm phenylmethanesulfonyl fluoride (PMSF) (section 2.2.1 of materials and methods). Protein estimation was carried out according to Bradford method using BSA as standard (section 2.2.2 of materials and methods). The proteins were resolved on 12% SDS-PAGE can be seen in Fig 11 (Laemmli 1970) (2.2.3 materials and methods).

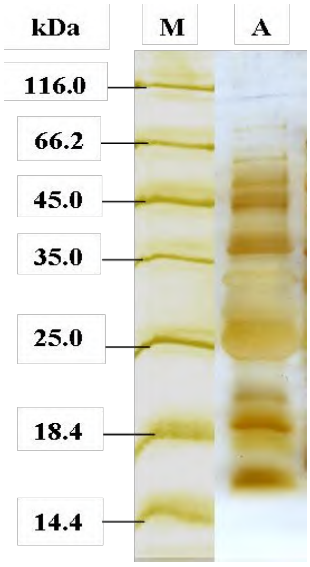


Fig 11. Total soluble protein fraction from pearl millet seedlings separated on 12% SDS-PAGE.

Lane M: Unstained protein marker Fermentas (# SM0431), Lane A: Total soluble protein fraction from pearl millet seedlings (20 µg of total protein extract was loaded onto the gel and the gel was silver stained).

Lane A was loaded with 20 µg of protein and the gel was stained with silver nitrate (section 2.2.3 (ii) of materials and methods). Total soluble protein fraction extracted from pearl millet showed several protein bands with molecular weight ranging between 16-100 kDa, the protein profile showed prominent bands of 45, 40, 35, 26, 24, 23 and 18 and 16 kDa as shown in the Fig 11.

3.3 Immunological detection of proteins.

The proteins were then electro transferred onto PVDF membrane (section 2.3.4 of materials and methods) and subsequently performed immuno blot analysis with anti-thaumatin antibody from Douglas fir (raised in rabbit) was carried out (section 2.4 of materials and methods), which showed cross reaction of 23 kDa protein band indicating that it belongs to thaumatin-like protein family (Fig 12).

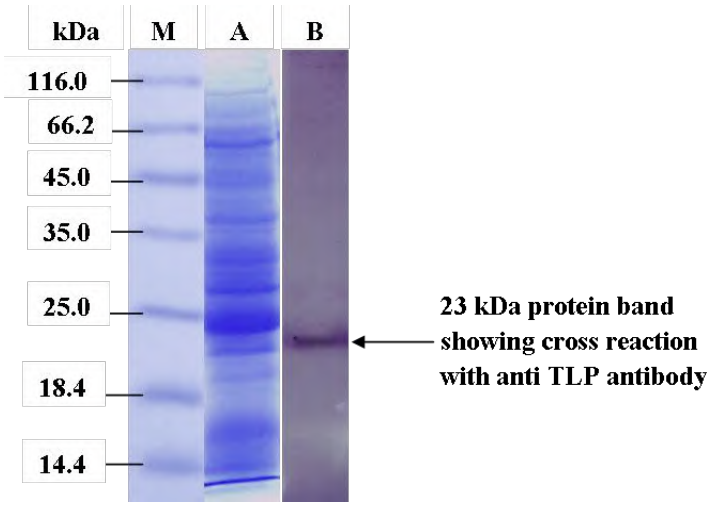


Fig 12. Immunological detection of TLP in pearl millet total soluble protein fraction.

Lane M: Unstained protein marker (Fermentas # SM0431), Lane A: Total soluble protein from pearl millet seedlings separated on 12% SDS-PAGE and stained with Coomassie brilliant blue, Lane B: 23 kDa protein showing cross reaction with anti-thaumatin antibody (dilution 1:5000) and antirabbit secondary antibody (raised in goat) tagged with alkaline phosphatase was used at a dilution of 1:20,000, 30 µg of total protein was loaded to both the lanes.

Part C- Results of biochemical assays.

3.4 Characterization of pearl millet TLP.

3.4.1 Glucan binding activity of pearl millet TLP.

It has been shown by the Trudel and coworkers (1998) that thaumatin-like proteins bind to β-1,3-glucans; glucans are important component of oomycete cell wall and binding of thaumatin-like proteins to glucans on the cell wall facilitates antifungal activity (Ibeas *et al*., 2000). The gelled curdlan (section 2.3.1 of materials and methods) was equilibrated in 50 mM sodium acetate (pH 5.0) and total soluble protein extract from pearl millet seedlings was incubated with curdlan to allow binding at 22°C (section 2.3.2 of materials and methods); unbound proteins were washed off with the same buffer and bound protein was eluted by boiling the sample in Laemmli sample loading buffer (2.3.3 materials and methods). The bound sample was analysed on 12% SDS-PAGE and stained with Coomassie brilliant blue (section 2.3.3 (i) of materials and methods). Analysis of curdlan bound protein fraction on 12% SDS-

PAGE showed a major band of 23 kDa with two other proteins falling in the same molecular weight range as seen in the Fig 13.

3.4.2 Immuno blot analysis of the curdlan bound protein.

Curdlan bound protein was eluted by boiling in Laemmli sample loading buffer (section 2.5.3 of materials and methods) analysed on 12% SDS-PAGE. The resolved proteins were electro transferred onto activated nitrocellulose membrane (section 2.3.4 of materials and methods) and subsequently immuno blotted against anti-thaumatin antibody from Douglas fir (section 2.4 of materials and methods).

Immunoblot analysis of the curdlan bound protein with anti TLP antibody revealed cross reaction of a protein band with apparent molecular weight of 23 kDa (Fig 13), confirming β-1,3-glucan binding activity of the pearl millet TLP. Primary antibody was used at a dilution of 1:5000 and antirabbit secondary antibody (raised in goat) tagged with alkaline phosphatase (Sigma) was used at a dilution of 1:20,000.

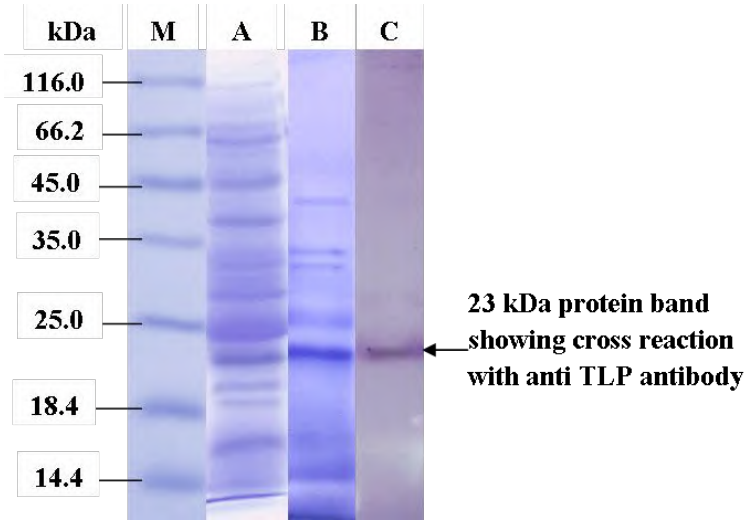


Fig 13. Detection of glucan binding activity of pearl millet TLP

Lane M: Unstained protein marker (Fermentas # SM0431), Lane A: Total soluble protein from pearl millet seedlings, Lane B: Curdlan bound protein, Lane C: 23 kDa curdlan bound protein showing cross reaction with anti-thaumatin antibody-dilution 1:5000. 20 µg of total protein was loaded onto each lane.

3.5 Inhibition of S. graminicola by pearl millet TLP

3.5.1 Inhibition of *S. graminicola* zoospore mobility with the curdlan bound protein.

Inhibition of *S. graminicola* zoospore mobility with the curdlan bound protein was observed under Leitz compound microscope (section 2.6 of materials and methods). Curdlan bound proteins were eluted with 0.1 mL of 0.1 M HCl (pH 2.2) and subsequently neutralized with 0.1 M HCl (section 2.5.3 of materials and methods). Living zoospores of *S. graminicola* ($4x10^4$ spores/mL) were suspended in sterile deionised water and were treated with curdlan bound protein (10 µg/mL). Zoospores suspended in deionised water served as control. The

zoospore suspension was observed under Leitz compound microscope at 200 x magnification (under bright field).

It was observed that most of the zoospores (>90%) lost mobility after treatment with curdlan bound protein, whereas the control zoospores showed no change in their mobility. This reduction in the mobility of zoospores is attributed to inhibitory activity of TLP found in the curdlan bound protein fraction. TLPs are known to permeabilise fungal membrane leading to ion leakage and death of fungus.

3.5.2 Microscopic study to detect membrane permeabilization using the fluorescent dye, SYTOX green.

To investigate membrane permeabilization, the assay based on the uptake of SYTOX Green, an organic compound that penetrates cells with compromised plasma membranes and fluoresces upon interaction with nucleic acids developed by Thevissen and coworkers in 1999 (Section 2.6.2 of materials and methods) was chosen. Sporangial suspension (4 X 10^4 zoospores/mL) harvested in ice cold deionised water was centrifuged and resuspended in 100 mM HEPES-KOH (pH 6.5) with 5 mM $MgCl_2$ and 0.2 mM Sytox Green; 90 µL of this sporangial suspension was treated with 10 µL of curdlan bound protein (10 µg/mL); Membrane permeabilization of *S. graminicola* by curdlan bound protein fraction from pearl millet can be seen in Fig 14.

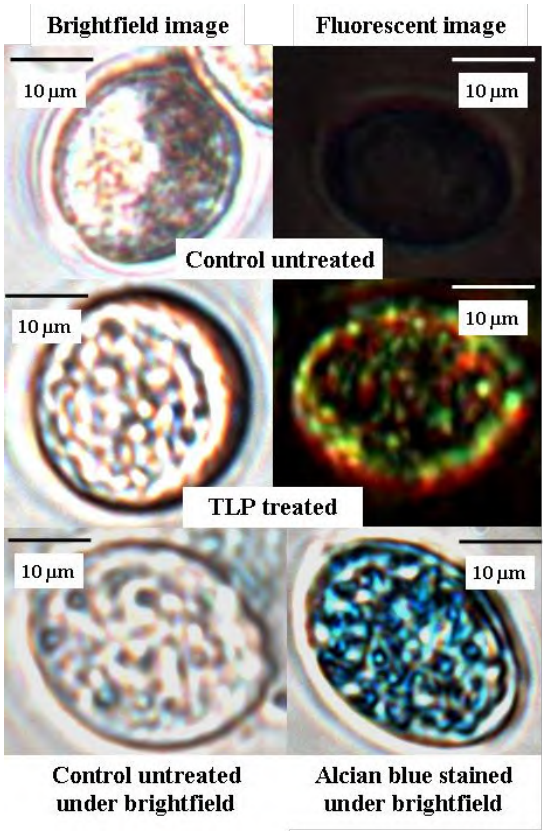


Fig 14. Membrane permeabilization of *S. graminicola* by curdlan bound protein fraction from pearl millet. Control untreated: Sporangia treated with buffer only. TLP treated: Sporangia treated with curdlan bound protein fraction containing TLP, stained with Sytox green showing fluorescence, Sporangium stained with Alcian blue. Magnification: Sporangium were viewed under compound microscope (200 x magnification).

It was observed that curdlan bound protein treated zoospores showed fluorescence. Fluorescence is observed only when the cells have a compromised cell membrane through which the dye can enter the cell and permeabilise the membrane. On treatment *of S. graminicola* zoosporangia with partially purified TLP from pearl millet indicated membrane permeabilization by the partially purified TLP which is one of its important biological features. The major component of curdlan bound protein was identified to be TLP from immuno blot analysis (section 3.4.2 of results). Untreated zoospores which served as control did not show any fluorescence; therefore they were photographed under bright field.

As supporting evidence, zoosporangia were stained with aqueous alcian blue solution. Alcian blue has the same binding site as that of TLP on fungal cell walls. Therefore positive staining with alcian blue indicates the presence of binding (receptor) sites for TLP on *S. graminicola* sporangial wall (Ibeas *et al.,* 2000) - Fig 14.

3.6 Purification of pearl millet chitinase.

Development of method of purification.

Purification of pearl millet chitinase by chromatographic method was optimized. The steps include induction of elevated levels of PR proteins in pearl millet seedlings by treatment with 1 mM $AgNO_3$ solution (section 2.1.5 of materials and methods), extraction of total soluble protein fraction (section 2.2.1of materials and methods), ammonium suphate fractionation of the total protein extract, ion exchange chromatography using DEAE cellulose column and CM sepharose column, chromatographic separation through hydroxyapatite column and FPLC using Mono Q sepharose column. The protein fractions eluted in each chromatographic step were analysed by SDS-PAGE, the fractions containing protein of interest were carried over to the next step and rest of the samples were discarded.

3.6.1 Induction of PR proteins in pearl millet by silver nitrate.

Pearl millet seedlings were induced to over express PR proteins by treating one week old seedlings with 1 mM solution of silver nitrate (Conejero & Granell, 1986 and Rahimi *et al*., 1993) (section 2.1.5 of materials and methods). The seedlings were sprayed once in a day with 100 µL of 1 mM silver nitrate solution/10 seedlings for five consecutive days. The treated seedlings were harvested on the sixth day and stored at -70°C. Seedlings sprayed with deionised water served as control. Total soluble protein was extracted from equal weights of treated and untreated seedlings separately (section 2.2.1 of materials and methods) and 20 µg of total soluble protein fraction from both the samples were resolved on 12 % SDS-PAGE in adjacent lanes and the gels were silver stained (section 2.3.3b of materials and methods) as shown in the Fig 15.

Induction of Proteins in pearl millet after treatment with 1mM silver nitrate solution.

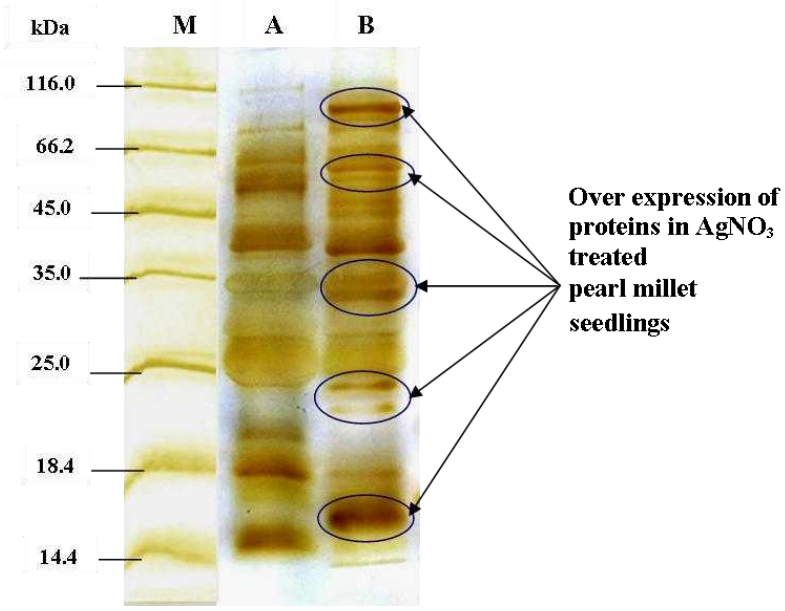


Fig 15. Induction of PR protein expression in pearl millet seedlings after treatment with 1 mM silver nitrate.

Lane M: Protein marker (Fermentas # SM0431), Lane A: Protein profile of untreated seedlings, Lane B: Protein profile of silver nitrate treated seedlings showing over expression of some of the proteins (indicated by arrows) 20 µg of protein was loaded into each lane.

As silver nitrate treated pearl millet seedlings showed expression of proteins at higher levels, silver nitrate seedlings were used for purification of chitinase. Purification of pearl millet chitinase was achieved through chromatographic a technique which is outlined schematically in Fig 16.

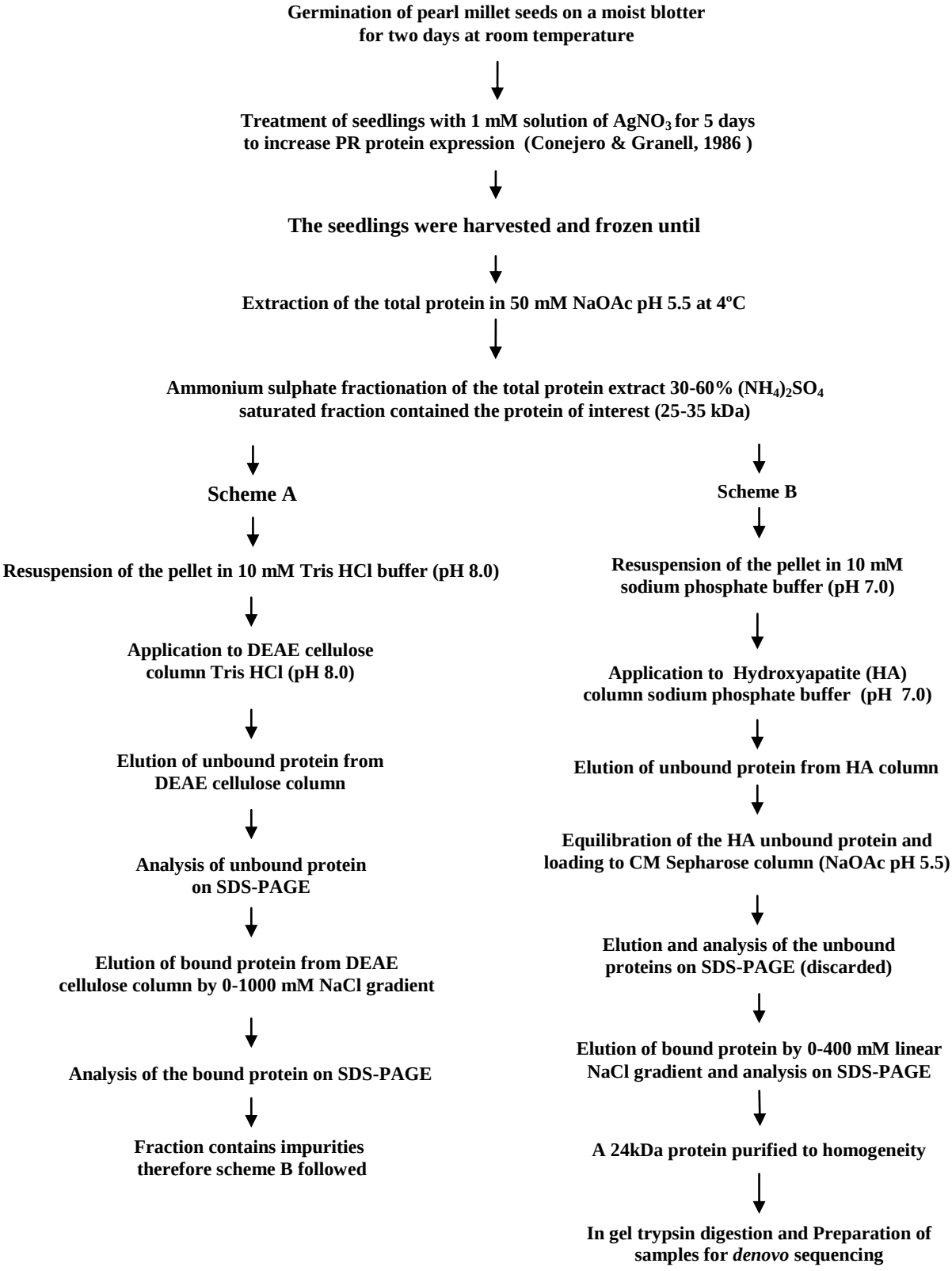


Fig 16. Schematic representation of purification of pearl millet chitinase by chromatography.

3.6.2 Ammonium sulphate fractionation of total soluble proteins from pearl millet after induction by silver nitrate.

Total soluble protein was extracted by crushing the frozen seedlings in 50 mM sodium acetate buffer (pH 5.5) and subjected to ammonium sulphate fractionation at 0-30%, 30-60% and 60-100% saturation (section 2.7.4 of materials and methods). The precipitated protein was divided into 2 parts and one part was desalted by dialyzing against 50 mM sodium acetate buffer (pH 5.5), the other part was dialyzes against 10 mM Tris-HCl buffer (pH 8.0) All the three fractions were analysed on 12% SDS-PAGE (Section 2.3 of materials and methods) to check the protein profile, seen in Fig 17.

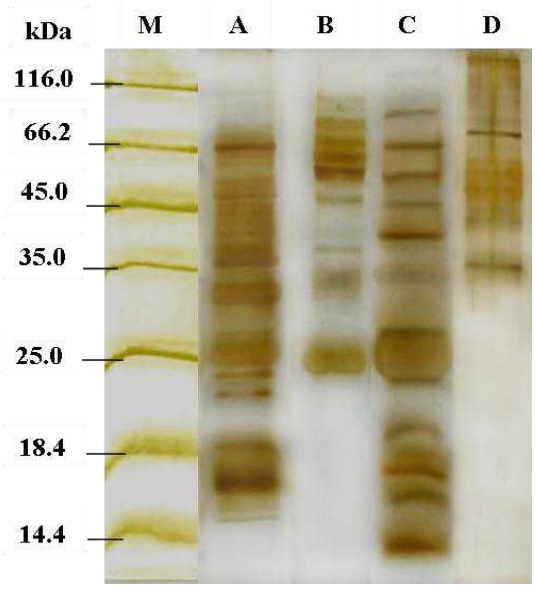


Fig 17. Ammonium sulphate fractionation of total soluble proteins from pearl millet.

Lane M: Protein marker (Fermentas # SM0431). Lane A: Total protein extract, Lane B: 0-30% Ammonium sulphate saturated protein fraction; Lane C: 30-60% saturated protein fraction; Lane D: 60-100% saturated protein fraction (15µg of protein was loaded into each lane).

Ammonium sulphate precipitated fraction at 0-30% saturation showed proteins with apparent molecular weight of 65-75 kDa, 50 kDa, 35 kDa and 25 kDa. 30-60% saturated fraction showed proteins with apparent molecular weight of 65-75 kDa, 55 kDa, 45 kDa, 35 kDa and 23-26 kDa 14-20 kDa. 60-100% saturated fraction showed proteins with apparent molecular weight of 65-75 kDa and 35-66 kDa as seen in the Fig 8. 0-30% ammonium sulphate saturated protein had the major proteins in the molecular weight range of 20-35 kDa (most of the known TLP and chitinases fall within this molecular weight range), and some higher as well as lower molecular weight proteins. Moreover, the 30- 60% ammonium sulphate saturated fraction showed enrichment of many of the proteins in the molecular weight range of 22-35 kDa. The ammonium sulphate fractions were subjected to immuno blot analysis with anti chitinase antibody and a strong cross reaction was observed with two protein bands of apparent molecular weight of 24 and 22 kDa in 30-60% ammonium sulphate saturated fraction. Therefore this fraction was desalted and used for further purification.

3.7 Purification by column chromatography.

Scheme of purification: Two different methods were followed in parallel to purify the protein of interest. Ammonium sulphate precipitated protein fraction (30-60% saturated) divided into two parts. One part was dialyzed against the 10 mM Tris-HCl (pH 8.0) buffer before loading onto DEAE Column equilibrated with the same buffer and the other part was dialysed against 10 mM sodium phosphate buffer (pH 5.0) and loaded onto HA column. The columns were washed with three bed volumes of the respective elution buffers before loading the protein sample.

3.7.1 Chromatographic separation of proteins on DEAE cellulose column.

The dialysed protein fraction was loaded onto DEAE cellulose column and the unbound proteins were collected (detailed explanation under section 2.7.6 of materials and methods) and analysed on SDS-PAGE (section 2.3 of materials and methods). The results obtained showed that there was no enrichment of proteins falling in the molecular weight range of (20-35 kDa) from the mixture as seen clearly in the Fig 18. The unbound protein fractions from DEAE cellulose column were collected and analysed on SDS-PAGE. The fraction contained proteins with their apparent molecular weight ranging between 32-55 kDa, 22 kDa, 19 kDa and 15 kDa.

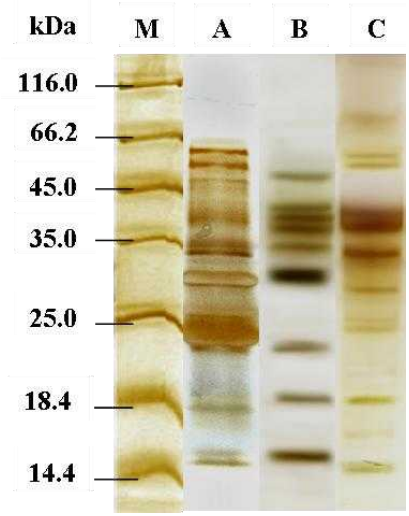


Fig 18. Chromatographic separation of pearl millet proteins with DEAE-Cellulose column.

Lane M: Protein marker (Fermentas # SM0431), Lane A: Ammonium sulphate precipitated proteins fraction (30-60%) that was loaded onto the column. Lane B: Unbound proteins eluted from DEAE cellulose column, Lane C: Bound proteins eluted from DEAE cellulose column (10µg of protein was loaded into each lane).

Elution of DEAE cellulose bound proteins.

The proteins bound to DEAE cellulose column were eluted linear 0-1000 mM NaCl gradient; however, there was no enrichment of proteins falling within the molecular weight range of 20-35 kDa as observed by SDS-PAGE analysis of the eluted proteins as seen in the Fig 18, Lane C. The DEAE cellulose bound fraction mainly contained proteins with an apparent

molecular weight of 30-45 kDa (and a few high as well as low molecular weight weak bands). Therefore ammonium sulphate fractionated protein fraction was chromatographed through hydroxyapatite column (BioRad) (section 2.7.7 of materials and methods).

3.7.2 Chromatography with hydroxyapatite column.

Since the separation of proteins by DEAE Column was not satisfactory, the proteins were chromatographed through hydroxyapatite column equilibrated with 10 mM sodium phosphate buffer (pH 7.0) (25 cm length, 1.25 cm radius, height of the bed was 10 cm). The unbound proteins from HA column with the same buffer, flow rate was adjusted to 0.4 mL/ min and the fraction volume was 2 mL. One hundred μL of each fraction was concentrated by SpeedVac and subsequently analysed on SDS-PAGE (section 2.3 of materials and methods). These HA unbound fractions contained proteins in the molecular weight range of 22-26 kDa, 28 kDa, 35 kDa (Fig 19). Therefore these fractions were pooled and carried over to the next step involving ion exchange chromatography using CM Sepharose.

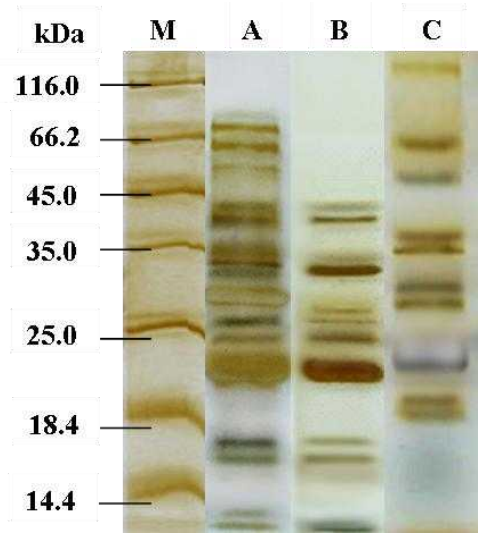


Fig 19. Chromatographic separation of pearl millet proteins with Hydroxyapatite (HA) column.

Lane M: Protein marker (Fermentas # SM0431). Lane A: Ammonium sulphate precipitated proteins fraction (30-60%) that was loaded onto the column, Lane B: Unbound proteins eluted from HA column, Lane C: Bound proteins eluted from HA column (10μg of protein was loaded into each lane).

The bound protein fraction from HA column contained proteins with an apparent molecular weight of 35 and 20-22 kDa, 26 kDa, 28-30 kDa, 36 kDa, 55 kDa and 66 kDa. However there was no enrichment of proteins, therefore the HA bond protein fraction were discarded. Depending on the physicochemical behavior of TLP no enrichment was expected and the HA column was used as negative chromatography (unbound proteins were collected) to minimize the impurities

3.7.3 Ion exchange chromatography involving CM sepharose.

The HA unbound protein fractions were pooled and concentrated using Vivaspin concentrator tubes and carried over to the next step involving ion exchange chromatography

involving CM Sepharose (section 2.7.8 of materials and methods). This concentrated protein fractions were dialysed against 10 mM sodium acetate buffer (pH 5.5) and loaded onto CM sepharose column pre equilibrated with the same buffer (25 cm length and 1.25 cm radius, height of the bed was 15 cm). Flow rate was adjusted to 0.4 mL/min. The CM sepharose unbound proteins were analysed on SDS- PAGE as seen in Fig 20.

Elution of unbound proteins from CM sepharose column.

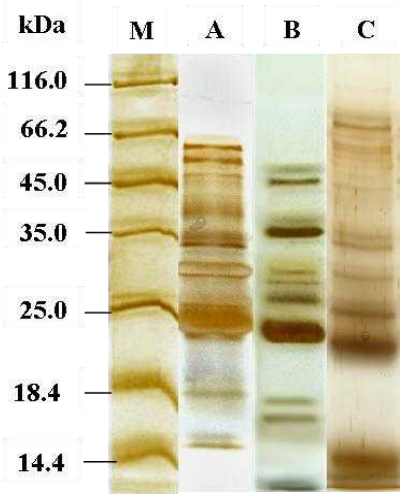


Fig 20. Chromatographic separation of pearl millet proteins with CM-sepharose column.

Lane M: Protein marker (Fermentas # SM0431), Lane A: Ammonium sulphate precipitated proteins fraction (30-60% saturation), Lane B: HA column unbound protein fractions pooled (which was loaded onto CM sepharose column), Lane C: CM sepharose unbound protein (10µg of protein was loaded into each lane).

The unbound protein fractions from CM sepharose column were eluted by washing the column with 10 mM sodium acetate buffer (pH 5.5); flow rate was adjusted to 0.4 mL/min, fraction size was 2 mL. One hundred µL of the collected protein fractions were concentrated on SpeedVac and separated on 12 % polyacrylamide gel (section 2.3 of materials and methods). The gels were silver stained as seen in the Figure 20. It was observed that none of the CM sepharose unbound protein fractions showed enrichment of proteins within the molecular weight range of 20-35 kDa. Moreover the samples showed many weak bands falling in the molecular weight range of 40-65 kDa and one major protein band of 24 kDa molecular weight, also a protein band with apparent molecular weight of 14 kDa was also observed. However there was no resolution of this 24 kDa protein from other higher molecular weight weak bands. Therefore these unbound protein fractions were discarded. The bound protein fractions from CM sepharose column were eluted by washing the column with 10 mM sodium acetate buffer (pH 5.5) under a linear salt gradient of 0-400 mM NaCl; flow rate was adjusted to 0.4 mL/min, fraction size was 2 mL. One hundred µL of the collected protein fractions were concentrated on SpeedVac and separated on 12 % polyacrylamide gel (section 2.3 of materials and methods). The gels were silver stained as seen in the Fig 21.

Elution of bound proteins from CM sepharose column under linear NaCl gradient.

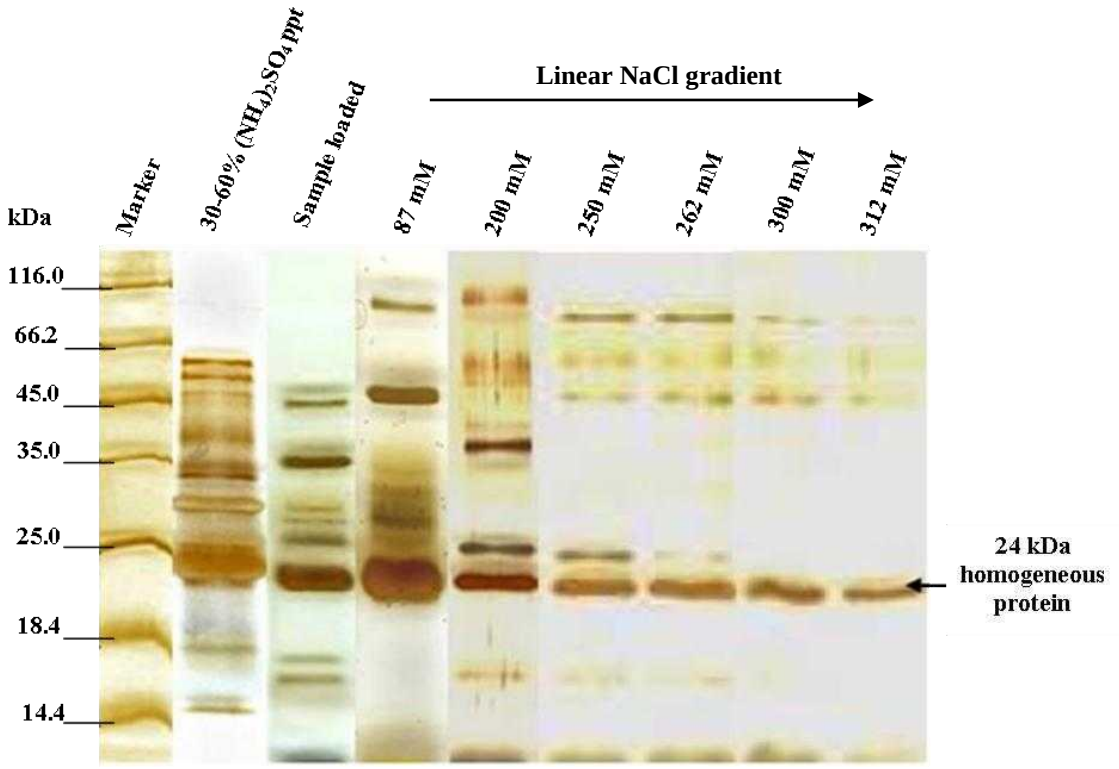


Fig 21. Elution of CM-sepharose bound proteins under 0-400 mM linear NaCl gradient.

The bound protein fractions from CM sepharose column were eluted by washing the column with 10 mM sodium acetate buffer (pH 5.5) under a linear salt gradient of 0-400 mM NaCl. The 1st fraction was had no NaCl and last fraction was fraction number 32 eluted at 400 mM NaCl concentration. Homogenous protein with apparent molecular weight of 24 kDa was eluted as the 25th to 28th fraction between approximately 312.5–350 mM NaCl concentration. At higher NaCl gradient (beyond 29th fraction) some impurities were seen, therefore those fractions were discarded. SDS-PAGE analysis of these CM sepharose bound protein fractions showed a clear resolution of proteins along the salt gradient as seen in Fig 21 above. Proteins eluted at 200 mM NaCl concentration showed separation of 24 and 25 kDa proteins along with a 35 kDa protein. As the salt concentration was increased the 25 and 24 kDa proteins separated out giving a homogenous 24 kDa protein towards the end of salt gradient as seen in the lane with 312 mM NaCl concentration as seen in Fig 21.

3.8 Identification of purified pearl millet protein (24 kDa).

3.8.1 Immuno blot analysis of the purified protein.

Purification was achieved by chromatography of the ammonium sulphate precipitated protein (30-60% saturation) through hydroxyapatite column followed by cation exchange chromatography using CM sepharose column. The purified protein had an apparent molecular mass of 24 kDa (reference Fermentas unstained protein marker # SM0431) which was resolved

on 12% SDS-PAGE and stained with Coomassie Brilliant Blue. The purified protein resolved on 12% SDS-PAGE was subsequently immuno blotted (Figure 22) with anti chitinase antibody from tobacco raised in rabbit (1:5000 dilution, section 2.4 of materials and methods). Secondary antibody was antirabbit antibody (raised in goat) tagged with alkaline phosphatase used at a dilution of 1:20000.

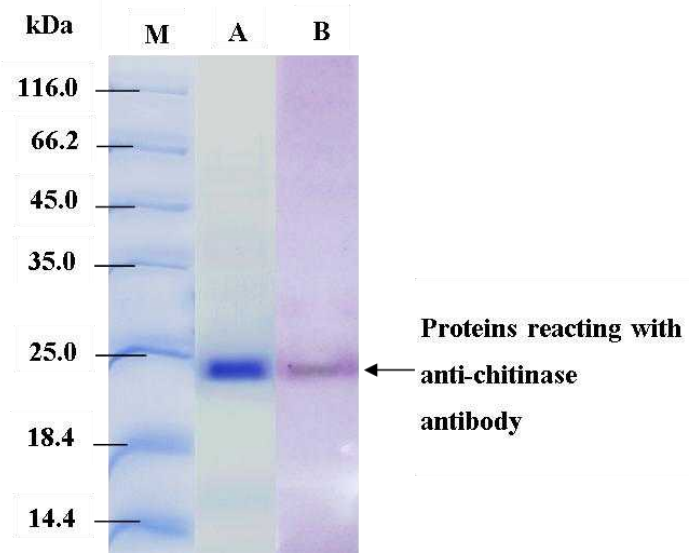


Fig 22. Immunoblot analysis of purified pearl millet chitinase 24 kDa with anti chitinase antibody.

Lane M: Unstained protein marker (Fermentas # SM0431), Lane A: Purified pearl millet chitinase, Lane B: Pure 24 kDa protein showing cross reaction with anti-chitinase antibody (10 µg of protein was loaded into each lane.

3.8.2 In-gel trypsin digestion of the excised protein band and *de novo* sequencing.

The pure 24 kDa protein band (Fig 22) was carefully excised from the Coomassie stained gel and subjected to in-gel trypsin digestion (Jensen *et al*., 1998). The peptides resulting from trypsin digestion were recovered from the gel and sequenced by ESI Q-TOF. The deduced peptide sequences are compiled in the form of a table below. The deduced peptide sequences show homology to maize endochitinase (Swiss prot accession number sp|P29022). The mass spectra of the peptide fragments are included under the appendix 1. The table 10 shows the mass and charge of the peptide fragments their deduced amino acid sequence and sequence similarity position in comparison with the maize endochitinase having Swiss prot accession number sp|P29022.

Table 10. Amino acid sequence of the peptide fragments generated by trypsin digestion of purified 24 kDa chitinase from pearl millet.

(*indicates oxidised methionine residues, the amino acids in upper case indicate highly accurate sequences generated automatically and the ones in lower case represent the amino acids that are predicted).

Band	Mass	Charge	Rel. Mass kDa	Derived amino acid sequence	Sequence similarity position	Organism	Possible function	Accession number
24kDa	475.63	2+	951.26	SAFLEAANK	SAFLEA 109 S+FLEA 113	*Zea mays*	chitinase A	Swissnew P29022
24kDa	525.46	3+	1050.92	TALWFWMNNVHR	ALWFWMNNVH 219 ALWFWMNNVH 228	*Tripsacum dactyloides*	chitinase	Sptrembl Q6JBL6
24kDa	530.78	3+	1592.34	TALWFWM*NNVHR	ALWFWMNNVH 219 ALWFWMNNVH 228	*Zea diploperennis*	chitinase	Sptrembl Q6JBP3
24kDa	532.12	3+	1596.36	YSGFAHGgTEVEgkR	YSGFAHGGTEVEGKR 117 YPGFAHGGTEVEGKR 132	*Oryza sativa*	chitinase	Sptrembl Q7Y1Z0
24kDa	582.64	2+	1165.28	VMPQGFGATTR	VMPQGFGATTR 230 VMPQGFGATIRA 240	*Oryza sativa*	chitinase	Sptrembl Q7Y1Z0
24kDa	590.65	2+	1181.30	VM*PQGFGATTR	VMPQGFGATTR 230 VMPQGFGATIR 240	*Oryza sativa*	chitinase	Sptrembl Q7Y1Z0
24kDa	632.15	4+	2528.60	asFLEAANKYSGFAHGGTEVEGKR	FLEAANKYSGFAHGGTEVEGKR 109 FLSAVNAYPGFAHGGTE VEGKR 132	*Tripsacum dactyloides*	chitinase	Sptrembl Q6JBL6
24kDa	787.70	2+	1575.40	TALWFWMNNVHR	TALWFWMNNVH 218 TALWFWMNNVH 228	*Zea mays*	chitinase A	Swissnew P29022
24kDa	855.24	2+	1710.48	ganvASVVTDAFFN gLK	GANVASVVTDAFFNGK 77 GANVANVVTDAFFNGIK 93	*Zea mays*	chitinase A	Swissnew P29022
24kDa	896.72	2+	1793.44	GPLQLSWNYNYGPAGR	GPLQLSWNYNYGPAGR 180 GPLQISWNYNYGPAGR 194	*Zea mays*	chitinase A	Swissnew P29022

3.8.3 Homology search for deduced amino acid sequences using MS Blast software.

Homology search of the deduced amino acid sequence showed that the purified 24kDa protein belongs to the class of chitinases. The peptides shared homology to maize endochitinase with Swiss prot accession number sp|P29022.

3.9 Substrate affinity chromatography

3.9.1 Purification of chitinase by affinity chromatography.

Chitinase was purified by an affinity procedure according to the method of (Hsu and Lockwood 1975) (section 2.7.9 of materials and methods). 30-60% ammonium sulphate saturated fraction was dialysed against 10 mM sodium phosphate buffer (pH 7.0) and loaded onto colloidal chitin column (15.0 cm height and 1.25 cm radius, height of bed 10 cm) equilibrated with the same buffer. Chitin bound sample was eluted with 10 mM acetic acid, the eluted protein fraction was dialysed against 10 mM sodium phosphate buffer (pH 7.0) and concentrated using Vivaspin sample concentrator tubes.

3.9.2 Immuno blot analysis of chitinase bound proteins.

The samples were analysed using SDS-PAGE as explained in section 2.3 of materials and methods and subsequently immuno blotted against anti-chitinase antibody from tobacco raised in rabbit (section 2.4 of materials and methods) Fig 23.

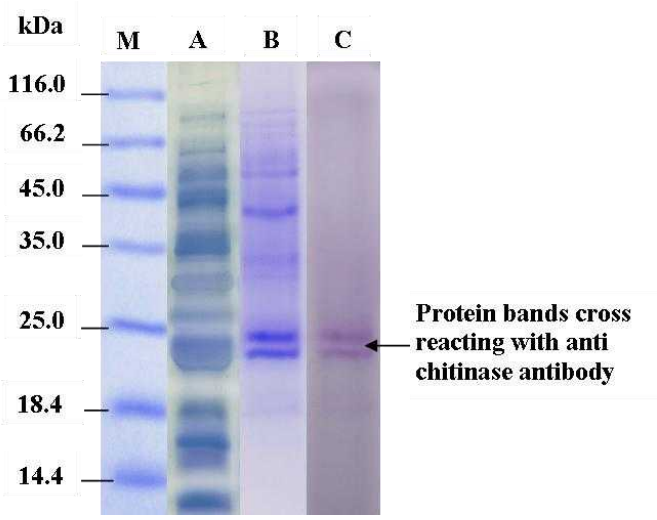


Fig 23. Purification of pearl millet chitinase by substrate affinity chromatography with colloidal chitin column.

Lane M: Unstained protein marker (Fermentas # SM0431); Lane A: 30-60% ammonium suphate saturated fraction (20 µg of protein/lane); Lane B: colloidal chitin bound protein fraction; Lane C: Colloidal chitin bound proteins showing cross reaction with anti-chitinase antibody (10µg of protein was loaded into each lane). The primary antibody was used at a dilution of 1:5000 and the anti rabbit secondary antibody (raised in goat) tagged with alkaline phosphatase was used at a dilution of 1:20,000.

For this, separating gel was prepared with 1% glycol chitin which is the substrate for chitinase. Thirty µg of total soluble protein fraction and 10 µg of the 24 kDa purified protein was electrophoretically separated on 12% polyacrylamide gel under denaturing conditions (2.3 of materials and methods). The proteins in the gel were renatured after electrophoresis by incubating in renaturation buffer at 37°C overnight (Potvin *et al.*, 1998, Audy *et al*., 1989) with gentle shaking. The gel was washed twice with 100 mM Tris HCl and stained with 100 mL of 0.5 M Tris-HCl, pH 9.0 containing 3-4 grains of fluorescent brightener (Fluorescent brilliant 28, Sigma # F343) for 5 min. The gel was rinsed twice with deionised water and observed under UV illuminator Fig 24.

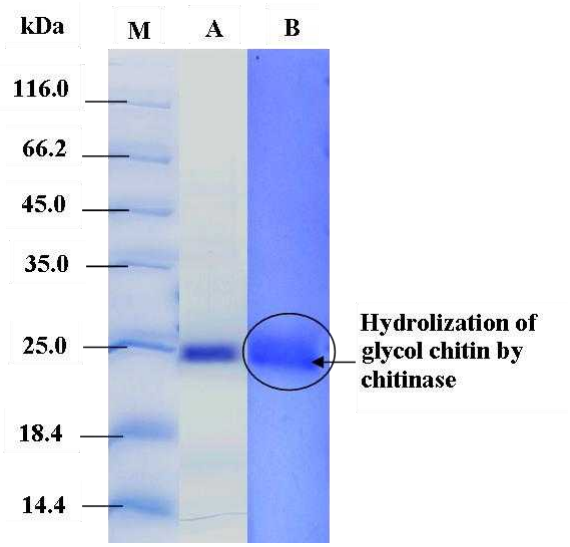


Fig 24. In gel assay to detect chitinolytic activity of the purified pearl millet chitinase.

Lane M: Protein marker (Fermentas # SM0431), Lane A: Purified pearl millet chitinase (24 kDa); Lane B: Hydrolization of glycol chitin by pearl millet chitinase (10µg of protein was loaded into each lane).

Glycol chitin (substrate for chitinase) in the gel hydrolyzed by purified chitinase band appeared as dark blue (slightly diffused) band against pale blue background of the gel under UV light as seen in Fig 24. This suggests chitinolytic activity of the purified pearl millet chitinase.

3.10 Partial purification of pearl millet proteins by size exclusion chromatography using FPLC (Fast Protein Liquid Chromatography) system.

This chromatographic method involves separation of proteins by the principle of size exclusion. Here a gel matrix with specific pore size serves as stationary phase; and the solvent (inorganic buffer solution or organic solvents) serves as the mobile phase. The small protein molecules can enter the gel beads and are eluted slowly from the column. The larger protein molecules cannot enter the gel beads because of their bigger size and hence flow through the space between gel particles and therefore move quickly through the column. Retention time of the protein molecule therefore is determined by its size; and by knowing the retention time of the protein molecule, size can be estimated.

In the present study Superose-12 column (Pharmacia, particle size 10 ± 2 μm) was used. The standard protein solutions used were of 1 mg/mL concentration in 20 mM sodium phosphate buffer (pH7.0); volume of the standards loaded to the column was 500 μL. Flow rate was adjusted to 0.5 mL/min. Volume of each fraction was 1 mL. Different protein standards used to calibrate the FPLC column with their corresponding molecular weights and retention time are listed in the table 2 below. Proteins were detected at 280 nm using a UV detector attached to FPLC.

Table 11. Standards used to calibrate Superose-12 FPLC column

Serial No.	Standard protein	Size in kDa	Retention time	Elution volume	Retention factor
1	Bluedextran	2000.0	17 min	8.5 mL	1.00
2	Ferritin	400.0	21 min	10.5 mL	0.81
3	Catalase	240.0	23 min	11.5 mL	0.74
4	BSA	66.0	25 min	12.5 mL	0.68
5	Ovalbumin	45.0	27 min	13.5 mL	0.63
6	Carboanhydrase	30.0	31 min	15.5 mL	0.55
7	Myoglobin	17.0	35 min	16.0 mL	0.53
8	Cytochrome C	12.4	39 min	14.5 mL	0.59
9	Pearl millet chitinase	24.0	25 min	12.0 mL	0.48

To estimate the molecular weight of purified pearl millet chitinase, it was passed through Superose 12 column using a FPLC system after calibrating the column with different protein standards mentioned above. CM sepharose bound proteins were eluted by applying 0-400 mM NaCl gradient. The fractions eluted at lower salt concentration of the gradient (showed strong protein bands of apparent molecular mass 22-35 kDa. These fractions were pooled, desalted by dialyzing against 20 mM sodium phosphate (pH 7.0) buffer and concentrated with Vivaspin concentrator tubes until 1 mg/mL concentration was achieved. Concentration was measured by Bradford method (section 2.2.2 of materials and methods). Five hundred μL of the concentrated sample (in 20 mM sodium phosphate buffer pH 7.0) was loaded onto Superose -12 column equilibrated with the same buffer. The fraction size was 1 mL and only the fraction containing protein as detected by the UV detector attached to FPLC (section 2.8.5 of material and methods) were analysed on 12% SDS-PAGE. Rests of the fractions were discarded. Only one major peak was observed at 25th min (retention time) and was eluted as 12th sample (elution volume 12 mL). The reason could be that the protein is aggregated and therefore of a higher molecular weight in contrary to 24 kDa as observed under SDS-PAGE analysis. Moreover, it can be observed that the pore size of Superose 12 column is not able to efficiently separate the three major proteins in the molecular weight range of 23-33 kDa. Therefore all the three bands elute together as a single fraction from the column without any resolution Fig 25.

Purification of protein fractions by Superose-12 column (FPLC)

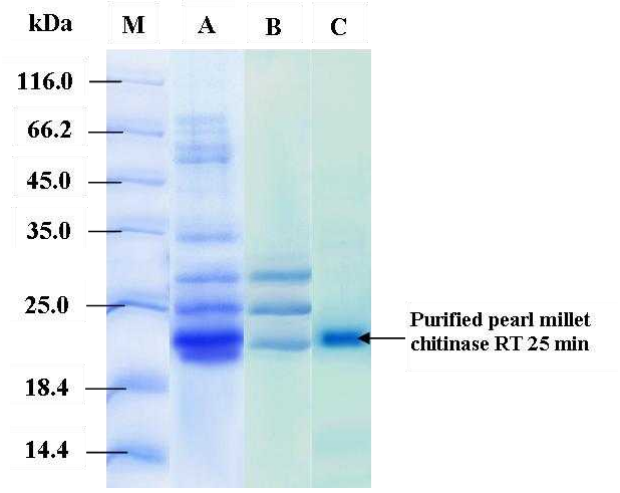


Fig 25. Purification of protein fractions by Superose-12 column (FPLC)

Lane M: Unstained protein marker (Fermentas # SM0431), Lane A: CM-Sepharose bound proteins pooled (which was loaded onto Superose 12), Lane B: Proteins eluted from Superose 12 column (retention time 25 min). Lane C: Purified pearl millet chitinase passed through FPLC as a separate fraction with 25 min retention time (RT).

CM sepharose bound fractions eluted at low salt gradient containing proteins in the molecular weight range of 20-35 kDa were pooled and desalted. This was loaded onto Superose 12 column (500 µL containing 1 µg/µL of protein). Separation of the CM sepharose bound protein fraction resulted in enrichment of three proteins in the molecular weight range on 23 kDa, 26 kDa and 28 kDa as shown in the Figure. All these three proteins were eluted as a single fraction since the three proteins do not differ much in their molecular mass and have the same retention time. However 35 kDa protein and the higher molecular weight impurities were not observed in this 25th fraction. Further separation of these three proteins (23 kDa, 24 kDa and 26 kDa) on Superose 12 column was not possible.

Purified pearl millet chitinase (500 µL containing 1 µg/µL of protein) was passed through the same column which eluted at 25 min.

3. 11. Identification of various PR proteins from pearl millet by *de novo* sequencing.

As it was not possible to achieve the goal of purifying pearl millet TLP by ion exchange chromatographic methods, the next strategy was to get the partial amino acid sequence of the protein and translate it to get the nucleotide sequence which would serve as reference for primer designing. Therefore different protein bands falling in the molecular weight range of 22-35 kDa were excised from the gel; the peptide fragments were generated by in-gel trypsin digestion and subjected to *de novo* sequencing by ESI Q-TOF. The protein bands considered for analysis are marked in the Fig 26.

Protein bands used for *de novo* sequencing by ESI-Q TOF.

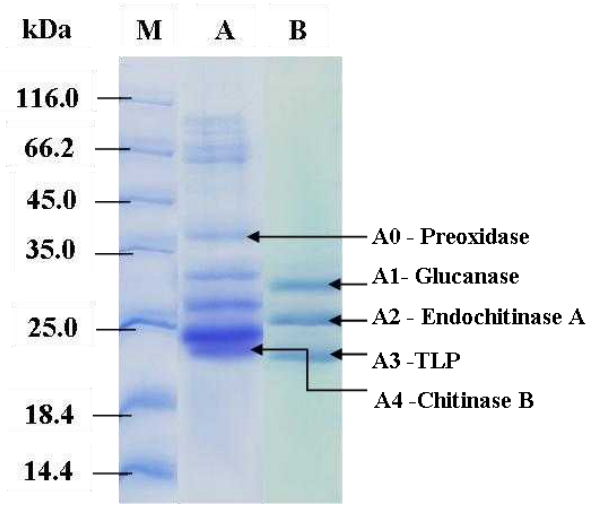


Fig 26. Protein fractions purified by Superose-12 column (FPLC) subjected to *de novo* sequencing.

Lane M: Unstained protein marker (Fermentas # SM0431), Lane A: CM-Sepharose bound proteins pooled (which was loaded onto Superose 12), Lane B: Proteins eluted from Superose 12 column (12th fraction, retention time 25 min) the bands subjected to de novo sequencing are marked. (Retention time 25 min).

The deduced peptide sequences were subjected to homology search using MS Blast tool from EMBL which is compiled briefly in separate tables listed below. The mass spectra and the detailed compilation of the data can be seen in appendix 1.

Band	Protein identified as	Deduced amino acid sequences compiled under
➢ Band A0	Peroxidase	Table 12
➢ Band A1	Glucanase	Table 13
➢ Band A2	Endochitinase A	Table 14
➢ Band A3	Thaumatin-like protein	Table 15
➢ Band A4	Chitinase B	Table 16

Table 12. Amino acid sequence of the peptide fragments generated by trypsin digestion of protein band A0.

(*indicates oxidised methionine residues, the amino acids in upper case indicate highly accurate sequences generated automatically and the ones in lower case represent the amino acids that are predicted).

Band	Mass kDa	Charge	Rel. Mass kDa	Derived amino acid sequence	Sequence similarity position	Organism	Possible function	Accession number
A0	468.18	2+	936.36	SFVQEAVR	SFVQEAVR 57 RKFVRDAVR 65	*Oryza sativa*	Peroxidase	sp|Q94DM0
A0	492.72	2+	985.44	DVGLAAGLLR	DVGLAAGLLR 67 DIGLAAGLLR 76	*Hordeum vulgare*	Peroxidase	sp|Q42854
A0	520.70	2+	1041.40	YYVNLVNR	YYVNLVNR 267 YYVNLVNR 274	*Triticum aestivum*	Peroxidase precursor	sp|Q8LK23
A0	529.72	2+	1059.44	VLTGSQGQLR	VLTGSQGQLR 320 VLTGSQGQVR 329	*Hordeum vulgare*	Peroxidase	sp|Q40069
A0	546.21	2+	1092.42	SFVQEAVrr	SFVQEAVR 57 RKFVRDAVR 65	*Zea mays*	Anionic peroxidase	sp|O04710
A0	566.70	2+	1144.40	GSLFDFYQR	FDFY 42 FDFY 45	*Oryza sativa*	Peroxidase	sp|Q94DM0
A0	597.70	2+	1195.40	(sd)VVASGG	VVASGG 149 VVASGG 154	*Oryza sativa*	Peroxidase	sp|Q94DM0
A0	682.22	2+	1364.44	AFFDQFGSSMVK	AFFDQFGSSMVK 303 AFFDQFAVSMVK 314	*Triticum aestivum*	Peroxidase precursor	sp|Q8LK23
A0	690.23	2+	1380.46	AFFDQFGSSM*VK	AFFDQFGSSMVK 303 AFFDQFAVSMVK 314	*Oryza sativa*	Peroxidase	sp|Q94DM0
A0	807.29	2+	1614.58	EGLFTSDQDLF	EGLFTSDQDLF 275 EGLFTSDQDLF 285	*Nicotiana tabacum*	Peroxidase	sp|Q94IQ1

The derived amino acid sequence of peptides recovered from in gel trypsin digestion of protein band A0 showed homology to Peroxidase from O*ryza sativa* with the accession number sptrembl|Q94DM0.

Table 13. Amino acid sequence of the peptide fragments generated by trypsin digestion of protein band A1.

(*indicates oxidised methionine residues, the amino acids in upper case indicate highly accurate sequences generated automatically and the ones in lower case represent the amino acids that are predicted).

Band	Mass kDa	Charge	Rel. mass kDa	Derived amino acid sequence	Sequence similarity position	Organism	Possible function	Accession number
A1	436.68	2+	873.36	QPVYPLR	PVYPLRFYDE 17 PVATLRYYDE 26	*Oryza sativa*	Endo-glucanase	spl|Q8GT15
A1	478.65	2+	957.30	FYDEQVR	FYDEQVR 1 FYDAKVS 6	*Oryza sativa*	Endo-glucanase	sp|Q8S9Q6
A1	497.19	2+	994.38	SEVVQLYR	-SEVVQLY 338 RSEVVQMY 345	*Oryza sativa*	Glucanase	sp|Q8W4V0
A1	513.18	2+	1026.36	YYQPLWR	-----YYQ 534 VQLYHFYQ 541	*Triticum aestivum*	Glucosidase precursor	sp|Q8GT15
A1	550.19	2+	1100.38	NFGLFYPNK	FGLFYPNK 615 FGLFYPNK 622	*Hordeum vulgare*	Glucanase	sp|O64938
A1	588.51	2+	1177.02	fqpTGTVR	fqpTGTVR 511 FQPGTTVR 518	*Oryza sativa*	Glucanase	sp|Q8GT15
A1	686.74	2+	1373.48	TYNQGLLDHVGR	YNQGLLDHVGR 573 RAYNQGLIDHVGR 584	*Oryza sativa*	Endo-glucanase	sp|Q8S9Q6
A1	719.23	3+	2157.69	YLAV	YLAV 116 RYIAV 119	*Oryza sativa*	Endo-glucanase	sp|Q8S9Q6
A1	757.81	2+	1515.62	NLVSALASAGLGGSGLK	ALASAGLGGS 118 ALVSAGLSSS 127	*Oryza sativa*	Glucanase	sp|Q8W4V0
A1	775.29	2+	1550.58	LYFPDQQALDALR	LYFPDQQALDALR 36 IYYPDKEALDALR 48	*Triticum aestivum*	Glucosidase precursor	sp|Q8GT15
A1	816.79	2+	1633.58	lgvC*YGVLGNNLPDK	gvCYGVLGNNLP 8 GVCYGVLGDNLP 15	*Oryza sativa*	Glucanase	sp|Q8W4V0
A1	863.31	2+	1726.62	dlSLGYATFQPGTTVR	dlSLGYATFQPGTTVR 188 DISLNYATFQPGTTVR 203	*Oryza sativa*	Glucanase	spl|Q8GT15

The derived amino acid sequence of peptides recovered from in gel trypsin digestion of protein band A1 showed homology to endo-1,3-beta- glucanase from *Oryza sativa* with the accession number sptrembl|Q8GT15.

Table 14. Amino acid sequence of the peptide fragments generated by trypsin digestion of protein band A2.

(*indicates oxidised methionine residues, the amino acids in upper case indicate highly accurate sequences generated automatically and the ones in lower case represent the amino acids that are predicted).

Band	Mass kDa	Charge	Rel. mass kDa	Derived amino acid sequence	Sequence similarity position	Organism	Possible function	Accession number
A2	475.69	2+	951.38	SAFLEAANKYSGFAHGGTEVEGKR	SAFLEAANKYSGFAHGGTEVEGKR 109 SAFLSAVNAYPGFAHGGTEVEGKR 132	*Zea mays*	Chitinase A	sp|P29022
A2	582.72	2+	1165.44	Y*GFAHGGTEVEGKR	Y*GFAHGGTEVEGKR 119 YPGFAHGGTEVEGKR 132	*Tripsacum dactyloides*	Chitinase A	sp|Q6JBL6
A2	896.83	2+	1793.66	GPLQLSWNYNYGPAGR	GPLQLSWNYNYGPAGR 179 RGPLQISWNYNYGPAGR 195	*Zea diploperennis*	Chitinase B	sp|Q6JBP3
A2	787.79	2+	1575.58	TALWFWMNNVH	TALWFWMNNVH 218 TALWFWMNNVH 228	*Zea mays*	Chitinase A	sp|P29022
A2	590.71	2+	1181.42	VMPQGFGATTR	VMPQGFGATTR 230 VMPQGFGATIR 240	*Zea mays*	Chitinase A	sp|P29022

The derived amino acid sequence of peptides recovered from in gel trypsin digestion of protein band A2 showed homology to chitinase A from *Zea mays* with swissprot accession number sp|P29022.

Table 15. Amino acid sequence of the peptide fragments generated by trypsin digestion of protein band A3.

(*indicates oxidised methionine residues, the amino acids in upper case indicate highly accurate sequences generated automatically and the ones in lower case represent the amino acids that are predicted).

Band	Mass kDa	Charge	Rel. mass kDa	Derived amino acid sequence	Sequence similarity position	Organism	Possible function	Accession number
A3	459.66	2+	919.32	C*AVDVNAR	C*AVDVNAR 145 CAVDVNAR 152	*Zea mays*	Thaumatin-like protein	sp|P33679
A3	475.68	3+	1427.04	n-AVDVNAR	AVDVNAR 146 AVDVNAR 152	*Oryza sativa*	Thaumatin-like protein	sp|Q75GX4
A3	511.50	3+	1534.50	PAELR	PAELR 154 PAELR 158	*Hordeum vulgare*	Thaumatin-like protein	sp|Q946Y8
A3	522.22	2+	1044.44	LSVPAGT(taar	LSVPAGTtaar 54 RITAPAGTTAAR 65	*Oryza sativa*	Thaumatin-like protein	sp|O04364
A3	786.24	2+	1572.48	tgdCGGVLAC*TGYGR	tgdCGGVLACTGYGR 84 TGDCGGVLQCTGYGR 98	*Hordeum vulgare*	Thaumatin-like protein	sp|Q946Y9
A3	881.75	2+	1763.50	ATSTFTC*PAGTNYK	ATSTFTCPAGTNYK 209 ATSTFTCPAGTNYK 222	*Oryza sativa*	Thaumatin-like protein	sp|Q75GX4
A3	650.19	2+	1300.38	GQC*PVSYSYPK	GQCPVSYSYPK 196 GQCPDAYSYPK 206	*Zea mays*	Thaumatin-like protein	sp|P33679
A3	714.21	2+	1428.42	TGDTGYGR	TGDT--------GYGR 83 RTGDCGGVLQCTGYGR 98	*Zea mays*	Thaumatin-like protein	sp|P33679

The derived amino acid sequence of peptides recovered from in gel trypsin digestion of protein band A3 showed homology to zeamatin from *Zea mays* with swissprot accession number sp|P33679.

Table 16. Amino acid sequence of the peptide fragments generated by trypsin digestion of protein band A4.

(*indicates oxidised methionine residues, the amino acids in upper case indicate highly accurate sequences generated automatically and the ones in lower case represent the amino acids that are predicted).

Band	Mass	Charge	Rel. Mass	Derived amino acid sequence	Sequence similarity position	Possible function	Organism	Accession number
A4	425.29	2+	850.58	SAFLNAVK	SAFLNAVK 115 SAFLSAVK 122	Chitinase	*Zea diploperennis*	UNIPROT. Q6JBP3
A4	432.67	2+	865.34	SLGFD(gl)R	SLGFDgl++++R 202 AIGFDGLGDPGR 213	Chitinase	*Sorghum bicolor*	XP002455593
A4	449.50	2+	899.00	SLGFD(glr)n(pd)r	SLGFDgl+np+r 202 AIGFDGLGDPGR 213	Chitinase	*Zea diploperennis*	UNIPROT. Q6JBP3
A4	513.87	2+	1027.74	PQGFGATTR	PQGFGATTR 238 PQGFGATTR 247	Chitinase	*Zea diploperennis*	UNIPROT. Q6JBP3
A4	534.70	2+	1069.40	AALWYWMK	AALWYWMK 224 AALWFWM 229	Chitinase B1	*Sorghum bicolor*	sp|Q93WT1
A4	537.23	2+	1074.46	VAQDPVVAFK	VA+D+VVAFK 214 VARDAVVAFK 223	Class IV chitinase	*Oryza sativa*	sp|O04138
A4	582.72	2+	1165.44	VMPQGF(ga)ttr	V+PQGFgattr 236 VVPQGFGATTR 246	Chitinase	*Zea diploperennis*	UNIPROT. Q6JBP3
A4	673.76	2+	1347.52	SLGFDG(lr)NPDR	SLGFDGlrNP+R 202 AIGFDGLGDPGR 213	Chitinase	*Sorghum bicolor*	XP002455593
A4	770.30	2+	1540.60	NVHGVVPQGFGATTR	NVHGVVPQGFGATTR 233 +VHGVVPQGFGATTR 246	Chitinase	*Zea diploperennis*	UNIPROT. Q6JBP3
A4	882.82	2+	1765.64	GPLQLSWNYNYGPAGK	GPLQLSWNYNYGPAGR 186 GPLQISWNYNYGPAG+ 199	chitinase-B1	*Sorghum bicolor*	sp|Q93WT1
A4	896.82	2+	1793.64	GPLKLSWNYNYGPAGR	GPLKLSWNYNYGPAGR 186 GPLQISWNYNYGPAG+ 199	Chitinase	*Zea diploperennis*	UNIPROT. Q6JBP3

The derived amino acid sequence of peptides recovered from in gel trypsin digestion of protein band A4 showed homology to Chitinase B from *Zea diploperennis* with Uniprot accession number Q6JBP3.

Part D-Molecular biology

3.12 Characterization of *chitinase* and *tlp* from pearl millet.

After obtaining partial amino acid sequence of pearl millet chitinase and thaumatin like protein, it was aimed to isolate the corresponding gene so that it could be cloned and over expressed in bacterial expression vector for further studies. Suitable primers were designed and PCR conditions were optimized to amplify the gene of interest.

3.12.1 Amplification of pearl millet *chitinase* gene (*chi*).

Neither nucleotide sequence nor protein sequences for pearl millet chitinase are available in the database. Therefore peptide sequences deduced from *de novo* sequencing of the purified 24 kDa protein was considered for primer designing.

3.12.1 i) Primer designing.

The deduced amino acid sequence of the peptide fragments generated from in-gel trypsin digestion of the purified 24 kDa protein band showed homology to chitinase from *Sorghum bicolor* (NCBI Accession number: gi|15529119|gb|AAK97763). Pair wise alignment of *S. bicolor* (NCBI Accession number: gi|15529119|gb|AAK97763) chitinase amino acid and the nucleotide sequence is given in Fig 27 below; Yellow boxes indicate the primer sequence and the grey boxes indicate the corresponding Amino acid sequence.

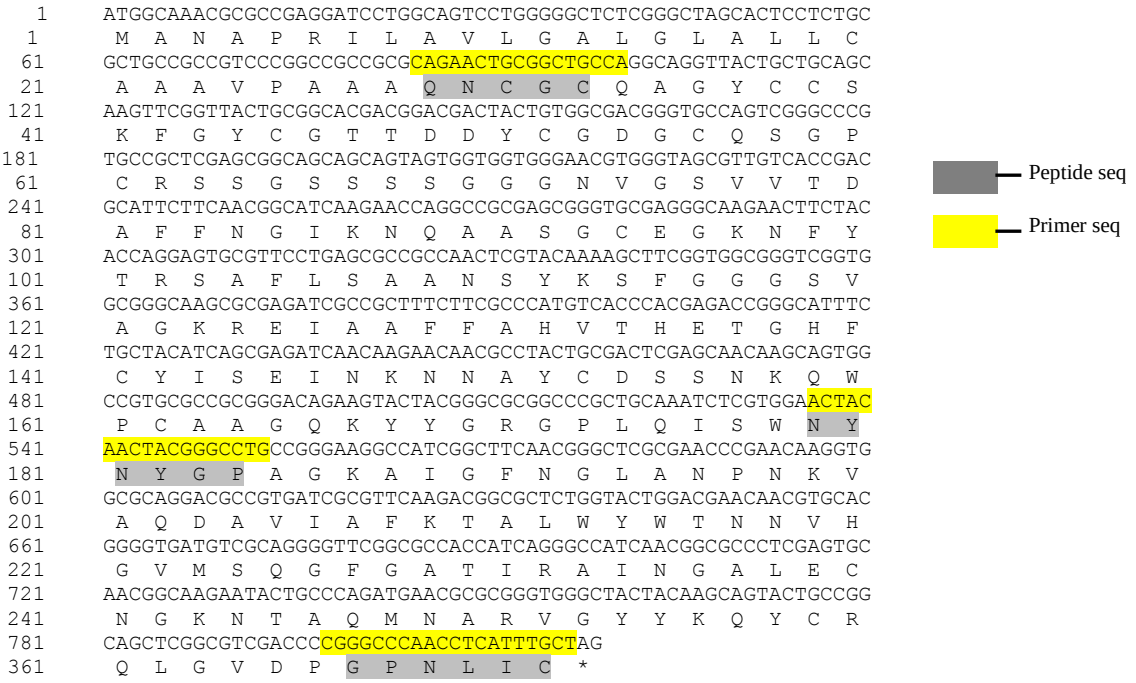


Fig 27. Primer design for amplification of chi gene from pearl millet based on homologous sequence of *Sorghum bicolor* chitinase (NCBI Accession number: gi|15529119|gb|AAK97763). Grey shaded boxes indicate amino acid sequences obtained by *de novo* sequencing of pearl millet chitinase and the primer sequence.

Primers were designed to amplify the gene of interest considering *S. bicolor* chitinase (NCBI Accession number: gi|15529119|gb|AAK97763) as reference are listed in table 17 below with their corresponding annealing temperatures.

Table 17. Primers designed to amplify chi gene from pearl millet based on homologous sequence of *Sorghum bicolor* chitinase (NCBI Accession number: gi|15529118|gb|AY047608.1).

Peptide sequence	Primer	Primer sequence	Annealing temperature
NCGCQ	1a) Sorg_Fwd	5′- cagaactgcggctgcca -3′	59°C
GPNLTC	1b) Sorg_Rev	5′- agcaaatgaggttgggcccg -3′	59°C

The primer pair 1a and 1b amplified approximately 800 bp fragment at 60°C annealing temperature using genomic DNA as template. Though the calculated annealing temperature was 59°C a strong band was amplified at 60°C. PCR product was separated on 1.5 % agarose gel with 100 bp ladder plus DNA marker as reference as illustrated in Fig 28.

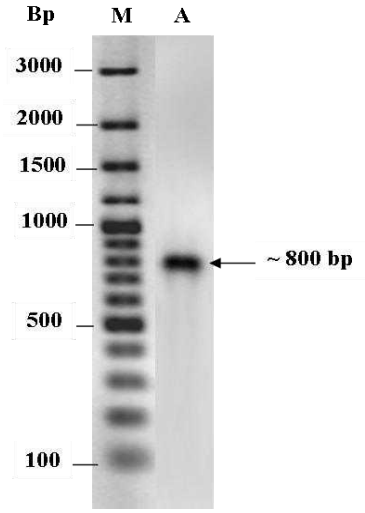


Fig 28. Amplification of ~800 bp product using primer pair 1a and 1b.

Lane M: 100 bp ladder plus (Fermentas # SM0321), Lane A: 790 bp product amplified by the primer pair 1a and 1b separated on 1.5% agarose gel.

The ~ 800 bp band was excised from the gel and purified using QIAEX II Gel Extraction Kit (Qiagen # 20021). Purified PCR product was cloned to pGEMT-Easy (Promega # A3600) TA-cloning vector and subsequently sequenced. The plasmid containing insert was sequenced in both the directions and the consensus sequence was subjected to homology search.

Nucleotide sequence of non specific product amplified by primer pair 1a and 1b

```
   1 NANCCACGTCAAGGCGAANNGNNTNGGNANNCANCNGACATACCTATCAGTNTGGNTCAG
  61 GTCGTAAGNCTTATCGNCNAGAGCCCGATTANCTGACGGAAAGCGCNAACNGGCGNCAAA
 121 TGAGGTTGGGCCCGCCAGAATACCCATTACAAGGGTTCGGAGGAAAACCAATCAAACCGG
 181 TTGGAAAAATATCACTCCCTGTTTCCTTCGAAAATCTTGACAATGCCAGAACAGAGAGTC
 241 TCACCTTCGATGTGGTTGATATCTATCACCTGTATCTCGCGATTTTCGGCAGAGGCTTTA
 301 TGAACAAGTTCGACGCGGTCATAAGGCAGCAGTTCTTATGTATGAAGATGCCTGCACCCA
 361 AGGGGGTTATCACAGTGTTCGGCAATCAACAAGAGGCCAGAAACATAGAAAAAGGCCATA
 421 CGCCCGGCCAGACGAACGTGTACCAGTTGAAAACAGCTGATGAGAAGAAGGAAACTTACG
 481 AAGAGGCCAAGCGGGATAAAGAAAAAATTGAAATAGCCGCAGATAGTGAAACCAAGAAAG
 541 TGTATCTGGATGACATGCCCGATAGAGCAGTCACAATAGGGGCTCACCTCAGTGCCGAAG
 601 AGGACAAAGACCTCATACAATTCCTGAACAAAAACAAAGATGTTTTCGCATGGTCAGCTA
 661 AGGACCTGCAAGGTGTTGACAGGGACATCATTGAACATGCCCTGGAAACAGATGAAAAAA
 721 TACCTCCCAAGAAACAGGAACTTCAGAAGATGTCCGAGGAAAAAGTGAAAGCAGTGGCAG
 781 CCGCAGTTCTGAATCACTAGTGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAA
 841 CGCGTTG
```

Fig 29. Consensus nucleotide sequence of 847 bp non specific product amplified by primer pair 1a & 1b.

This 847 bp fragment showed similarity to polyprotein *Sorghum bicolor* (NCBI Accession number: gb|AAD27571 gi|4680208) and polyprotein from *Zea mays* (NCBI Accession number: gi|18568275|gb|AAL76007). Since the primers 1a and 1b designed using *S. bicolor* chitinase as reference sequence amplified a non specific product, maize *endochitinase* (NCBI Accession number: gi|195627426 gb|ACG35543) with which purified pearl millet chitinase shares homology was used as reference to design new set of primers.

Pair wise alignment of *Zea mays endochitinase* (NCBI Accession number: gi|195627425 gb|EU963425.1) amino acid and nucleotide sequence; amino acid sequences deduced by *de novo* sequencing of purified pearl millet chitinase are indicated by grey shaded boxes and the yellow boxes indicate the primer sequence in Fig 30.

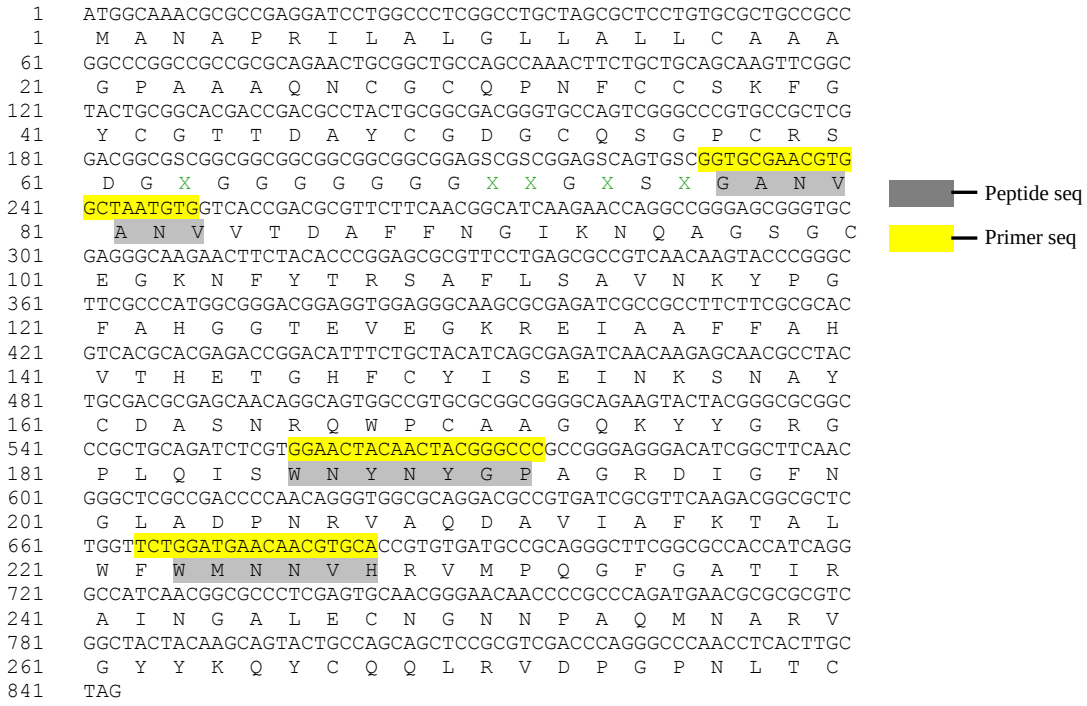


Fig 30. Primer design for amplification of *chi* gene from pearl millet based on homologous sequence of *Zea mays endochitinase* (NCBI Accession number: gi|195627425|gb|EU963425). Grey shaded boxes indicate amino acid sequences obtained by *de novo* sequencing of pearl millet chitinase and the primer sequence.

The primers designed considering *Zea mays* endochitinase (NCBI Accession number: gi|195627426|gb|ACG35543.1) as reference are listed in table 18 below along with their annealing temperatures.

Table 18. Primers designed to amplify *chi* gene from pearl millet based on homologous sequence of *Zea mays endochitinase* (NCBI Accession number: gi|195627425).

Peptide sequence	Primer sequence	Primer	Annealing temperature
GANVAN	5′-GGTGCGAACGTGGCTAA-3′	2a)Chi_252 Fwd	57
WMNNVH	5′-GTGCACGTTGTTCATCCAGA-3′	2b)Chi_684 Rev	58
ANVANV	5′-GTGCGAACGTGGCTAATGTG-3′	3a)Chi_230 Fwd	58
NYNYGP	5′-GGGCCCGTAGTTGTAGTTCC-3′	3b)Chi_709 Rev	57

The primer pair 2a and 2b was used to amplify the pearl millet *chitinase* gene as the next effort. However, no product was amplified by this primer pair as seen in Fig 31 below. Both cDNA as well as genomic DNA were used as templates and separate PCRs were performed; temperature gradient PCR was carried out with annealing temperatures ranging between 45-55°C and 55-65°C. Different PCR additives like PCR additives like DMSO (0-2%), Formamide (0-1%), Betaine (0-0.6 M) and $MgCl_2$ (0-2 mM) were also used. Positive control was ~ 350 bp (*nonspecific lipid transfer protein- nsltp*) product amplified by the primers Remo forward primer

5′-GGGCTGGTCCTCGAATTC AC-3′ Remo reverse primer 5′-GTGTATGAGTAATGGATC CG-3′ using 100 ng of genomic DNA from *Malus domestic* cv. Remo as template.

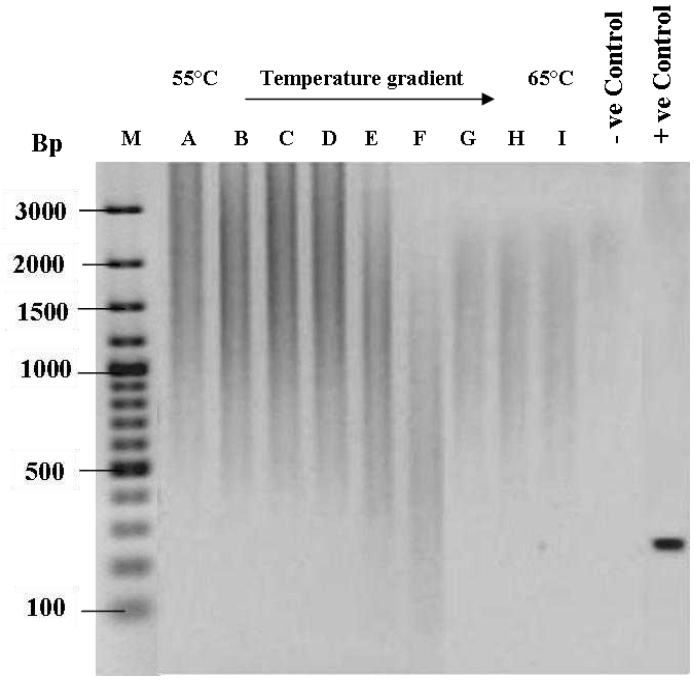


Fig 31. Amplification of pearl millet *chi* using primer pair 2a and 2b.

Lane M: 100 bp ladder plus (Fermentas #SM0321), Lane A-I: Amplification at different annealing temperatures (55-65°C) showing no product, negative control is PCR carried out without any template DNA.

No products were obtained with primer pair 2a and 2b, under varied PCR conditions tried. Therefore the next primer pair was used.

The primer pair 3a (Chi_Fwd primer_230) and 3b (Chi_Rev Primer_709) did not amplify any products with *Taq* DNA polymerase. Several efforts were made to optimize the PCR conditions. Temperature gradient PCR was carried out in the range of 45-65°C. Stepdown PCR was carried out with initial 10 cycles at 65°C annealing temperature, followed by 20 step down cycles with every cycle lower from the previous cycle by one degree annealing temperature. No PCR products were amplified under these conditions. Therefore PCR additives like DMSO and Betaine were added; and a weak band of approximately 500 bp was amplified at 59°C annealing temperature with DMSO (1%v/v) Betaine (0.6 M) and $MgCl_2$ (1.5 mM) along with a background smear. Genomic DNA was used as template (100 ng/ 25 µL reaction volume). Hot start PCR was carried out with initial denaturation at 96°C for 30 sec followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 59°C for 30 sec, elongation at 72°C for 30 sec and final elongation for 2 min at 72°C. Amplification with the primer pair 3a and 3b is illustrated in Fig 32.

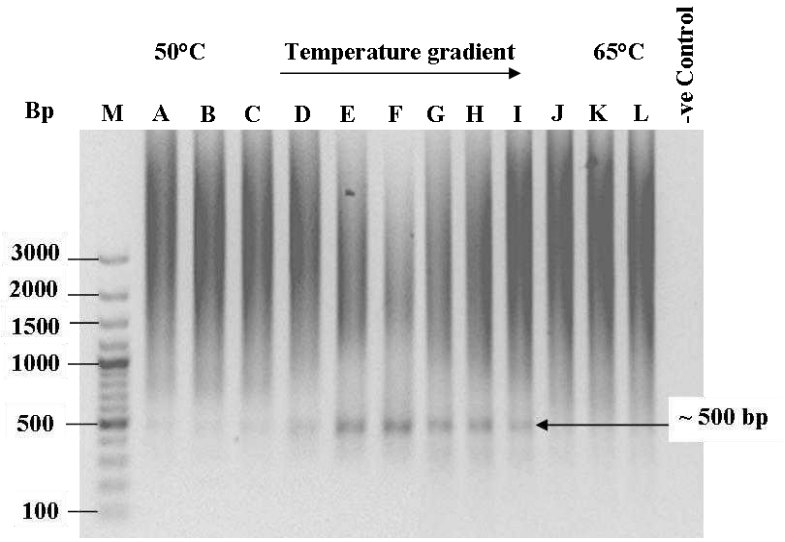


Fig 32. Temerature gradient PCR with primer pair 3**a and 3b and genomic DNA as template.**
Lane M: 100 bp ladder plus (Fermentas #SM0321), Lane A-L: Amplification at different annealing temperatures (50-65°C) using Dream*Taq*, separated on 1.5% agarose gel. Negative control is PCR without any template DNA.

However, it was not possible to get a clean band, the background smear could not be minimized under the conditions used for amplification; even the Reamplification of the product did not yield any satisfactory results. The product was not homogenous and could not be sequenced directly. As the next option KAPA2GRobust DNA polymerase kit (Peqlab # 07-KK5004-01) was used as it is known to give better amplification because of the presence of PCR enhancers which are the component of the kit.

A clear strong band was obtained with the primer pair 3a and 3b when KAPA2GRobust DNA polymerase (Peqlab # 07-KK5004-01) was used in combination with GC buffer, Enhancer 1 and Enhancer 2 at 1X concentration (components of KAPA2GRobust DNA PCR kit). PCR was carried out as per manufacturer's specification. Amplification was observed at 60-63°C annealing temperature as illustrated in the Fig 33.

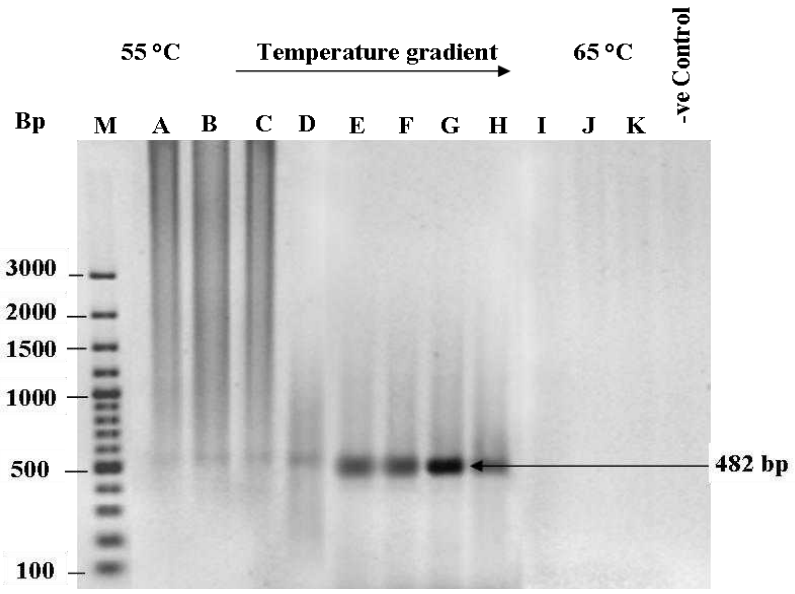


Fig 33. Pearl millet *chi* with the primer pair 3a and 3b using KAPA2GRobust DNA polymerase kit.

Lane M: 100 bp Ladder plus (Fermentas # SM0321) Lane A-K: Temperature gradient PCR, amplification of 482 bp product observed at 59-61°C using KAPA2GRobust DNA polymerase kit (Peqlab 07-KK2100-02). Last lane is the negative control with no template DNA.

The strong 482 bp band amplified at 63°C annealing temperature was excised from the agarose gel, purified using QIAEX II Gel Extraction Kit (Qiagen # 20021) and directly sequenced.

Nucleotide sequence of pearl millet *chitinase* (partial length) amplified by the primer pair 3a and 3b 482 bp.

```
  1 CCGCACGGGGAGCACGCCGGCAGCGGTAAAGGCGCGAACGTGGCCAGCGTCGTCACCGAC
 61 GCGTTCTTCAACGGCATCAAGAACCAGGCCGGGGGCGGGTGCGAGGGCCGGAACTTCTAC
121 ACGCGGAGCGCGTTCCTGGAGGCCGCCAACAAGTACTCCGGCNTCGCCCACGGCGGAACG
181 GAGGTCGAGGGCAAGCGCGAGATCGCCGCCTTCTTCGCGCACGTCACGCACGAGACCGGA
241 CGTAAGTTGGATGAATAGCTAAGCTAGCNCTCCAATCCGCCTCTCTACTACTGCAAACTC
301 CAATATTTCTTCTGCTGAACTAAATGTTCACGGCTTCATACCGACAGATTTCTGCTACAT
361 CAGCGAGATCAACAAGAACAACGCCTACTGCTCGAGCAACGCGCAGTGGCCGTGCGCCGC
421 GGGGAAGAAGTACTACGGGCGCGGGCCGCTGCAGATCTCGTGGAACTACAACTACGGGCC
481 CA
```

Fig 34. Nucleotide sequence of pearl millet *chi* (482 bp) amplified by the primer pair 3a and 3b.

The 482 bp sequence was subjected to homology search and it was observed to share homology with Maize *chitinase* (NCBI Accession number: gi|195627425). It was also observed that the pearl millet *chitinase* gene is interrupted by a 107 bp intron at 241 bp position to 348 bp position. Amplification was also carried out using KAPA HiFi DNA polymerase (Peqlab 07-

KK2100-01) with the same primer combination. Two clear bands were obtained at annealing temperature 55-57°C, no amplification was observed at higher annealing temperatures. Products of PCR were separated on 1.5% agarose gel as seen in Fig 35.

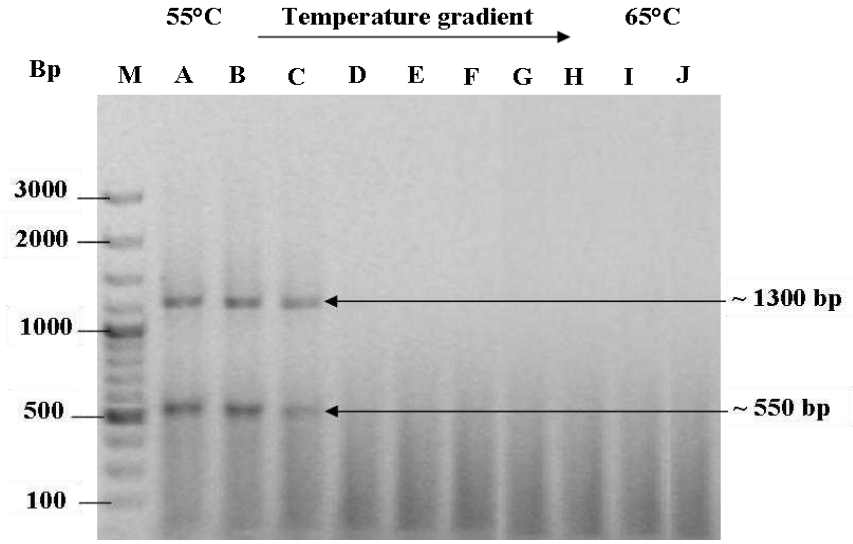


Fig 35. Amplification of pearl millet chi using Phusion HiFi DNA polymerase (NEB # F-530S) with primer pair 3a and 3b.

Lane M: 100 bp ladder plus (Fermentas #SM0321); Lane A-J: Amplification at different annealing temperatures (55-65°C).

The two bands were excised from the gel, purified using QIAEX II Gel Extraction Kit (Qiagen # 20021). Phusion HiFi DNA Polymerase (NEB # F-530S) produces blunt end PCR products. Several efforts were made to clone the two products to CloneJet PCR cloning kit (Fermentas # K1231). Strangely *E. coli* cells transformed with these fragments developed ampicillin resistance but were devoid of plasmids as illustrated in Fig 36.

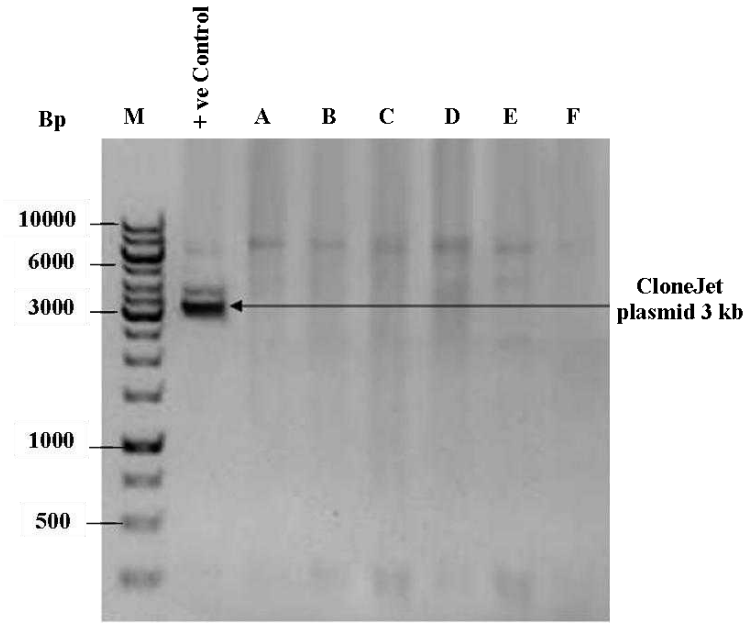


Fig 36. Plasmid isolation by HB lysis from ampicillin resistant transformants (cloning vector used CloneJet).
Lane M: 1kb DNA ladder (Fermentas # SM0311); Positive control: CloneJet plasmid - 3kb (Fermentas # K1231), Lane A-F Ampicillin resistant colonies devoid of plasmids indicating integration of plasmid into genomic DNA.

This lead to the conclusion that the ampicillin resistance gene from the plasmid along with the insert was getting cloned into genomic DNA of *E. coli* conferring antibiotic resistance to the transformants. Because of this reason the products were directly sequenced. Sequencing showed that these bands were not homogenous; therefore the bands were run on agarose gel for 2 hrs for better separation, then excised, purified and sent for sequencing. Though the band seemed visibly homogenous, sequencing revealed that the samples were not a homogenous band. Reamplification and purification of the product also did not yield any homogenous band. Increasing the annealing temperature above 58°C did not amplify any product. Therefore it was concluded that the Phusion HiFi DNA polymerase (NEB # F-530S) was not useful in this case, with genomic DNA as template. RT-PCR (Reverse transcriptase PCR) using total RNA as template did not yield any product. Therefore Phusion HiFi DNA polymerase was not used for PCRs with this primer pair.

3.12.2 Amplification of pearl millet *tlp* (thaumatin-like protein) gene.

Neither nucleotide sequence nor protein sequences for pearl millet genes encoding thaumatin-like protein were available in the database. Therefore peptide sequences obtained from *de novo* sequencing were considered for primer designing.

3.12.2 i) Primer designing

The deduced amino acid sequence of the peptides generated from in gel trypsin digestion of the protein band (Band A3 Fig 26) showed homology to maize zeamatin (NCBI Accession number: gi|459170). Pair wise alignment of maize TLP amino acid and nucleotide (NCBI Accession number: gb|AAA92882.1, gi|459170) sequence is seen in Fig 36 below. Yellow boxes indicate the primer sequence and the grey shaded box indicate corresponding amino acid sequence in Fig 37.

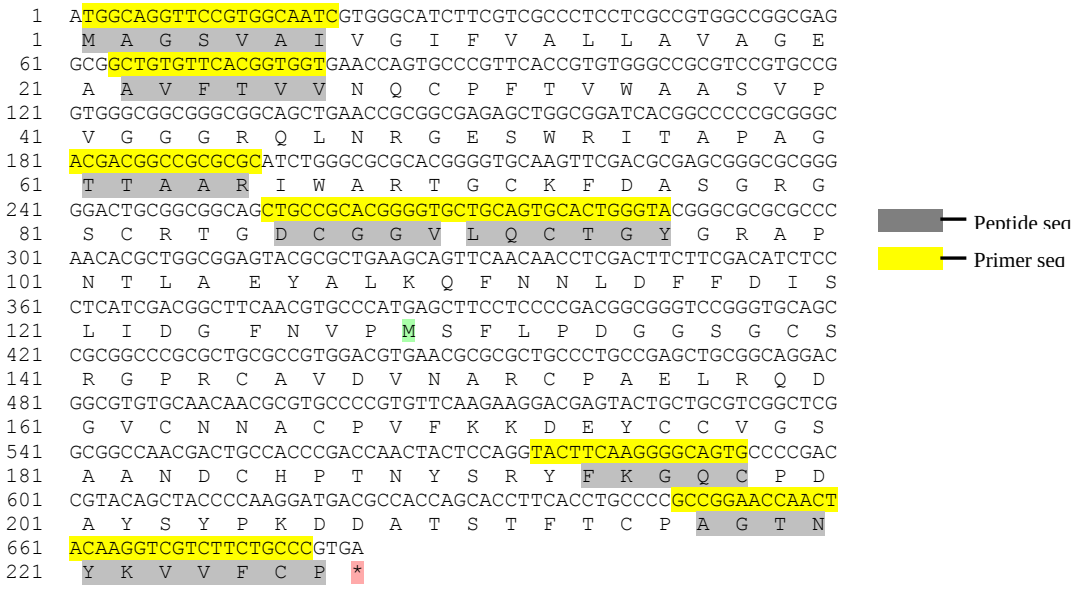


Fig 37. Primer design for amplification of *tlp* gene from pearl millet based on homologous sequence of zeamatin from maize (NCBI Accession number: gb|AAA92882.1, gi|459170). Grey shaded boxes indicate amino acid sequences obtained by *de novo* sequencing of pearl millet TLP and the primer sequence.

Primers designed to amplify pearl millet *tlp* gene with their corresponding annealing temperatures are compiled in the table 19.

Table 19. Primers designed to amplify *tlp* gene from pearl millet, based on homologous sequence of *zeamatin* from maize (NCBI Accession number: gi|459169|gb|ZMU06831).

Peptide sequence	Primer sequence	Primer	Annealing temp.
AVFTVV	5′-GCTGTGTTCACGGTGGT-3′	1a) Zea Fwd primer	59
YKVVFCP	5′-GGCAGAAGACGACCTTGTAG-3′	1b) Zea Rev primer	59
LQCTGY	5′-CTGCAGTGCACTGGGTA-3′	2a) Fwd primer_271	58
AGTNYK	5′-TGTAGTTGGTTCCGGCG-3′	2b) Rev primer_667	58
LQCTGY	5′-CTGCAGTGCACTGGGTA-3′	3a) Fwd primer_271	57
YFKGQC	5′-GCACTGCCCCTTGAAGTA-3′	3b) Rev primer_594	57
DCGGVL	5′-GACTGCGGCGGCGTGCT-3′	4a) Fwd primer_256	58
AGTNYK	5′-TGTAGTTGGTTCCGGCG-3′	4b) Rev primer_667	58
MAGSVAI	5′-ATGGCAGGTTCCGTGGCAATC-3′	5a) FL Fwd primer_92	58
YFKGQC	5′-GCACTGCCCCTTGAAGTA-3′	5b) Rev primer_594	58
MAGSVAI	5′-ATGGCAGGTTCCGTGGCAATC-3′	6a) FL Fwd primer_92	58
AGTNYK	5′-TGTAGTTGGTTCCGGCG-3′	6b) Rev primer_667	58
TTAAR	5′-ACGACGGCCGCGCGC-3′	7a) Pep Fwd	59
YFKGQC	5′-GCA CTGCCCCTTGAAGTA-3′	7b) Rev primer_594	58
TTAAR	5′-ACGACGGCCGCGCGC-3′	8a) Pep Fwd	59
AGTNYK	5′-TGTAGTTGGTTCCGGCG-3′	8b) Rev primer_667	58

Amplification of *tlp* gene using various primer combinations and nucleotide sequence of the corresponding amplicons are also given below.

The primer pair 1a and 1b amplified a 483 bp non-specific product at 59°C annealing temperature using Dream*Taq* polymerase from Fermentas (# EP0701) as seen in Fig 38.

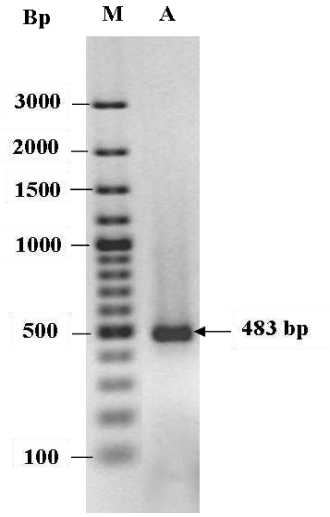


Fig 38. Amplification of 483 bp product by primer pair 1a and 1b

Lane M: 100 bp ladder plus (Fermentas # SM0321), Lane A: Product amplified by the primer pair 1a and 1b separated on 1.5% agarose gel.

PCR product was purified by gel extraction and was cloned into pGEMT-Easy (Promega # A3600) TA cloning vector and subsequently sequenced. Homology search of the 483 bp nucleic acid sequence showed no homology to known *tlp* sequences in the database, indicating the primer pair amplified a nonspecific product. Therefore new primers were designed to amplify *tlp* gene. Therefore the next set of primers was used.

The primer pair 2a (Fwd primer_271, 5′-CTGCAGTGCACTGGGTA-3′) and 2b (Rev primer_667, 5′- TGTAGTTGGTTCCGGCG-3′) amplified ~ 300 bp product at 58°C annealing temperature using Dream*Taq* polymerase from Fermentas (# EP0701) as illustrated in Fig 39.

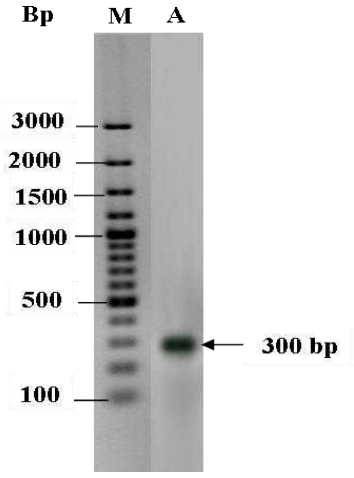


Fig 39. Amplification of pearl millet *tlp* gene using primer pair 2a and 2b.

Lane M: 100 bp ladder plus (Fermentas # SM0321), Lane A: 300 bp Product amplified by the primer pair 2a and 2b separated on 1.5% agarose gel.

Nucleotide sequence of 300 bp product amplified by the primer pair 2a and 2b.

```
  1 GCACGCTGGCCGAGTACGGGCTGAACAAGTACCAGAACCTCGACTTCTTCGACATCTCGC
 61 TGGTGGACGGGTTCAACGTGCCCATGGACTTCCTCCCGGCGGGCAGCGGCGCCGGGTGCC
121 CCAAGGGCGGGCCGCGGTGNNGCGNCNACGTCACGGGGCAGTGCCNCNNCNCGCTGCGCG
181 CCACCNGCGGCTGCAACAACCCGTGNACGGTGTTCAAGANGGACNANTACTGCTGCACGG
241 GGTCGGNGGCGAACAGCTGCGGCGCCACCGACTACTCGAGGTACTTCAAGGGGNAGTGCA
```

Fig 40. Nucleotide sequence of 300 bp PCR product amplified with primer pair 2a & 2b.

The nucleotide sequence shared homology with *Sorghum bicolor tlp* (gi|242038634:61-756) and maize *tlp* (NCBI Accession number: gi|226505631:49-741 *Zea mays* LOC100281135) along with many other known *tlp*s from rice barley etc., when homology search was performed using Blastx.

The primer pair 3a and 3b amplified 226 bp product at 59°C annealing temperature.

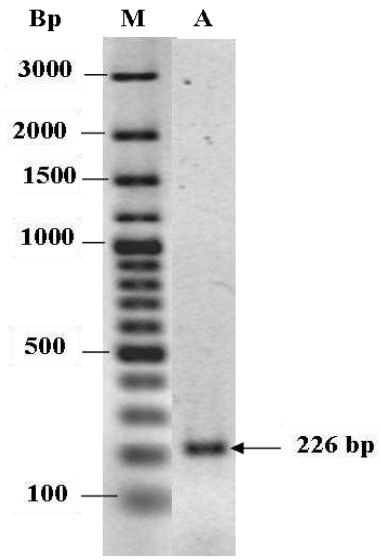


Fig 41. Amplification of pearl millet *tlp* gene with the primer pair 3a and 3b.

Lane M: 100 bp ladder plus (Fermentas #SM0321), Lane A: 226 bp Product amplified by the primer pair 3a and 3b separated on 1.5% agarose gel.

PCR product was separated on 1.5% agarose gel and the band was excised and purified using QIAEX II Gel Extraction Kit (Qiagen # 20021) subsequently sequenced.

Nucleotide sequence of 226 bp product amplified by the primer pair 3a and 3b.

```
  1 AACNGCNNNNANNGCCNCNGGGCNGTATAGCAACGGCCGCCGAGACTCAGGGAANAAAGC
 61 CCGCAGACTCGNCGNNCATNAAGTANNNNGNGNANCGATAGCCNNCCTNTGGCNNCTCTN
121 ATNGGGGGCCNACCNNNNNNTACTGGTACTNATCCANTNCNGGTATGNTGTNCTNTAGANA
181 TCCNAANNAAGCNTNTTCCANTNTAGNTANACCTGNNTNCNNNNNN
```

Fig 42. Nucleotide sequence of 226 bp PCR product amplified with primer pair 3a & 3b.

Homology search of the 226 bp nucleotide sequence revealed that the sequence does not share homology with any known tlps. The reason could also be poor quality of the sequence. Also the expected size of the amplicon was higher than 300 bp and therefore this primer pair was not considered for any further work and the next set of primers was used.

The primer pair 4a and 4b did not amplify any product when Dream*Taq* polymerase (Fermentas # EP0701) was used. PCR additives DMSO (1% v/v), Betaine (0.6 M) and $MgCl_2$ (2 mM) were used along with reaction buffer. However, no products were obtained under annealing temperature ranging from 45-65°C as illustrated in the Fig 42 below. Positive control was ~ 350 bp (nonspecific lipid transfer protein- nsltp) product amplified by the primers Remo forward primer 5′-GGGCTGGTCCTCGAATTCAC-3′ Remo reverse primer 5′-GTGTATGA GTAATGGATCCG-3′ using 100 ng of genomic DNA from *Malus domestic* cv. Remo as template.

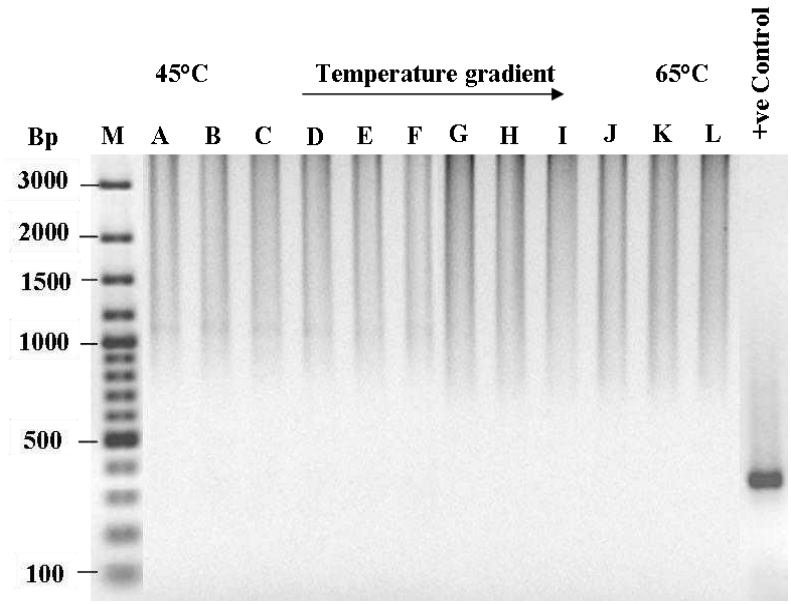


Fig 43. Amplification of pearl millet *tlp* gene with the primer pair 4a and 4b.

Lane M: 100 bp ladder plus (Fermentas #SM0321), Lane A-L: Amplification at different annealing temperatures (45-65°C) showing no product, the last lane is positive control.

Therefore KAPA2G Robust PCR Kit Peqlab Germany (# 07-KK5004-01) which contains second generation polymerase which amplifies GC rich gene fragments was used. The kit contained GC buffer, Enhancer 1 and 2; all these were used at 1X end concentration. PCR reaction conditions were as per manufacturers specifications. This resulted in amplification of ~ 422 bp product at 65°C. PCR products were separated on 1.5% agarose gel, stained with ethidium bromide and visualized under UV imager as seen in Fig 44.

Amplification of pearl millet *tlp* gene with the primer pair 4a and 4b using KAPA2G Robust PCR Kit.

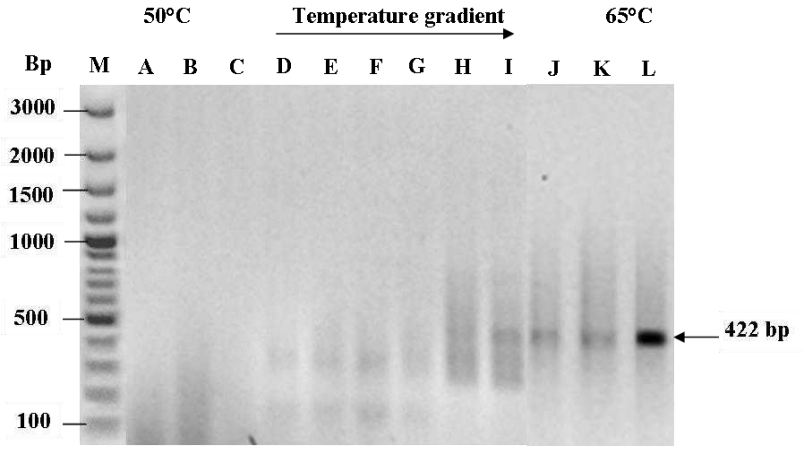


Fig 44. Amplification of pearl millet *tlp* gene with the primer pair 4a and 4b using KAPA2G Robust PCR Kit
Lane M: 100 bp ladder plus (Fermentas #SM0321), Lane A-L: Amplification at different annealing temperatures (45-65°C) showing 422 bp pearl millet tlp at 65°C.

The 422 bp band was reamplified under same conditions; separated on agarose gel, 422 bp band was excised from the gel; purified using QIAEX II Gel Extraction Kit (Qiagen # 20021) and directly sequenced. Reamplification of 422 bp product is seen in Fig 45.

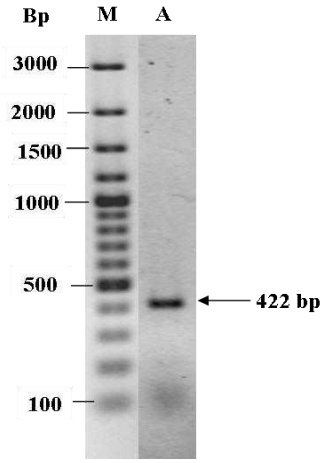


Fig 45. Reamplification of 422 bp pearl millet *tlp* fragment.
Lane M: 100 bp ladder plus (Fermentas #SM0321); Lane A: 422 bp product amplified by the primer pair 4a and 4b.

Nucleotide sequence pearl millet *tlp* (partial length) 422 bp fragment.

```
  1   GGGGACTGCGGCGGCGTGCTGCAGTGCACTGGGTACGGGCGCGCGCCCAACACGCTGGCG
 61   CNAGTCGGGCTGACNAGTACCANAACCTCGACTTCTTCGACATCTCGCTGGTGGACGGGT
121   TCAACGTGCCCATGGACTTCCTCCCGGCGGGCAGCGGCGCCGGGTGCCCCAAGGGCGGGC
181   CGCGGTGCCGCGCCGACGTCACGGGGCAGTGCCCCGCCGCGCTGCGCGCCACCGGCGGCT
241   CAACAACCCGTGCACGGTGTTCAAGACGGACCAGTACTGCTGCACGGGGTCGGCGGCGAT
301   ACAGCTGCGGCGCCACCGACTACTCGAGGTACTCAAGGGGAANTGCCCGGACGCCTNCAG
361   CTACCCCAAGGACGACGCCACCNNCNCCTACACTTGNNNCGNCGGAANNNACTACAAGGT
421   TT
```

Fig 46. Nucleotide sequence of 422 bp PCR product amplified with primer pair 4a & 4b.

Homology search of the obtained nucleotide sequence (422bp) showed strong homology to maize (*Zea mays* subsp. *Parviglumis*) *zeamatin-like protein* (NCBI Accession number: EU.725365.1) and *Sorghum bicolor* hypothetical protein similar to pathogenesis-related *tlp* (NCBI Accession number: gi|242038634|ref|XM. 002466667) and *Zea mays* antifungal *tlp –zeamatin* (NCBI Accession number gi|459169|gb|U06831|ZMU06831).

Since none of the amplicons sharing homology with known *tlps* were complete genes, 2 more primers were designed, one corresponding to a peptide fragment close to the upstream region of the gene next to signal peptide, named Pep_Fwd (5′-ACGACGGCCGCGCGC-3′) and another corresponding to the beginning of the gene, named FL_92 Fwd (5′-ATGGCAGGTTCCGTG GCAATC-3′) with the aim of isolation of full length gene. These were used in combination with the reverse primers Rev_Primer 594 and Rev_Primer 667. However, these primers did not lead to the amplification of any product with Dream *Taq* along with PCR additives like DMSO (0-1%) and Betaine (0-600 mM) and under $MgCl_2$ concentration gradient (0-2 mM); genomic DNA was used as template. Surprisingly the primer combination FL_Fwd+Rev_594 and FL_Fwd+Rev_667 gave several products when the PCR was carried out with KAPA HiFi PCR kit from Peqlab (# 07-KK2100-01). The polymerase kit contained GC buffer containing PCR enhancers for the amplification of GC rich templates. Amplification with the above mentioned primer pairs are given below.

The primer pair 5a and 5b amplified two products namely 1110 bp and a 900 bp product at 56-60°C annealing temperatures as seen in Fig 47.

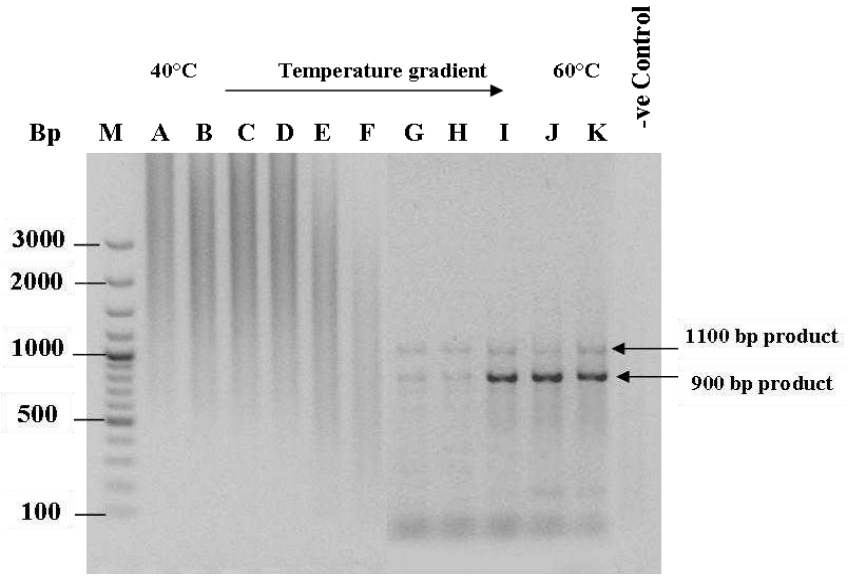


Fig 47. PCR using KAPA HiFi PCR kit using primer pair 5a and 5b

Lane M: 100 bp ladder plus (Fermentas #SM0321); Lane A-K Temperature Gradient PCR (40-60ºC annealing temperature) with primer pair 5a and 5b using KAPA HiFi PCR kit (Peqlab # 07-KK2100-01) and GC buffer. Negative control was PCR without any template DNA.

The PCR product of approximately 1 kbp size was excised from the gel, purified by gel extraction and directly sequenced.

Nucleotide sequence of 1014 bp product amplified with primer pair 5a and 5b.

```
   1 NAGAGGTNCTCAGTCCACGTCGATCATCATGACCTCCTGCCCAGCTGGTGGATGNGCTGG
  61 GACAGTGGTCGGTGGCGCCGGCTCCGGGATGGATGGCGCGTGAAGCTCNTGGACGAAGAC
 121 ACCCGCTGGTACTTGAGCGCGNGTNGATCCAAGCTTCGAGAGGACGTCCGCTGCAACGTT
 181 GTTGTCGCGGACGATGTGATGGAACTCGAGACCATAGAANTTGTTCTCGAGCTTGCGGAC
 241 TTCTAGGCAGTAGGCGTCCATGTTCTCCTTGTTGCGGTCCCAGGACTTGTTGACTTGGTT
 301 GATGACCACTGCGGAGTCGCCGTAGACTAGTAGCCGCTTGATGCCCAGTGAAGCCGCGAG
 361 GCGGAGCCCGTGTAGGAGAGCTTCGTACTCGGCTTCGTTGTTTGAGACCTTCCAGAAGAT
 421 CTGCAGGACGTACTTGAGTTGTTCGCCCGTTGGAGAGATCAGGAGTACCCCTGCNCCTGC
 481 GCCCTCGAGGTTGAGTGAGCCGTCAAAGTACATCACCCAATGCTCTGGTCGCACGGTGGG
 541 AGTAGGTAGCTGNTTCTCCCGCCACTCTGCCATGAAGTCGACTAGTGCNTGCGACTTGAT
 601 GGCTGTGCGTGGCTTGAAGTCNATGGTGAGGGCGCCGAGNTCGACNGCCCACTTGGAGAT
 661 TCTTCCAGTNGCATCTTGGTTCCTCAGGATGTCGCCGAGCGGNTAGTCNGTGACAACGGA
 721 AACCNANTACTCCTGGAAGTAGTGTCGTANCTTCCNCGAGGTGATGAGNACTGCNNTACA
 781 GNANCTTCTGCACNGGNTGANANCNAGCCTTGNANTCTAANAGAACTCNNTTGANNAAAN
 841 NAANTGGNNNTTGACNGNNANGNNTGNCNTNTTNTTCCTTCCAANACANTGGANTANNNN
 901 CCNCTGATTATNCCNACNAGTAAANCANTATNCTTTTTTTAANNNNNNNNNNNNNNNNN
 961 NNTNNNNNNNNNNNNNTNNNNNNNTNNNNNNNNNTNNNNNNTNNNNNNNNNN
```

Fig 48. Nucleotide sequence of 1014 bp product amplified with primer pair 5a and 5b using KAPA HiFi PCR kit (Peqlab # 07-KK2100-01) and GC buffer.

This 1014 bp fragment shared homology with hypothetical protein from seedlings of *Pennisetum glaucum* exposed to drought stress (NCBI accession number gi|32277246|gb|CD726399).

The primer pair 6a and 6b amplified two products namely 650 bp product at annealing temperature of 55-58°C annealing temperature and 7650 bp product at 60°C annealing temperature as seen in the Fig 49. No amplification was seen beyond 60°C.

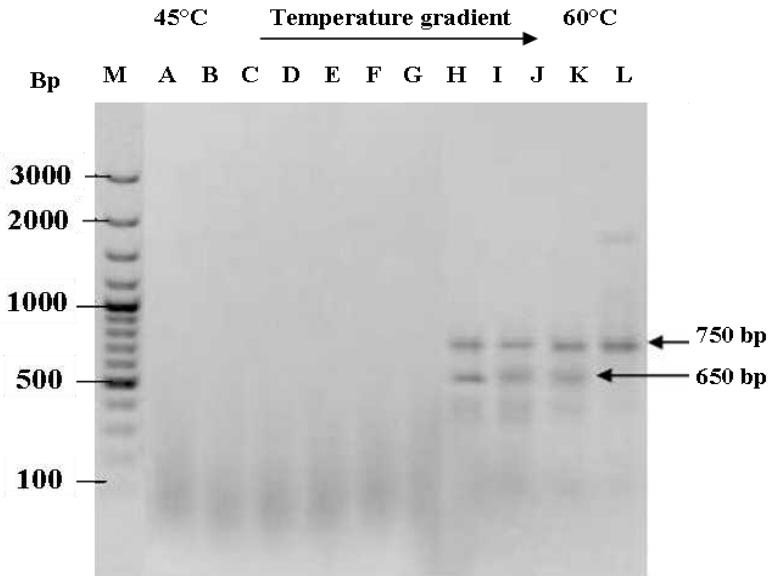


Fig 49. PCR using KAPA HiFi PCR kit using primer pair 6a and 6b

Lane M: 100 bp ladder plus (Fermentas #SM0321); Lane A-L: Temperature Gradient PCR (40-60ºC annealing temperature) with primer pair 6a and 6b using KAPA HiFi PCR kit (Peqlab # 07-KK2100-01) and GC buffer.

Both the fragments were sequenced directly without cloning into a vector as several efforts to clone these fragments into a plasmid and re isolation of plasmids containing inserts from the transformed cells were unsuccessful. Because of direct sequencing using the primers used for amplification, the primers used are not found in the sequence, moreover the length of the sequences are slightly shorter than the size of the amplified products.

Nucleotide sequence of approximately 650 bp product amplified by the primer pair 6a and 6b.

```
  1 CGACANAAGNTTGCCTCCAGNTCACGATCAATCATCATGAACCTCCTGCCCAGCTGGNGG
 61 ATGNGCTGGNTACAGTGGTCGGAGGCGCCGGCTCCGGGATGGATGGCGCGTGAAGCCTCG
121 TGGACGAAGACACCCGCTTGNTACTTGAGCGCGAGTGGATCCAATCCTTCNAGAGGACGT
181 CCGCTGCAACGATTGTTGTCGCGGACGATGTGATGGAACTCGAGACCNTAGAANTTGTTC
241 TCGAGCTTGCGGACTTCTAGGCAGTAGGCGTCCATGTNCTCCTTGTTGCGGTCCCANGAC
301 TTGTTGACTTGGTTGATGACCACTGCNGAGTCGCCGTANACTAGTAGCCGCTTGATGCCC
361 AGTGAAGCCGCGAGGCGGANCCCGTGTAGGAGAGCTTCNNTACTCGGCTTCNTTGTTTGA
421 GACCTTCCANAAGATCTGCANGACGTACTTGAGTTGTTCGCCCGTTGGAGAGATCAGGAG
481 TACCCCTGCNCTTTGCNCCCTCGAGGTTTGAGTGAGCCGTCGAAGTACANCACCCANTGC
541 TCTNGCNGCTCGNTTNGGAGNNNGTNNCTGAATTCTCCNNNNNNNNNNNNNNNNNNNNN
601 NNNNNNNNNNNN
```

Fig 50. Nucleotide sequence of approximately 612 bp product amplified by the primer pair 6a and 6b.

The 650 bp product amplified with primer pair 6a and 6b showed homology to hypothetical protein from seedlings of *Pennisetum glaucum* exposed to drought stress (NCBI accession number gi|32277246|gb|CD726399).

Nucleotide sequence of approximately 750 bp product amplified by the primer pair 6a and 6b.

```
  1 NNNNNNNNNNNACCGACCGGCACGAATAGTGAGACCAGCATCAGCGCAAGTTCCCCCCG
 61 CNACTCGGCAGCTTGTCATCGGCAGAGCGANGGGTTCCGTCTTGAGATCTCGGTGGAGN
121 GAACTTCCCTTCCAAGAGTTCTTGGTGAGAGGATGGCTCCTTTCACAAGTAAGTGTGAG
181 GTCTCGAATCAAATGGTTCGGGGATCTTAGAAACCTTCGGTTTGGGTAATTTCTCAGGG
241 ACTGCGAGTGGAATTCTTCCTTCGGCTAGCGAAAACAAAAAAAAATGCTGGAGCCTCGA
301 CTAAGAGAATTAGGCCTAAACTCTTGACATCCCATTGACTGAGTGCTTAGGAAAATGTC
361 CTCAGTGTCATAGNGTCTTGGAAGACAGANAAGTTCACAACAATCCTTATCGGTTGATC
421 TGCTTCACTCTTACGCAAGGCTGATGAAAGTTCATCCCCCTCTCTGGAGAATTCCGGCA
481 TTTCGCCCCAGATCATTAGGAGGTTTAACAATGACACTTATTTCTAACTGAAGATCCCT
541 TGAAAGGTGATCGTACCCCTCGGTTGAGGAACTAGAANTTTTACCATGTCACCCTTGAC
601 GGATCGGAGCTTCTCCTTTGCTCAAATTGTCAAAGAAGGTCGGGTGGTGTAAGTGCTCT
661 TTACCATAGAANGTTTTTTTNNNNNNTTNNNNGNGNNNTANGNNCNNAACCNNNNA
```

Fig 51. Nucleotide sequence of approximately 750 bp product amplified by the primer pair 6a and 6b.

This approximately 750 bp product does not share homology with any known plant proteins. As these (650 and 750 bp) PCR products were directly sequenced, few base pairs on both the ends are missing and the length of the fragments appears shorter for the same reason. The primer pair 7a and 7b amplified approximately 800 bp band and primer dimer at lower annealing temperatures, as seen in the Fig 52.

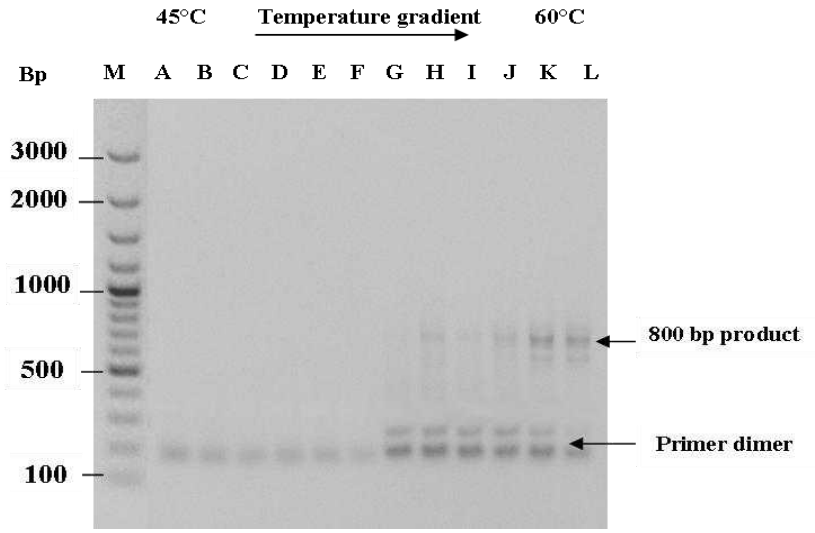


Fig 52. PCR using KAPA HiFi PCR kit using primer pair 7a and 7b

Lane M: 100 bp ladder plus (Fermentas #SM0321); Lane A-L: Temperature Gradient PCR (45-60ºC annealing temperature) with primer pair 7a and 7b using KAPA HiFi PCR kit.

The approximately 800 bp fragment could not be cloned to the cloning vector as transformants using this amplicon were ampicillin resistant but were devoid of plasmids. Sequencing of this amplicon directly revealed that the 861 bp product shares homology with retrotransposon protein from *Oryza sativa* (NCBI accession number: gi|77552546|gb|ABA95343. This explained the failure of cloning; since the fragment is a retrotransposon, the fragment was incorporated into the genome of *E. coli* cells along with the vector. Therefore the *E. coli* cells showed ampicillin resistant though they seemed devoid of plasmids. Plasmid along with the insert was integrated into the genome of *E. coli* making them ampicillin resistant.

Nucleotide sequence of approximately 861 bp product amplified by the primer pair 7a and 7b.

```
  1 NAGAGGTNCTCAGTCCACGTCGATCATCATGACCTCCTGCCCAGCTGGTGGATGNGCTGG
 61 GACAGTGGTCGGTGGCGCCGGCTCCGGGATGGATGGCGCGTGAAGCTCNTGGACGAAGAC
121 ACCCGCTGGTACTTGAGCGCGNGTNGATCCAAGCTTCGAGAGGACGTCCGCTGCAACGTT
181 GTTGTCGCGGACGATGTGATGGAACTCGAGACCATAGAANTTGTTCTCGAGCTTGCGGAC
241 TTCTAGGCAGTAGGCGTCCATGTTCTCCTTGTTGCGGTCCCAGGACTTGTTGACTTGGTT
301 GATGACCACTGCGGAGTCGCCGTAGACTAGTAGCCGCTTGATGCCCAGTGAAGCCGCGAG
361 GCGGAGCCCGTGTAGGAGAGCTTCGTACTCGGCTTCGTTGTTTGAGACCTTCCAGAAGAT
421 CTGCAGGACGTACTTGAGTTGTTCGCCCGTTGGAGAGATCAGGAGTACCCCTGCNCCTGC
481 GCCCTCGAGGTTGAGTGAGCCGTCAAAGTACATCACCCAATGCTCTGGTCGCACGGTGGG
541 AGTAGGTAGCTGNTTCTCCCGCCACTCTGCCATGAAGTCGACTAGTGCNTGCGACTTGAT
601 GGCTGTGCGTGGCTTGAAGTCNATGGTGAGGGCGCCGAGNTCGACNGCCCACTTGGAGAT
661 TCTTCCAGTNGCATCTTGGTTCCTCAGGATGTCGCCGAGCGGNTAGTCNGTGACAACGGA
721 AACCNANTACTCCTGGAAGTAGTGTCGTANCTTCCNCGAGGTGATGAGNACTGCNNTACA
781 GNANCTTCTGCACNGGNTGANANCNAGCCTTGNANTCTAANAGAACTCNNTTGANNAAAN
841 NAANTGGNNNNNNNNNNNNN
```

Fig 53. Nucleotide sequence of approximately 861 bp product amplified by the primer pair 7a and 7b.

3.12.2 ii) Cloning of PCR products

KAPA HiFi Polymerase generates blunt end products. The major bands from these PCR reactions were purified by gel extraction. The purified bands were cloned to CloneJet cloning vector. Electro competent *E. coli* cells were transformed by electroshock and the transformants were grown on SOC medium for 60 min at 37°C. The cells were then plated on LB agar plates containing ampicillin. The transformants were picked and grown on LB liquid medium overnight at 37°C. The cells were harvested and subjected to HB lysis to isolate the plasmids. Strangely ampicillin resistant colonies did not contain any plasmid; some colonies contained plasmid but colony PCR of these colonies did not give any product, moreover these plasmids were around 6 kbps in size (size of CloneJet cloning vector is ~3 kbps) as seen in the Fig 54.

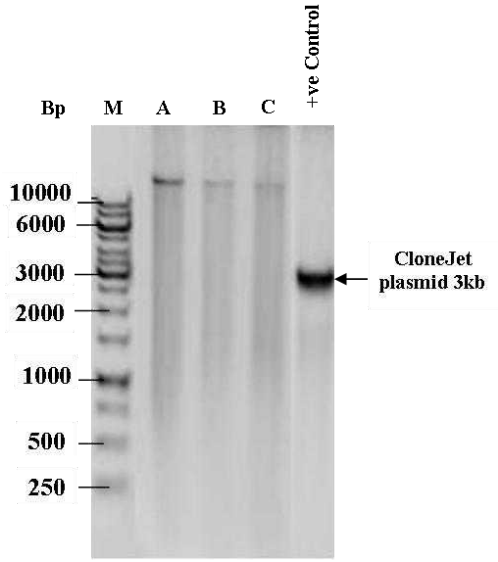


Fig 54. Plasmid isolation from transformed colonies-Ampicillin resistant colonies devoid of plasmid. Lane M: 1 Kbp DNA ruler Lane A, B and C: Transformants containing no plasmids, Positive Control: CloneJet plasmid (3 kbp).

Electro transformation was repeated with electro competent DH5α, NM522 and XLBlue-1 strains of *E. coli* cells. Heat shock transformation was also performed with the above mentioned ultra competent cells of *E coli* strains. But was it not possible to isolate plasmid from the transformants.

This lead to the conclusion that the transformants contain the ampicillin resistant gene from CloneJet cloning vector integrated into *E. coli* genome. This is one explanation for the formation of ampicillin resistant colonies without plasmid. The colonies with plasmid but showing no amplification of the insert on colony PCR were grown and their plasmid was isolated. The plasmids were linearised to check for the presence of insert. But it was observed that these were just self ligated plasmids with no insert.

The primer pair 8a and 8b gave only a primer dimer of approximately 200 bp as seen in the Fig 54 below. No amplification was seen beyond 60°C. No amplification was seen beyond 60°C. This concluded that the primer combination FL_Fwd+Rev_594, and FL_fwd+Rev_667

generated several nonspecific products even at higher annealing temperature with KAPA HiFi polymerase. Therefore the primer FL-Fwd 92 was not used for further amplifications.

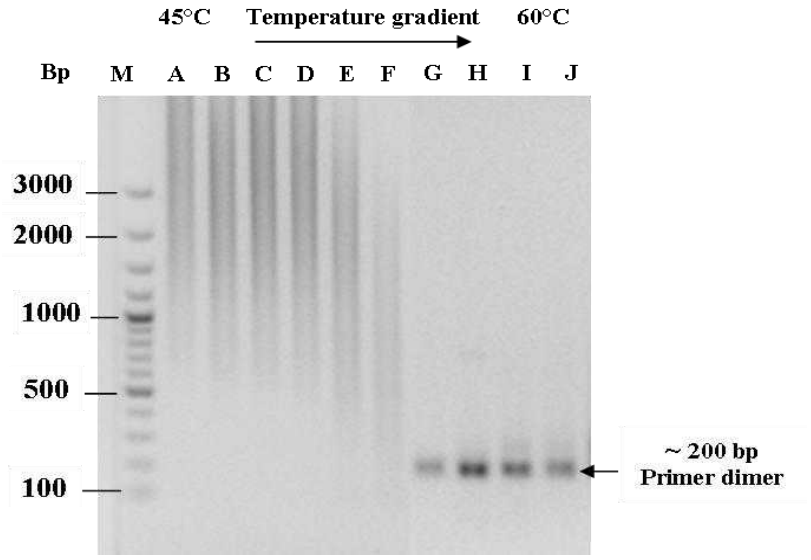


Fig 55. PCR using KAPA HiFi PCR kit using primer pair 8a and 8b showing primer dimer formation. Lane M: 100 bp ladder plus (Fermentas #SM0321) Lane A-J: Temperature Gradient PCR (45-60 ºC annealing temperature) with primer pair 8a and 8b using KAPA HiFi PCR kit (Peqlab # 07-KK2100-01) and GC buffer.

3.13 Inverse PCR and adaptor ligation-mediated genome walking in pearl millet genome to amplify unknown flanking regions of pearl millet chitinase and tlp gene.

Large genome size and multiploidy in plants is a big challenge in case of PCR-based genome walking in plants. To gain high sensitivity in ligation-mediated –PCR applications, it is important that nearly 100% of all genomic DNA fragments resulting from the initial restriction digest have the adaptor attached to both ends. This enlarges the pool of the desired PCR template. Nevertheless, this cannot be ensured while amplifying unknown flanking regions of a gene because of the lack of nucleotide sequence data that does not allow spotting of restriction site of the enzyme (used to construct genomic DNA libraries) on the gene of interest. This could lead to failure of the technique due to two possible reasons. Firstly, the distance from the primer to the restriction site is greater than the capability of the genome walking system (~6 kb) or the target gene might have restriction site just outside the known sequence separating the unknown flanking region of the gene and thereby not leaving any good length of unknown sequence to be walked by the primer. Or in case of multiploid plants with large genomes, probability of adaptor ligation to the target DNA is highly reduced. Previous studies (Farrelly *et al*., 1995; Hoelzel and Green, 1992) have explained the failure of PCRs in large genomes with two most possible reasons, the first being a reduction in target DNA relative to the total content of template DNA in a single PCR serves to reduce amplification efficiency. As genome sizes increase, this serves to dilute the proportion of available target DNA in a given volume of template DNA (Farrelly *et al*., (1995); because typically, template concentration is considered in terms of total amount of genomic DNA (Hoelzel and Green, 1992). Secondly, as genome size

increases, the number of regions complementary to any 20-bp region (the average size of primers) also increases. A larger genome would then increase the amount of nonspecific binding of primers, a concurrent diminishing of the primer pool available for PCR (Garner, 2002). If genome size has a negative effect on amplification success at a single locus then, by extension, the success rate of primer sets tested on organisms with large genomes should be lower than the success rate of primer sets tested on organisms with small genomes. Nonetheless, in this study many pairs of primers were designed considering the partial length of the gene and no specific products were observed with any of the primers combinations used. Though increasing the length of primers would improve the specificity, longer primers could not be designed due to high GC content of the genes (482 bp pearl millet *chitinase* 61% - GC content and 422 bp pearl millet *tlp* 69% - GC content). Higher GC content of the primers is not recommended for long distance PCRs and high GC content lead to poor results with normal PCRs in amplification of pearl millet *chitinase* and *tlp* genes in this study as well. This explains that the available sequence data on pearl millet *chitinase* and *tlp* need to be enriched in order to employ techniques like adaptor ligation mediated genome walking successfully. The results of inverse PCR and adaptor ligation mediated genome walking in pearl millet genome to amplify unknown flanking regions of pearl millet is therefore included under appendix 2.

3.14 Amplification of *actin* (housekeeping) gene.

In order to study the gene expression levels, a house keeping gene which serves as control was required. Pearl millet *actin* was used in the present study as control. As the nucleotide sequence of pearl millet *actin* was not available in the database, *actin* gene (partial length) was isolated from pearl millet and sequenced. The primer sequence to amplify pearl millet *actin* was obtained from the work of van den Berg and coworkers (2004) and is summerised in the table 20.

Table 20. Primers to amplify pearl millet *actin* (van den Berg *et al.*, 2004).

Primer	Primer sequence	Annealing temperature
Pm_act_Fwd	5'-ACCGAAGCCCCTCTTAACCC-3'	60°C
Pm_act_Rev	5'-GTATGGCTGACACCATCACC-3'	60°C

The primer pair Pm_act_Fwd and Pm_act_Rev amplified a 267 bp product. The amplicon was blunt ended product as proof reading polymerase was used. Product of the PCR reaction was separated on 1.5% agarose gel (section 2.11.3 section) and stained with EtBr and visualized under UV imager as seen in fig 56.

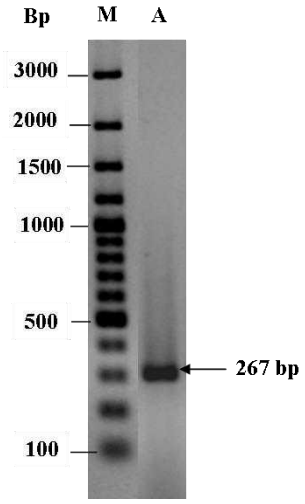


Fig 56. Amplification of pearl millet *actin* (partial length 267 bp).
Lane M: 100 bp ladder plus (Fermentas # SM0321), Lane A: Pearl millet *actin* 267 bp.

The 267 bp band was excised from the gel and purified using QIAEX II Gel Extraction Kit (Qiagen # 20021) (section 2.13.3 of materials and methods) and cloned into CloneJet blunt end cloning vector (Fermentas # K1231) (section 2.14.2 of materials and methods). Ultra competent *E. coli* cells of (XL-Blue1 strain) were transformed by heat shock (section 2.13.4. of materials and methods).

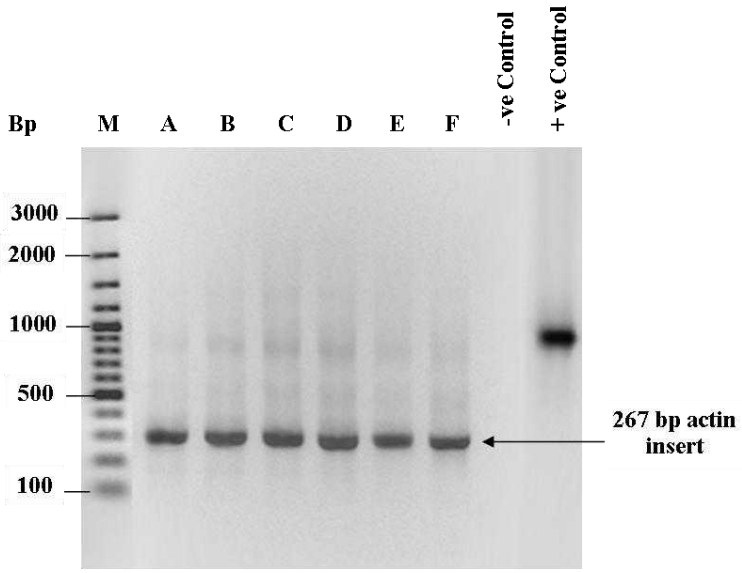


Fig 57. Colony PCR with CloneJet primers (Fermentas # K1231) to screen the transformants.

Lane M: 100 bp ladder plus (Fermentas # SM0321), Lane A-F: transformants containing pearl millet *act* (267 bp) insert amplified with cloneJet forward and reverse primer from Fermentas, Negative control without any template DNA; Positive control with 967 bp insert (component of the CloneJet blunt end cloning kit used).

The transformants were allowed to multiply on SOC medium without any antibiotics for 60 min at 37°C. The cells were then plated out on Amp^{100} LB agar plates (section 2.15.2 of materials and methods). The colonies were grown overnight at 37°C and screened by colony PCR (section 2.12.3. of materials and methods). Result of colony PCR showing positive clones is illustrated in Fig 57.

The transformants containing the insert were cultured overnight in LB medium. Plasmid DNA was extracted from the transformants using GeneJET plasmid mini prep kit (section 2.16.2 of materials and methods) and plasmids containing inserts were selected and sent for sequencing (Sequence Lab, Göttingen, Germany). Nucleotide sequence of 267 bp Pearl millet *actin* fragment is given in Fig 58.

Nucleotide sequence of 267 bp Pearl millet *actin* amplified by the primer pair Pm_act_Fwd and Pm_act_Rev.

```
  1 CTCGAGTTTTTCAGCAGATGTATGGCTGACACCATCACCAGAGTCGAGCACAATACCTGC
 61 AGGAAAAATATTTCTCACTTGCACTGCAGGTTGAGTATTGACATGAAAAAGAGGCTCTCA
121 TCATAGTGATCATACCAGTTGTTCGACCACTAGCGTACAGGGAAAGAACAGCCTGGATGG
181 CAACATACATTGCTGGGCAGTTGAATGTCTCGAACATGATCTGGGTCATCTTCTCCCTGT
241 TTGCCTTTGGGTTAAGAGGGGCTTCGGT
```

Fig 58. Nucleotide sequence of pearl millet *actin* (267 bp partial length).

The product was subjected and homology search of the nucleotide sequence showed homology to maize *actin* (NCBI accession number: gi|1498383|gb|U60508.1|ZMU60508).

Nucleotide sequence alignment of pearl millet *actin* (267 bp-partial length) with maize actin (NCBI accession number: ZMU60508) with which it shares homology.

```
Z. mays    380 ACCGAGGCTCCTCTGAACCCCAAGGCAAACAGGGAGAAGATGACCCAGATTATGTTCGAG  439
               ||||| || ||||| ||||| |||||||||||||||||||||||||||||| |||||||||
P.glaucum  268 ACCGAAGCCCCTCTTAACCCAAAGGCAAACAGGGAGAAGATGACCCAGATCATGTTCGAG  209

Z. mays    440 ACATTCAACTGCCCAGCAATGTATGTGGCCATCCAGGCTGTTCTTTCCCTGTACGCCAGT  499
               |||||||||||||||||||||||||| |||||||||||||||||||||||||||||| |||
P.glaucum  208 ACATTCAACTGCCCAGCAATGTATGTTGCCATCCAGGCTGTTCTTTCCCTGTACGCTAGT  149

Z. mays    500 GGTCGAACAACGGGTATG-TTACT-GCATTAAA---CCTGTTTCATATCAGCATTCGAAT  554
               |||||||||||| |||||| | |||   || | |    || |||||| |||  | || |
P.glaucum  148 GGTCGAACAACTGGTATGATCACTATGATGAGAGCCTCTTTTTCATGTCAATACTCAACC  89

Z. mays    555 AATAGTGTAGTGAGTGACAGGAATCTTT-CTGCAGGTATTGTGCTCGACTCTGGTGATGG  613
               |||| |   ||||| |   || ||| ||||||||||||||||||||||||||||||
P.glaucum  88  TGCAGTGCA---AGTGAGAAATATTTTTCCTGCAGGTATTGTGCTCGACTCTGGTGATGG  32

Z. mays    614 TGTGAGCCACAC  625
               ||| ||||| ||
P.glaucum  31  TGTCAGCCATAC  20
```

Fig 59. Nucleotide sequence alignment of pearl millet (Pennisetum glaucum) *actin* (267 bp-partial length) with maize actin (NCBI accession number: ZMU60508) with which it shares homology.

3.15 Bioinformatics.

Different bioinformatics tools were used in this study for Primer designing and sequence analysis. The Bioinformatics tools used include DNASTAR, Clonemanger and Primer packages for primer designing. NCBI database (http://www.ncbi.nlm.nih.gov) was used for homology search and detection of vector contamination. Protein similarity among several plant species was analysed by multiple alignment with ClustalW (**http://www.ebi.ac.uk/Tools/msa/clustalw2**). To predict the signal peptide cleavage site PSORT program (http://psort.ims.u-tokyo.ac.jp/form.html) was used. To find out the restriction sites NEB cutter 2 from New England Biolabs (**http://tools.neb.com/NEBcutter2**) was used.

3.15.1 Sequence evaluation of Chitinase.

The 482 bp sequence was subjected to homology search and it was observed to share homology with Maize *chitinase* (NCBI Accession number: gi|195627425). It was also observed that the pearl millet *chitinase* gene is interrupted by a 107 bp intron at 241 bp position to 348 bp position. The obtained nucleotide sequence was translated into a partial amino acid sequence that shares homology to the chitinase from Maize (NCBI Accession number: gi|195627425). The sequence alignment revealed the homology between 73 to aa 189 of the maize chitinase (Fig 60).

Amino acid sequence alignment of pearl millet *chitinase* (114 aa, gi|195627425) with maize endochitinase (NCBI accession number: gi|195627425) with which it shares homology.

```
Z.mays    MANAPRTLAVLALGLALLCAAAGPAAAQNCGCQPNFCCSKFGYCGTTDDYCGDGCQSGPC 60
P.glaucum ------------------------------------------------------------

Z.mays    RSGGGGGGGGSGGANVANVVTDAFFNGIKNQAGSGCEGKNFYTRSAFLSAVNKYPGFAHG 120
P.glaucum -------------ANVASVVTDAFFNGIKNQAGGGCEGRNFYTRSAFLEAANKYSGXAHG 47
                      ****.***************.****:*********.*.***.* ***

Z.mays    GTEVEGKREIAAFFAHVTHETGRFCYISEINKSNAYCDASNRQWPCAAGQKYYGRGPLQI 180
P.glaucum GTEVEGKREIAAFFAHVTHETGHFCYISEINKNNAYCS-SNAQWPCAAGKKYYGRGPLQI 106
          **********************:*********.****. ** *******:**********

Z.mays    SWNYNYGPAGRDIGFNGLADPNRVAQDAVIAFKTALWFWMNNVHRVMPQGFGATIRAING 240
P.glaucum SWNYNYGP---------------------------------------------------- 114
          ********

Z.mays     ALECNGNNPAQMNARVGYYKQYCQQLRVDPGPNLTC 276
P.glaucum  ------------------------------------
```

Fig 60. Amino acid sequence alignment between partial pearl millet chitinase (NCBI Accession number ADG65345) and maize chitinase (NCBI Accession number gi|195627425).

The pearl millet chitinase has 90% identity and 94% similarity to the chitinase from maize and belongs to the class I chitinase which is a member of the lysozyme like super family. Since the high homology between maize and pearl millet chitinase the further sequence characterization was performed with the chitinase from maize. The latter revealed the presence of a signal peptide sequence that is cleaved between aa 27 and 28. After cleavage of the signal sequence the mature protein consists of 209 aa and encodes for a protein with a calculated molecular mass of 26.48 kDa and calculated isoelectric of point of 8.60.

3.15.2 Sequence evaluation TLP.

The 422 bp sequence was subjected to homology search and it was observed to share homology with *Sorghum bicolor* similar to thaumatin like protein (NCBI Accession number: gi|242038635). The obtained nucleotide sequence was translated into a partial amino acid sequence that shares homology to the hypothetical protein (231 aa) from *Sorghum bicolor* (similar to thaumatin like protein NCBI Accession number: gi|242038635). The sequence alignment revealed the homology between 111-210 aa of the *Sorghum bicolor* TLP (see Fig 61).

Amino acid sequence alignment of pearl millet TLP (149 aa) with *Sorghum bicolor* TLP (NCBI accession number: gi|242038635) with which it shares homology.

```
S.bicolor MASVLHLLPLVVVVVFLAAAAADAATFTVTNKCQYTVWAAAVPGGGQQLDNGQTWSINVP 60
P.glaucum ----------------------SXXXXVX----------------------XWGLRRR 14
                               :    *                          *.:.

S.bicolor AGTTGGRVWARTGCSFDGSGNGQCQTGDCGGVLRCTQYGQPPNTLAEFGLNQYQGLDFID 120
P.glaucum AAVH----WVRARAQHAGASR------------------------ADX----YXNLDFFD 42
          *..     *.*: ... *:..                         *:     * .***:*

S.bicolor ISLVDGFNVPMDFLPAGDGSGCPKGGPRCDADVTSQCPAALKATGGCNNPCTVFKTDEYC 180
P.glaucum ISLVDGFNVPMDFLPAGSGAGCPKGGPRCRADVTGQCPAALRATGGCNNPCTVFKTDQYC 102
          *****************.*:********* ****.******:***************:**

S.bicolor CTGSAANTCGPTDYSKFFKGLCPDAYSYPKDDATSTYTCPGGTNYNVVFCP 231
P.glaucum CTGSAANSCGATDYSRYSRGXARTPXATPR--TTPPXPTLXXXEXTTRF-- 149
          *******:**.****:: :* .  . : *:  :*.. .     : .. *
```

Fig 61. Amino acid sequence alignment between partial pearl millet TLP (NCBI Accession number: XP_002466712) and maize chitinase (NCBI Accession number gi|242038635).

The pearl millet TLP belongs to family of Thaumatin like protein which is a member of the lysozyme like super family.

Since the high homology between maize and pearl millet chitinase the further sequence characterization was performed with the TLP from *Sorghum bicolor* (NCBI Accession number: gi|242038635). The latter revealed the presence of a signal peptide sequence that is cleaved between amino acid 22 and 23. After cleavage of the signal sequence the mature protein consists of 231 amino acid and encodes for a protein with a calculated molecular mass of 24.54 kDa and calculated isoelectric of point 4.36.

3.15.3 Multiple sequence alignment of amino acid sequence of class I chitinase from different plant species and construction of phylogenetic tree.

3.15.3 i) Multiple sequence alignment of amino acid sequences of Class I chitinases

Multiple sequence alignment of chitinase from different plant species revealed that pearl millet chitinase shares homology with other plant chitinases belonging to 25 different (monocotyledonous as well as dicotyledonous) plant species considered for comparison (see Fig 62). Class I chitinases are composed of three domains: a Cys rich chitin-binding domain, a Pro-rich hinge region, and a highly conserved catalytic domain. Of interest are the residues

important in catalysis or implicated in maintaining the active site geometry which include active site residues Y of the NYNYG motif, the second N of the NYNY motif, the first T of the SHETTGG motif, the second G and the Q of the GRGPIQL motif (Verburg *et al.,* 1992). Grey shaded boxes indicate conserved amino acid residue and red boxes indicate the position of conserved catalytic domains and dashes denote spaces required for optimal alignment (see Fig 61). Class I chitinases have an N-terminal cysteine-rich, chitin-binding domain which is separated from the catalytic domain by a proline and glycine-rich hinge region. Structural analysis reveals that Class I chitinases (belonging to Glycoside hydrolase family 19) are similar to several lysozymes including the one from T4-phage.

NCBI Accession number of sequences considered for multiple sequence alignment are: *Arabidopsis thaliana* gi|2597826, *Brassica rapa* (Chinese cabbage) gi|116267545, *Capsicum annuum* (Chilly) gi|222159963, *Cicer arieti*num (Chickpea) gi|3892724, *Citrus sinensis* (Orange) gi|1220144, *Daucus carota* (Carrot) gi|1549331, *Glycine max* (Soya bean) gi|255631536, *Gossypium hirsutum* (Cotton) gi|1729760, *Hevea brasiliensis* (Rubber tree) gi|14575525, *Hordeum vulgare* gi|18972, *Linum usitatissimum* (Linseed/Fax) gi|76056895, *Malus domestica* (apple) gi|20149280, *Mangifera indica* (mango) gi|189014948, *Musa accuminata* (Banana) gi|17932712, *Nicotiana tabacam* (Tobacco) gi|121663827, *Phaseolus vulgaris* (Common beans) gi|52548196, *Prunus dulcis* (Almond) gi|148807189, *Pseudotsuga menziesii* (Douglas-fir) gi|227937465 *Pennisetum glaucum* (Pearl millet) gi|296011301, *Saccharum Saccharum officinarum* (Sugar cane) gi|15213848, *Sorghum bicolor* gi|242076344, *Tripsacum dactyloides* (Gama grass) gi|48093316, *Triticum aestivum* (Wheat) gi|4741842, *Vitis vinifera* (Grape wine) gi|2306813.

```
Pearl millet     ------------------------------------------------------------
Gama grass       ----MANAPR--ILAVLALGLALLCAAGPPAAAQNCGCQPNFCCSKYGYCGTSDEYCGDG 54
Maize            ------------------------------------------------------------
Sugar            ---------------LVALGLALVCAAG-PAAAQNCGCQPNYCCSKFGYCGTTDDYCGDG 44
Sorghum          ----MASSPT--ILTVLALGLLALLCAVGPAAAQNCGCQPDFCCSQYGYCGQTSEYCGTG 54
Rice             ----MANSPTPTMLAFLALGLALLLSATGQASAQNCGCQSNMCCSKWGYCGTGKDYCGDG 56
Wheat            ----MAKSPM----AILALGLAAVVLSGAGLASGDP----------------------- 28
Linseed          ------------------------------------------------------------
Chinese cabbage ---------MALTKLSLVLFLCFLGLYSETVKSQNCGCAPNLCCSQFGYCGSTDAYCGTG 51
Mango            ------------------------------MSQNCDCAPNLCCSQFGYCGTGEAYCGLG 29
Grape wine       ------------MAAKLLTVLLVGALFGAAVAQNCGCASGLCCSKYGYRGTGSDYCGDG 47
Soya             ---MSKLLLIRVAGFVATFVTMVMMMVPKNVSAQNIG---------------------- 34
Almond           ------------------------------------------------------------
Arabidopsis      ---MLTPTISKSISLVT--ILLVLQAFSNTTKAQNCGCSSELCCSQFGFCGNTSDYCGVG 55
Tobacco          ---MNFSSRKQFIFLIA--LTIVLVVVPRTILAQNCGCAESLCCSKWGFCGTGNDFCGQG 55
Carrot           -----------MKTSFATKFIFLIFIAAPLVSAQNCNCAAGLCCSKYGYCGTTSDYCGEG 49
Douglas-fir      -------MGKTGGEKWVMGLVVVLLLVGVSVNAQNCGCASGLCCSKYGYCGTTSAYCGTG 53
Barley           ------------------------------------------------------------
Cotton           ------MKFLALMVVLILASALRASADIS------------------------------ 23
Banana           ---MPRAAGIRHEVIFTLASSLGAFAEQCGRQAGGALCPGGLCCSQFGWCGNTDPYCGKD 57
Rubber           -------------------------EQCGRQAGGALCPGGLCCSQYGWCANTPEYCGSG 34
Beans            MKKNRMMIMICSVGVVWMLLVGGSYGEQCGRQAGGALCPGGNCCSQFGWCGSTTDYCGKD 60
Chilly           ----MRLSEFSFFSLLFAVLLLAVSAEQCGSQAGGALCAAGLCCSKFGWCGNTNDYCGAG 56
Apple            ------------------------------------------------------------
Chickpea         ---MKMKLAIQFPILIVIVLVGWCWAEQCGKQAGGALCPGGLCCSKFGWCGSTSDYCGDG 57
Orange           ----MRLIGSLLIFSLVLSFVLGGSAQNCG--------------SGVVYGGRDTGHGTHG 42

Pearl millet     --------------------A--------NVASVVTDAFFNGIKNQAGGG-CEGRN-FYT 30
Gama grass       CRSGPCHSGGGGGGGGGGSGA--------NVANVVTDAFFNGIKNQAGSG-CEGKN-FYT 104
Maize            -----------------SGGA--------NVANVVTDAFFNGIKNQAGSG-CEGKN-FYT 33
Sugar            CQSGPCRSGGG----ASSGGG--------NVASVVTDAFFNGIKNQAGNG-CEGKN-FYT 90
Sorghum          CRSGPCTGTGSGGSSGSGSGA--------NVGNVVTDAFFNGIKNQAGSG-CEGSN-FYS 104
Rice             CRSGPCYGGGGGGGGGGGGGGGGGGSGVSVESVVTEAFFNGIKNQAPNG-CAGKN-FYT 114
Wheat            ------------------------------VESVVTDAFFDGIKSQAADGGCPGKS-FYT 57
Linseed          ------------------------------------------------------------
Chinese cabbage CRSGPCRSPGGTPSPP--GGG--------SVGSIVTQAFFNGIINQAGGG-CAGKN-FYT 99
Mango            CKGGPCTSTPSTPSPTPTGGG--------SVASIVTADFFDGIKNQAAAS-CAGKS-FYT 79
Grape wine       CQSGPCDSGSGSGS---------------SVSDIVTQAFFDGIINQAASS-CAGKN-FYT 90
Soya             -------------------------------DDIVTQDFFNSIISQADDG-CAGKN-FYS 61
Almond           ------------------------------------------------------------
Arabidopsis      CQQGPCF-APPPANGV-------------SVAEIVTQEFFNGIISQAASS-CAGNR-FYS 99
Tobacco          CQGGPCFSASLSSNGG-------------SVADVVSNAFFNGITDQAAST-CEGKG-FYT 100
Carrot           CQAGPCTNSAPSGGGN-----------AVSVADIVTDDFFNGIISQATGD-CDGKN-FYT 96
Douglas-fir      CQSGPCSSSSGGSPSGGGG----------SVGTIISESIFNGLAGGAASS-CEGKG-FYT 101
Barley           ------------------------------------------------------------
Cotton           -------------------------------SLISQDMFNEMLKHRNDGNCPGKG-FYT 50
Banana           -CQSQCGGSGGSGGGS-----------GGSVGSIISSSLFEQMLKHRNDAACPGKG-FYT 104
Rubber           -CQSQCDGGGGGEDG------------GIDLGSIISRSTFEEMLKHRNDAACPAKG-FYT 80
Beans            -CQSQC---GGPSPA------------PTDLSALISRSTFDQVLKHRNDGVCPAKG-FYT 103
Chilly           NCQSQCPGDSGPTG--------------DLGSIISNSMFDEMLKHRNDNACQGKNNFYS 101
Apple            ------------------------------------------------------------
Chickpea         -CQSQCGSGGGGVG------------------SIISRDTFNQMLKHRDDSGCEGKG-FYT 97
Orange           ----------------------------GELGKIISREMFDDLLEYRNDERCPARG-FYT 73
```

Fig 62. Multiple sequence alignment of amino acid sequences of chitinase from different plant species using ClustalW2.

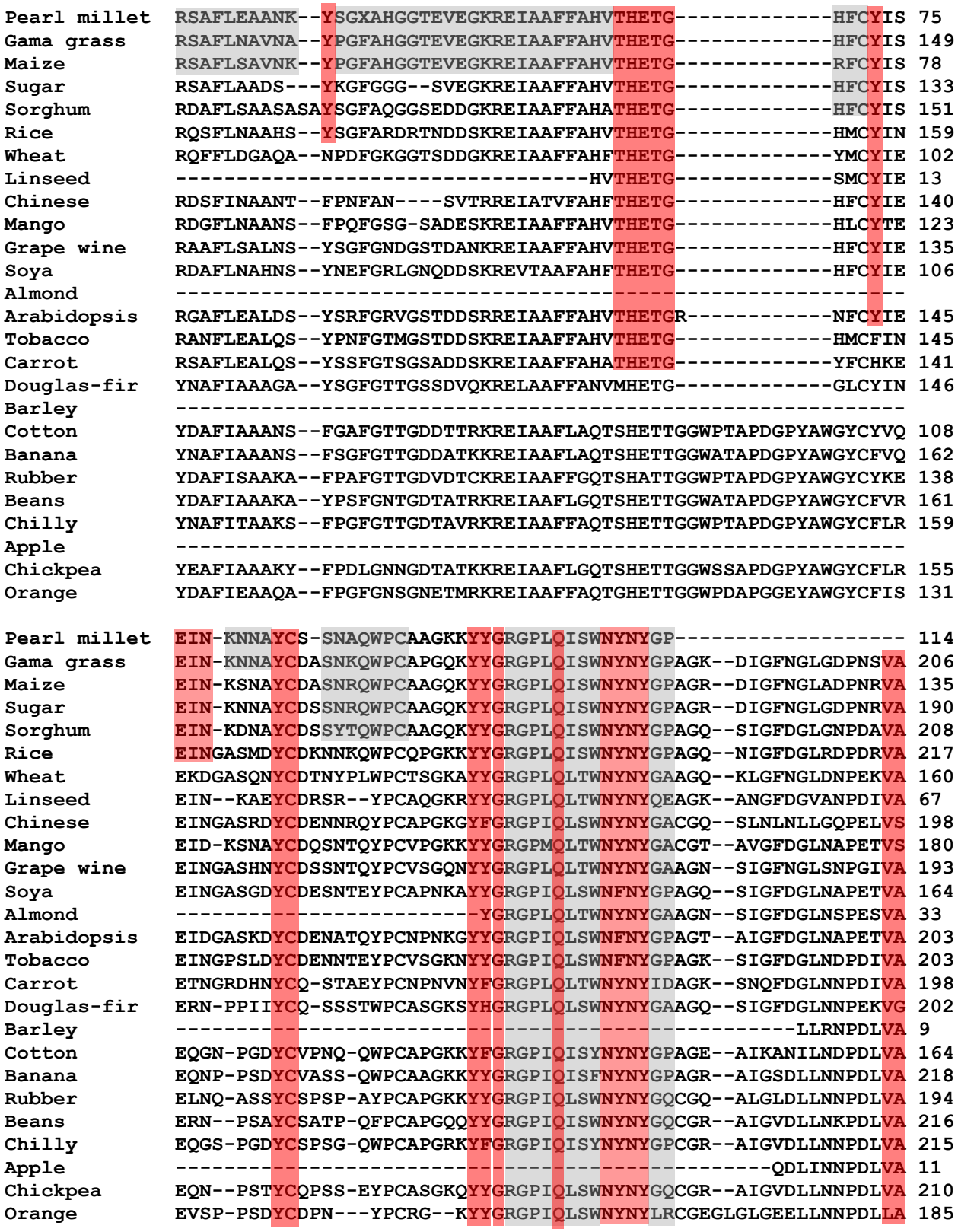


Fig 62. Multiple sequence alignment of amino acid sequences of chitinase from different plant species using ClustalW2.

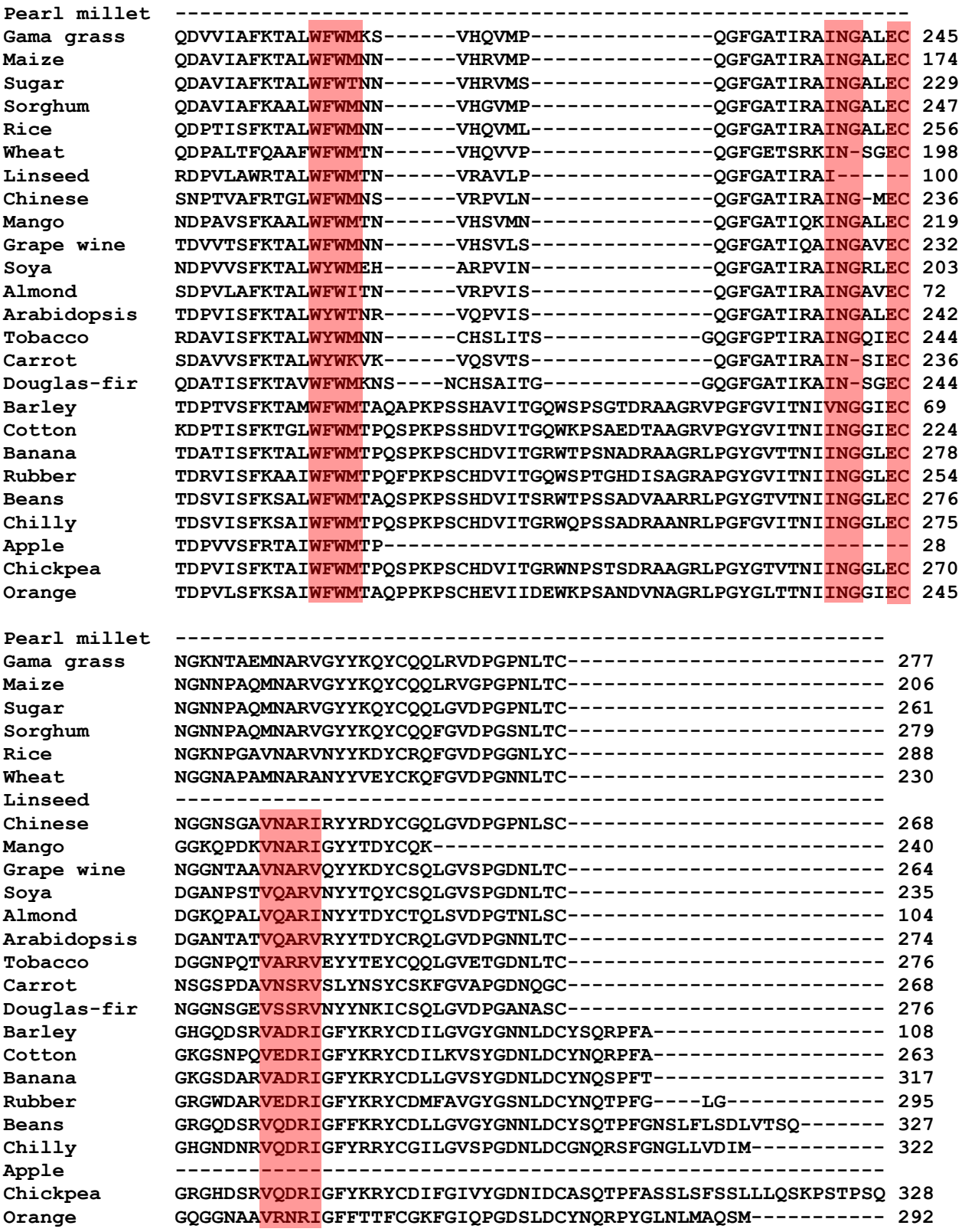


Fig 62. Multiple sequence alignment of amino acid sequences of chitinase from different plant species using ClustalW2.

3.15.3 ii) Phylogenetic tree of amino acid sequence of chitinases from different plant species.

The phylogenetic analysis of the aligned amino acid sequence of chitinase within 25 different plant species was created using ClustalW2 tool from EMBL. The phylogenetic tree represented in Fig 63 shows three major groups based on the degree of homology of amino acid sequence. The first major group includes pearl millet, gama grass, maize, sugar cane, sorghum, rice and wheat (Monocotyledonous) along with linseed, Chinese cabbage, mango and grape wine. Interestingly pearl millet is separated from barley chitinase which falls into the second major group along with banana, cotton, rubber tree, common bean, chilly, apple chick pea and orange. The third major group consists of dicotyledonous plants like carrot, Arabidopsis thaliana, soya bean and almond along with a gymnosperm douglas-fir.

Phylogenetic tree of amino acid sequence of chitinases from different plant species.

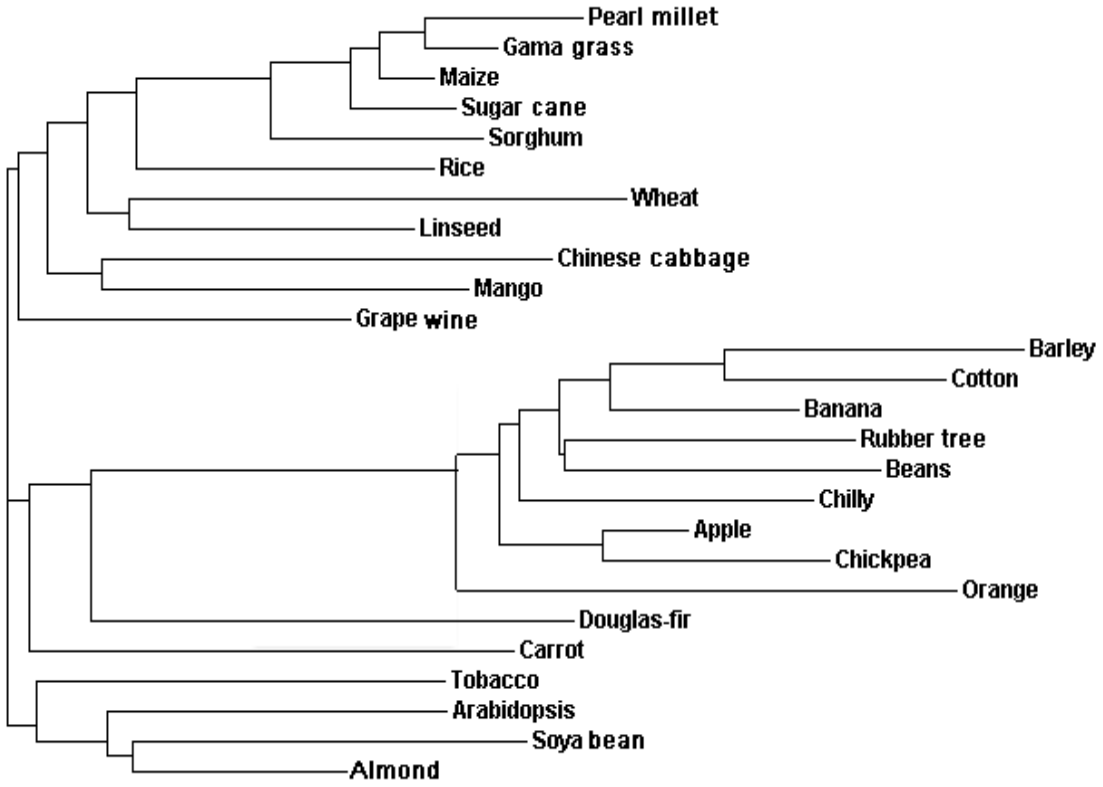


Fig 63. Phylogenetic analysis of the aligned amino acid sequence of pearl millet (*Pennisetum glaucum*) chitinase and chitinases from 25 different plant species. The horizontal lengths of branches are proportional to the relative homology between sequences. Constructed using ClustalW2.

NCBI Accession number of sequences considered for the construction of phylogenetic tree are: *Arabidopsis thaliana* (gi|2597826), *Brassica rapa* (Chinese cabbage, gi|116267545), *Capsicum annuum* (Chilly, gi|222159963), *Cicer arieti*num (Chickpea, gi|3892724), *Citrus sinensis* (Orange, gi|1220144), *Daucus carota* (Carrot, gi|1549331), *Glycine max* (Soya bean, gi|255631536), *Gossypium hirsutum* (Cotton, gi|1729760), *Hevea brasiliensis* (Rubber tree, gi|14575525), *Hordeum vulgare* (Barley, gi|18972), *Linum usitatissimum* (Linseed/Flax, gi|76056895), *Malus domestica* (Apple, gi|20149280), *Mangifera indica* (Mango, gi|189014948), *Musa accuminata* (Banana, gi|17932712), *Nicotiana tabacam* (Tobacco, gi|121663827), *Phaseolus vulgaris* (Common beans, gi|52548196), *Prunus dulcis* (Almond, gi|148807189), *Pseudotsuga menziesii* (Douglas-fir, gi|227937465), *Pennisetum glaucum* (Pearl millet,gi|296011301), *Sacccharum officinarum* (Sugar cane, gi|15213848), *Sorghum bicolor* (Sorghum, gi|242076344), *Tripsacum dactyloides* (Gama grass, gi|48093316), *Triticum aestivum* (Wheat, gi|4741842), *Vitis vinifera* (Grape wine, gi|2306813).

3.15.4 Multiple sequence alignment of amino acid sequence of TLPs from different plant species and construction of phylogenetic tree.

3.15.4 i) Multiple sequence alignment of amino acid sequence of TLPs.

Multiple sequence alignment of TLPs from different plant species revealed that pearl millet TLP shares homology with TLPs and related antifungal proteins like osmotin and zeamatin (TLP from maize) belonging to 24 different (monocotyledonous as well as dicotyledonous) plant species considered for comparison. Grey shaded boxes indicate well conserved amino acid residues and red boxes marked with asterisks indicate the position of 16 highly conserved cysteine residues which form disulphide bridges in all TLPs and dashes denote spaces required for optimal alignment (Fig 64). Of interest are the 16 conserved cysteine residues involved in the assemblage of eight disulfide linkages. These linkages not only help stabilize the molecule and allow for correct folding but also make TLPs resistant to protease degradation and may be essential for antifungal activity (Krebitz *et al.,* 2003; Roberts and Selitrennikoff, 1990). Disruption of these disulfide bonds will result in loss of the tertiary structure of the thaumatin molecule, and loss of sweetness (Van der Wel and Loeve, 1972). The 3-D crystal structure of PR-5 proteins such as zeamatin from maize and osmotin from tobacco have been used as model templates to determine the occurrence of a negatively charged surface cleft structure on related proteins (Batalia et *al.,* 1996; Hoffmann-Sommergruber, 2002). In TLPs this acidic cleft is comprised of five amino acids (arginine, glutamic acid, and three aspartic acid residues R, E and three Ds) and is believed to be involved in a PR-5-fungal cell interaction that permeabilizes the fungal cell membrane, disrupting the osmotic balance inside hyphal cells leading to cell rupture (Batalia *et al.,* 1996). These sequences are indicated by yellow shaded boxes. Blue shaded boxed indicate the conserved tryptophan among solanaceous members important in carbohydrate binding activity of TLPs.

NCBI Accession number of TLPs considered for multiple sequence alignment are: *Avena sativa* (Oats, gi|1373392), *Camellia sinensis* (Tea, gi|90995395), *Capsicum annuum* (Chilly, gi|11321161), *Citrus sinensis* (Orange, gi|2854101), *Coffea Arabica* (Coffee, gi|158668024), *Gossypium hirsutum* (Cotton, gi|34538351), *Helianthus annuus* (Sunflower, gi|20385169), Hordeum vulgare (barley, gi|14164983), *Mirabilis jalapa* (gi|46949200), *Musa acuminata* (Banana, gi|2586149), *Nepenthes alata* (gi|165292440), *Nicotiana tabacum* (Tobacco, gi|1167854), *Oryza sativa* (Rice, gi|41469595), *Piper colubrinum* (gi|161375756), *Pseudotsuga menziesii* (Douglas-fir, gi|35396766), *Solanum lycopersicum* (Tomato) gi|20750097, *Solanum tuberosum* (Potato, gi|53830847), Sorghum bicolor gi|242038635, *Thaumatococcus daniellii* (gi| 121945718), *Theobroma cacao* (Cacao tree, gi|88193285), *Vitis vinifera* (Grapewine, gi|225426803), *Zea mays* (Maize, gi|226505632), *Zingiber zerumbet* (Shampoo ginger, gi|220683825).

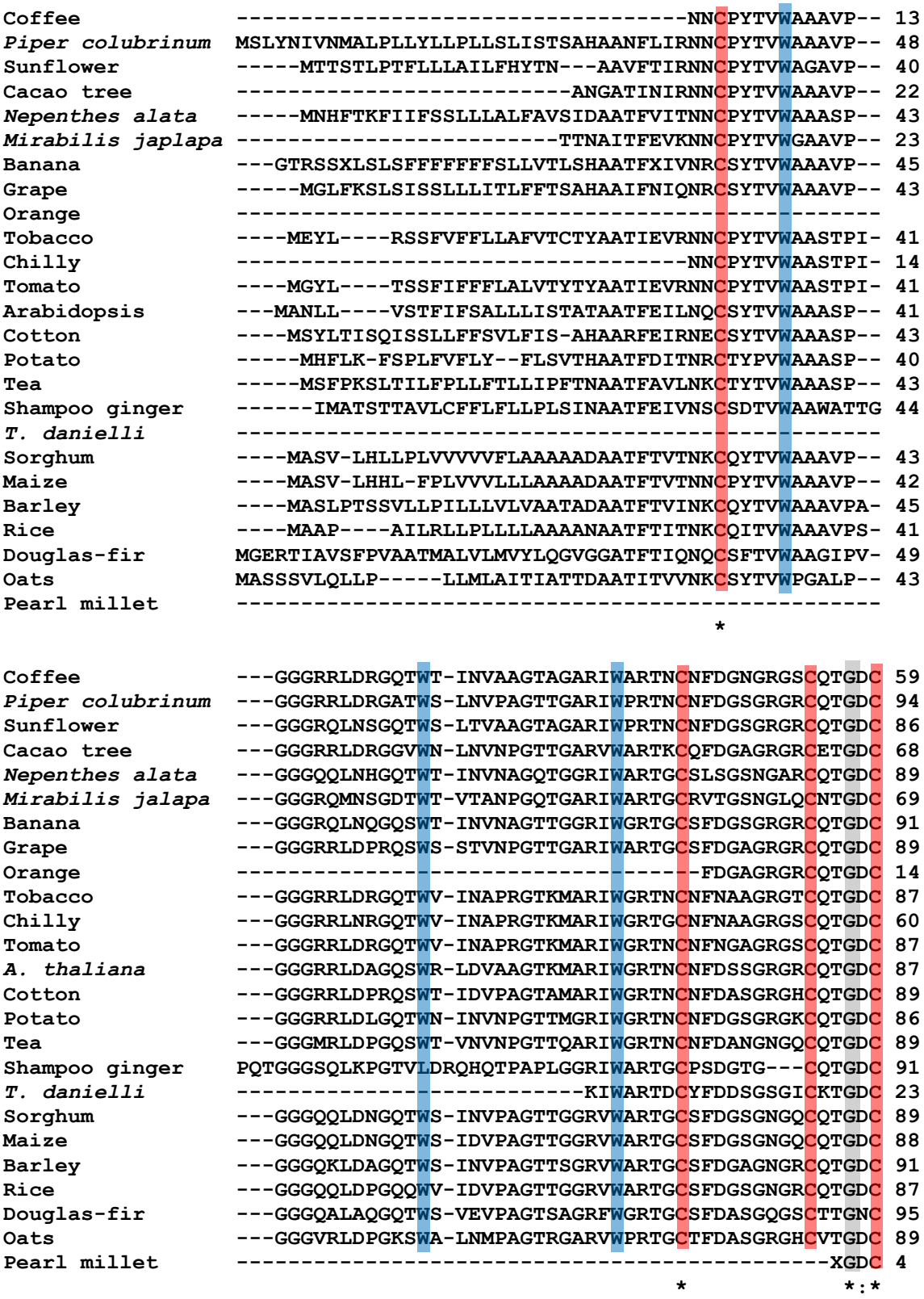


Fig 64. Multiple sequence alignment of amino acid sequences of TLP from different plant species using ClustalW2.

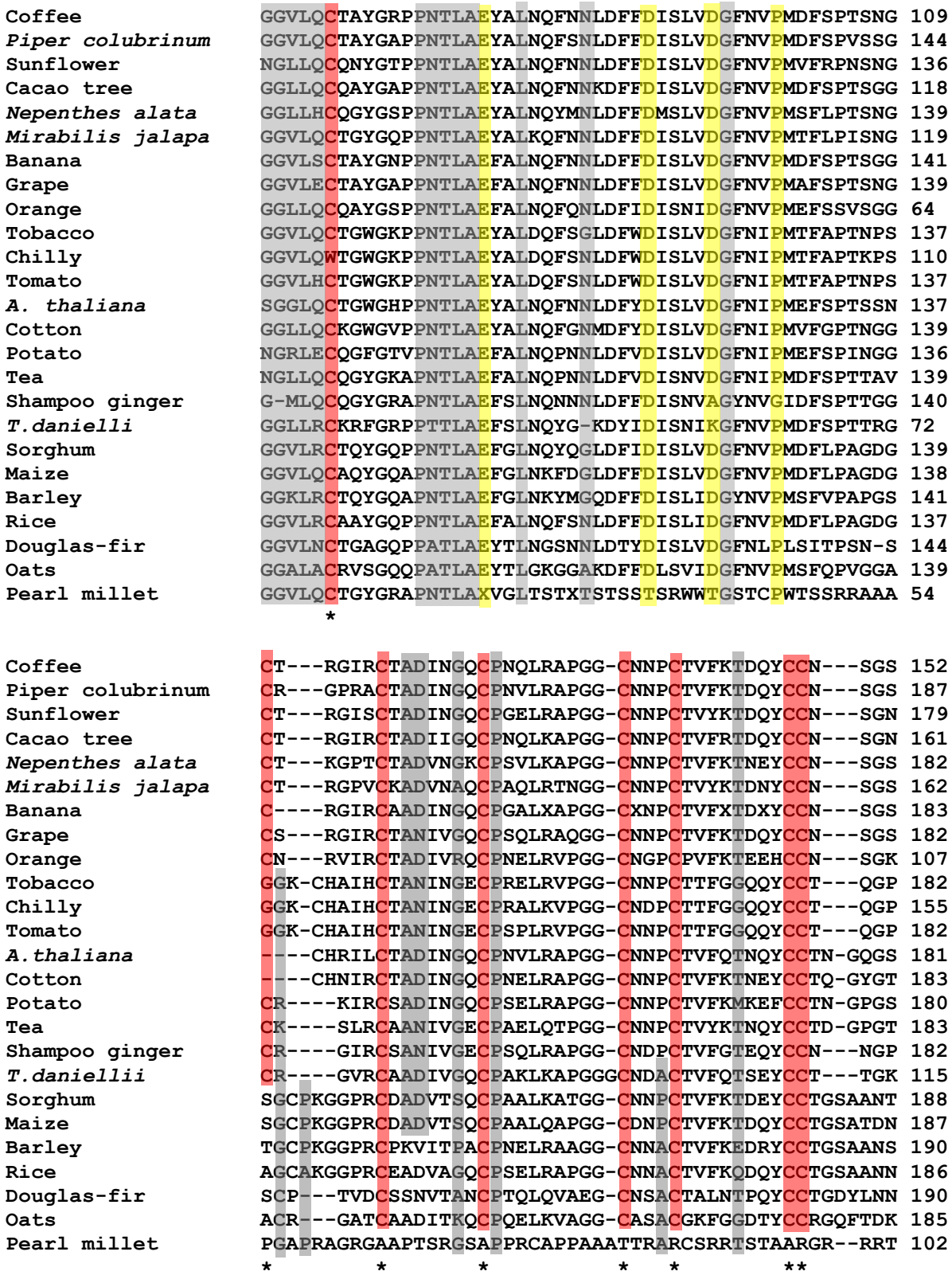


Fig 64. Multiple sequence alignment of amino acid sequences of TLP from different plant species using ClustalW2.

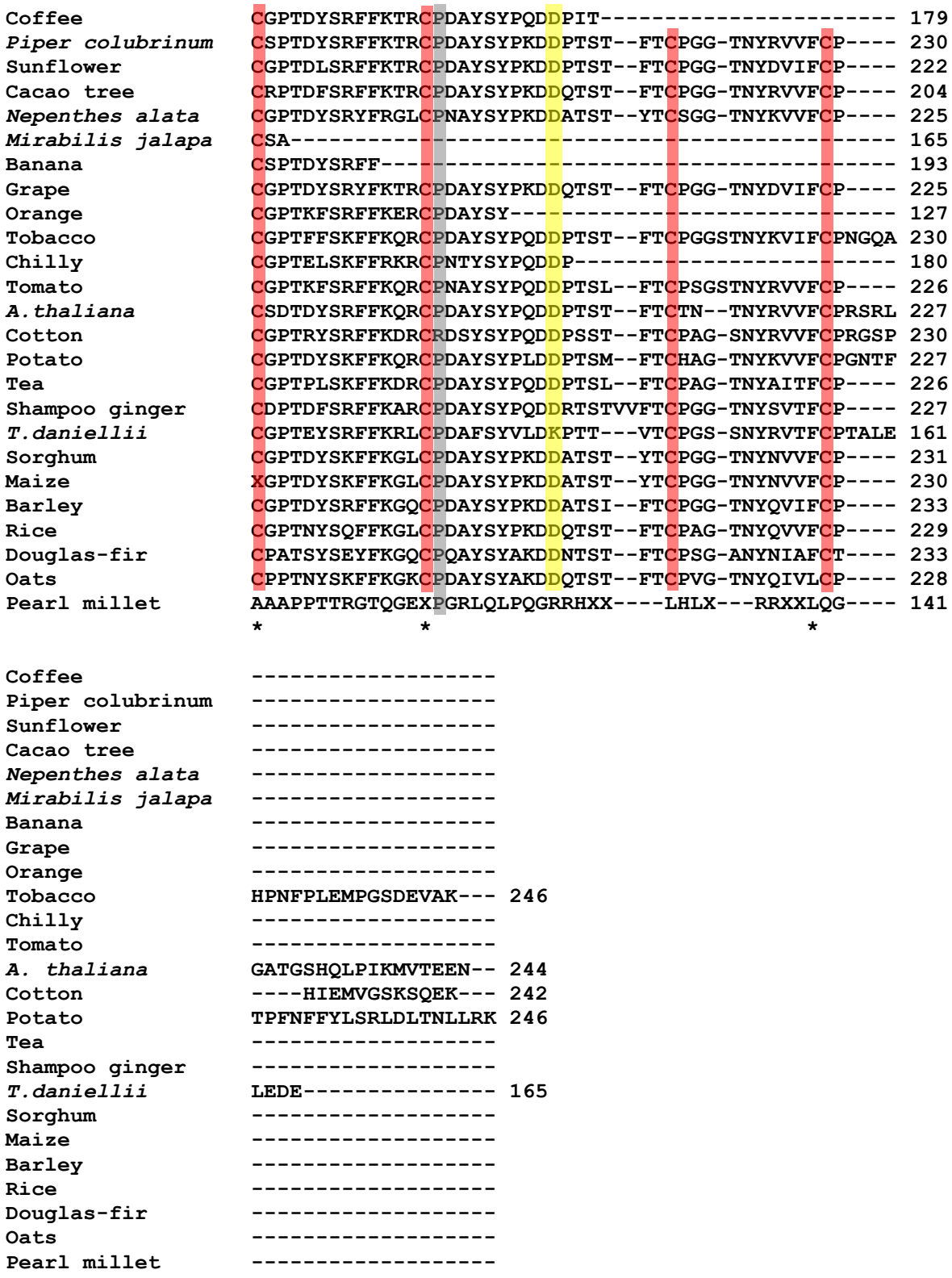


Fig 64. Multiple sequence alignment of amino acid sequences of TLP from different plant species using ClustalW2.

3.15.4 ii) Phylogenetic tree of amino acid sequences of TLP from different plant species.

The phylogenetic analysis of the aligned amino acid sequence of TLP within 24 different plant species was created using ClustalW2 tool from EMBL. The phylogenetic tree represented in Fig 65 shows three major groups and based on the degree of homology of amino acid sequence. The first sub group includes pearl millet, douglas-fir and oats. Interestingly pearl millet is separated from other monocotyledonous plants like sorghum, maize, barley and rice belonging to the second subgroup. The next subgroup consists of shampoo ginger and Thaumatococcus danielli. Next subgroup includes potato and tea, this is followed by the next subgroup consists of solanaceous members tobacco chilly and tomato. Orange is separately placed in the next subgroup. The next subgroup consists of banana, *Nepenthes alata* and *Mirabilis jalapa* and grapewine falling into the next minor sub group. Cacao tree is separately placed on the next subgroup. The next major sub group consists of coffee and *Piper colubrinum*. The last major group consists only of sunflower.

Phylogenetic tree of amino acid sequences of TLP from different plant species

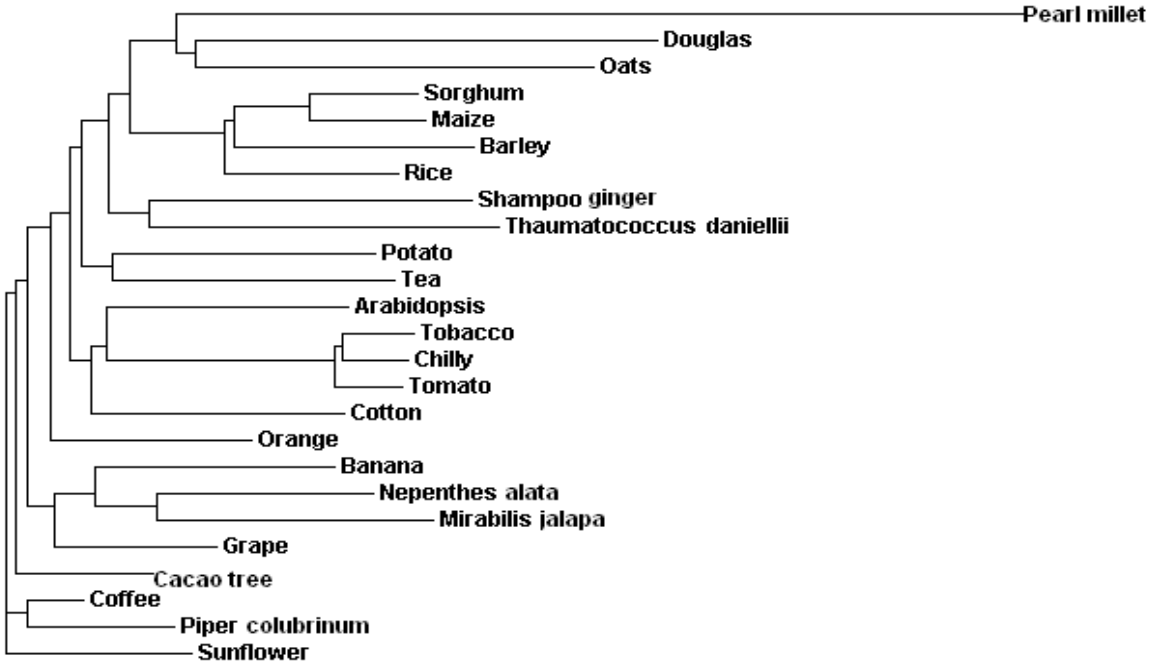


Fig 65. Phylogenetic analysis of the aligned amino acid sequence of pearl millet (*Pennisetum glaucum*) TLP and TLPs from 24 different plant species. The horizontal lengths of branches are proportional to the relative homologies between sequences. Constructed using ClustalW2.

NCBI Accession number of TLPs considered for multiple sequence alignment and construction of phylogenetic tree are: *Avena sativa* (Oat, gi|1373392), *Camellia sinensis* (Tea, gi|90995395), *Capsicum annuum* (Chilly, gi|11321161), *Citrus sinensis* (Orange, gi|2854101),*Coffea Arabica* (Coffee, gi|158668024), *Gossypium hirsutum* (Cotton, gi|34538351), *Helianthus annuus* (Sunflower, gi|20385169), *Hordeum vulgare* (barley, gi|14164983), *Mirabilis jalapa* (gi|46949200), *Musa acuminata* (Banana, gi|2586149), *Nepenthes alata* (gi|165292440), *Nicotiana tabacum* (Tobacco, gi|1167854), *Oryza sativa* (Rice, gi|41469595), *Piper colubrinum* (gi|161375756), *Pseudotsuga menziesii* (Douglas-fir) gi|35396766, *Solanum lycopersicum* (Tomato, gi|20750097), *Solanum tuberosum* (Potato, gi|53830847), *Sorghum bicolor* (Sorghum, gi|242038635), *Thaumatococcus daniellii* (gi| 121945718), *Theobroma cacao* (Cacao tree, gi|88193285), *Vitis vinifera* (grapewine, gi|225426803), *Zea mays* (maize, gi|226505632), *Zingiber zerumbet* (shampoo ginger, gi|220683825).

3.15.5 Phylogenetic tree of nucleotide sequences of chitinase from different plant species.

The phylogenetic analysis of the aligned amino acid sequence of chitinase within 25 different plant species was created using ClustalW2 tool from EMBL. The phylogenetic tree represented in Fig 66 shows three major groups and based on the degree of homology of nucleotide sequence. The first sub group includes pearl millet which is separated from the other monocotyledonous plants like maize, gama grass, sugar cane sorghum, rice and wheat which fall under the second subgroup along with the gymnosperm douglas fir. This indicates that though pearl millet chitinase belongs to the same major subgroup of other monocotyledonous plants it is separated from them forming a separate subgroup and it is also separated from barley which belongs to second major subgroup. The next subgroup consists barley, banana, cotton, common beans, rubber tree, chickpea, orange, chilly and apple. The next subgroup consists of turnip, linseed, soya bean and mango. The next subgroup consists of grape wine, almond, Arabidopsis and tobacco. The last major group consists only of carrot.

Phylogenetic tree of nucleotide sequences of chitinase from different plant species

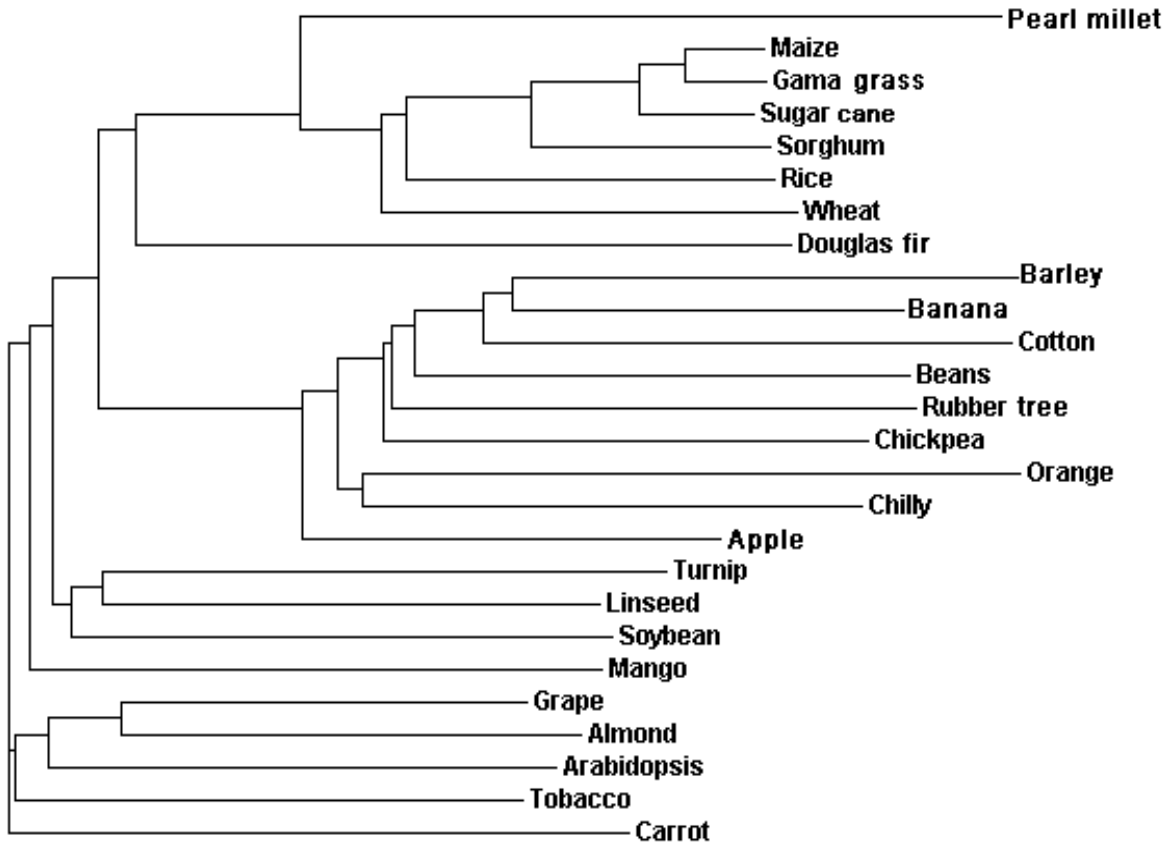


Fig 66. Phylogenetic analysis of the aligned nucleotide sequence of pearl millet (*Pennisetum glaucum*) chitinase and chitinases from 25 different plant species. Constructed using ClustalW2.

NCBI Accession number of sequences considered for multiple sequence alignment are: *Arabidopsis thaliana* gi|2597826, *Brassica rapa* (Chinese cabbage) gi|116267545, *Capsicum annuum* (Chilly) gi|222159963, *Cicer arieti*num (Chickpea) gi|3892724, *Citrus sinensis* (Orange) gi|1220144, *Daucus carota* (Carrot) gi|1549331, *Glycine max* (Soya bean) gi|255631536, *Gossypium hirsutum* (Cotton) gi|1729760, *Hevea brasiliensis* (Rubber tree) gi|14575525, *Hordeum vulgare* gi|18972 (Barley), *Linum usitatissimum* (Linseed/Flax) gi|76056895, *Malus domestica* (Apple) gi|20149280, *Mangifera indica* (Mango) gi|189014948, *Musa accuminata* (Banana) gi|17932712, *Nicotiana tabacam* (Tobacco) gi|121663827, *Phaseolus vulgaris* (Common beans) gi|52548196, *Prunus dulcis* (Almond) gi|148807189, *Pseudotsuga menziesii* (Douglas-fir) gi|227937465 *Pennisetum glaucum* (Pearl millet) gi|296011301, *Sacccharum Saccharum officinarum* (Sugar cane) gi|15213848, *Sorghum bicolor* gi|242076344, *Tripsacum dactyloides* (Gama grass) gi|48093316, *Triticum aestivum* (Wheat) gi|4741842, *Vitis vinifera* (Grape wine) gi|2306813.

3.15.6 The phylogenetic tree of nucleotide sequences of TLP from different plant species.

The phylogenetic analysis of the aligned nucleotide sequences of tlps within 24 different plant species was created using ClustalW2 tool from EMBL. The phylogenetic tree represented in Fig 67 shows three major groups and based on the degree of homology of nucleotide sequence. The first sub group includes only pearl millet which is separated from the other monocotyledonous plants like sorghum, maize, rice and barley and oats belonging to the second subgroup. This indicates phylogenetic divergence of pearl millet form maize rice and sorghum. The second major subgroup includes banana, shampoo ginger *Thaumatococcus danielli, Piper colubrinum* and grape wine. The next subgroup consists of cacao tree and sunflower. The next subgroup consists of coffee potato and orange followed by a subgroup consisting mainly of solanaceous members tobacco, tomato and chilly along with *Arabidopsis thaliana* and cotton. The last subgroup consists of *Nepenthes alata* and *Mirabilis jalapa* and douglas fir. The last major group consists only of tea.

The phylogenetic tree of nucleotide sequences of TLP from different plant species.

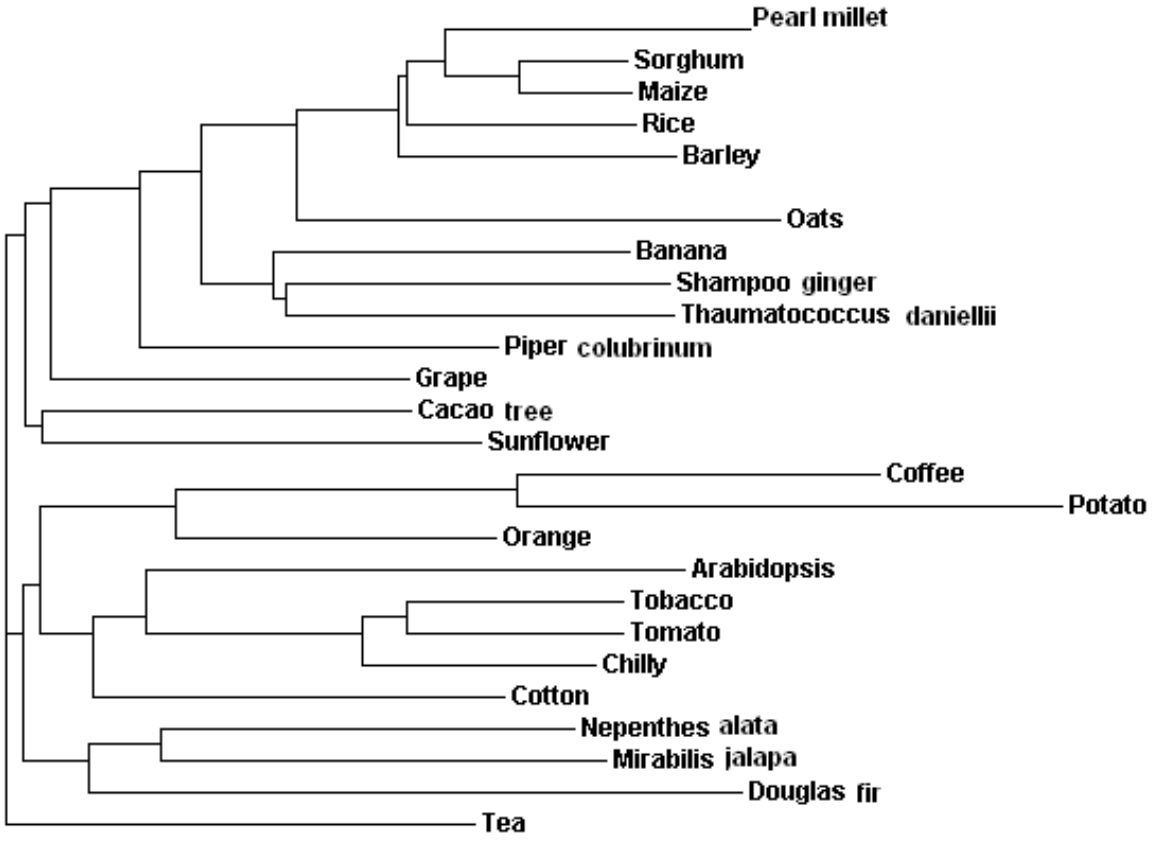


Fig 67. Multiple sequence alignment of nucleotide sequences of tlp from different plant species using ClustalW2. Constructed using ClustalW2.

NCBI Accession number of TLPs considered for multiple sequence alignment are: *Avena sativa* (Oats, gi|1373392), *Camellia sinensis* (Tea, gi|90995395), *Capsicum annuum* (Chilly, gi|11321161) *Citrus sinensis* (Orange, gi|2854101), *Coffea Arabica* (Coffee, gi|158668024), *Gossypium hirsutum* (Cotton, gi|34538351), *Helianthus annuus* (Sunflower, gi|20385169), *Hordeum vulgare* (Barley, gi|14164983), *Mirabilis jalapa* (gi|46949200), *Musa acuminata* (Banana, gi|2586149), *Nepenthes alata* (gi|165292440,) *Nicotiana tabacum* (Tobacco, gi|1167854), *Oryza sativa* (Rice, gi|41469595), *Piper colubrinum* (gi|161375756), *Pseudotsuga menziesii* (Douglas-fir, gi|35396766), *Solanum lycopersicum* (Tomato, gi|20750097), *Solanum tuberosum* (Potato, gi|53830847), *Sorghum bicolor* (Sorghum, gi|242038635), *Thaumatococcus daniellii* (gi| 121945718), *Theobroma cacao* (Cacao tree, gi|88193285), *Vitis vinifera* (grapewine, gi|225426803), *Zea mays* (maize, gi|226505632), *Zingiber zerumbet* (shampoo ginger, gi|220683825).

3.15.7 Northern Blot analysis.

In case of northern blot analysis using radioactive ^{32}P, total RNA extracted from downy mildew susceptible and resistant pearl millet plants at different time intervals after infection with the downy mildew pathogen *Sclerospora graminicola* was separated on agarose gel on agarose gel in different lanes. The resolved total RNA was transferred onto nylon membrane overnight hybridization with radioactive p32 labeled DNA probes was carried out (Alwine *et al.,* 1977 with modifications) as explained under section 2.18 of materials and methods. Concentration of total RNA was measured with spectrophotometer and equal amount of total RNA (10 µg/lane) was loaded onto all the lanes as seen in Fig 68.

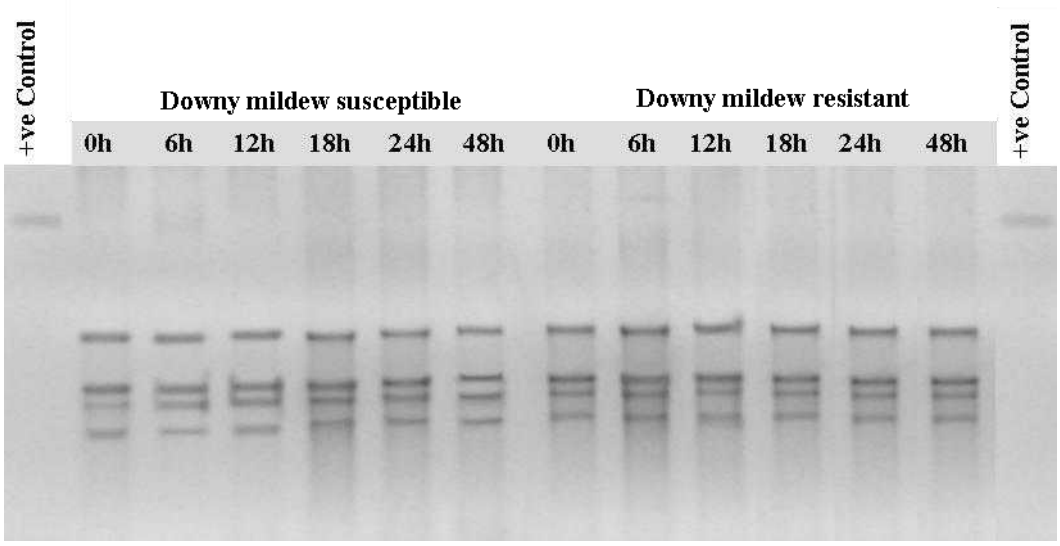


Fig 68. Electrophoresis of total RNA from down mildew susceptible and resistant seedlings.

First and the last lane is positive control which is pearl millet chitinase 482 bp used for radioactive ^{32}P labeling. First 6 lanes show total RNA from downy mildew susceptible seedlings and the next six lanes are total RNA from downy mildew resistant seedlings harvested 0-48 hrs after inoculation with the downy mildew pathogen (10 µg of total RNA was loaded to each lane).

3.15.7 i) Northern blot analysis with radioactive ^{32}P labeled pearl millet chitinase probe.

Hybridization of the blots was carried out as explained under section 2.18 of materials and methods. After hybridization the blots were washed with wash buffer and sealed between thin transparent plastic sheets and kept facing the autoradiography film overnight and scanned using phosphor image (Raytest autoradiography equipment) the next day. Autoradiogram of northern blot analysis using ^{32}P labeled chitinase probe is seen in the Fig 69.

Northern blot analysis using radioactive ^{32}P labeled pearl millet *chitinase* probe.

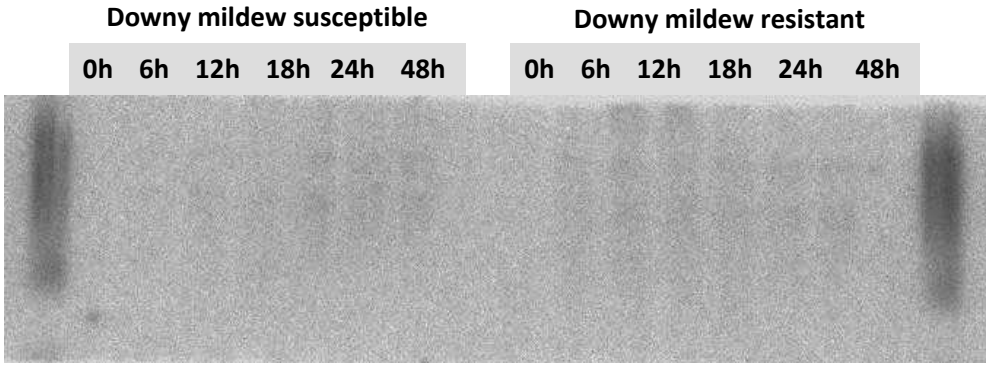


Fig 69. Northern blot analysis using radioactive ^{32}P labeled pearl millet chitinase (483 bp) probe. First and the last lane is positive control which is pearl millet chitinase 482 bp used for radioactive P32 labeling. First 6 lanes show total RNA from downy mildew susceptible seedlings and the next six lanes are total RNA from downy mildew resistant seedlings harvested 0-48 hrs after inoculation with the downy mildew pathogen.

As observed in the Fig 69 above the positive control showed strong signal whereas the signals from test samples were not conclusive. A very weak hybridization was observed at 55°C hybridization temperature. Hybridization carried out at 60°C did not show any signals. The experiment was repeated thrice; however, with no concrete and conclusions could be drawn from the results obtained.

3.15.7 ii) Northern blot analysis with radioactive P32 labeled pearl millet *tlp* probe.

The radioactivity from pearl millet chitinase labeled blot was stripped by incubation with the stripping solution as explained under section 2.18.8 of materials and methods and the same blot was hybridized with radioactive ^{32}P labeled pearl millet tlp probe. Autoradiogram of Northern blot analysis with radioactive ^{32}P labeled pearl millet tl*p* probe is shown in Fig 70.

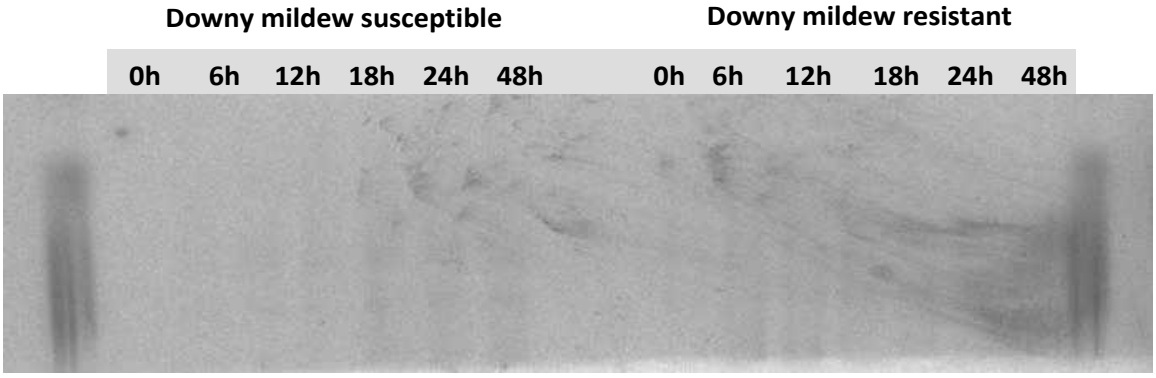


Fig 70. First and the last lane is positive control which is pearl millet *tlp* 422 bp used for radioactive ^{32}P labeling. First 6 lanes show total RNA from downy mildew susceptible seedlings and the next six lanes are total RNA from downy mildew resistant seedlings harvested 0-48 hrs after inoculation with the downy mildew pathogen.

Hybridization carried out at 55, 58 and 60°C did not show any strong signals as seen in Fig 69. The experiment was repeated thrice; however, with no concrete and conclusions could be drawn from the results obtained.

3.15.8 Southern blot analysis.

To study the copy number of thaumatin like protein and chitinase gene in down mildew susceptible and resistant pearl millet seedlings, southern blot analysis was carried out. Partial length gene fragments of tlp and chitinase were labeled with radioactive ^{32}P.

3.15.8 i) Southern blot analysis using radioactive *chitinase* probe.

Genomic DNA from downy mildew susceptible and resistant cultivars was isolated by CTAB method (Murray and Thompson, 1980 with modifications) and subjected to restriction digestion with different enzymes separately (*Alu*I, *Eco*RV, *Bam*HI, *Hind*III, *Sau*3AI and *Bam*HI+*Eco*RI).

Southern blot analysis with radioactive ^{32}P labeled pearl millet chitinase probe

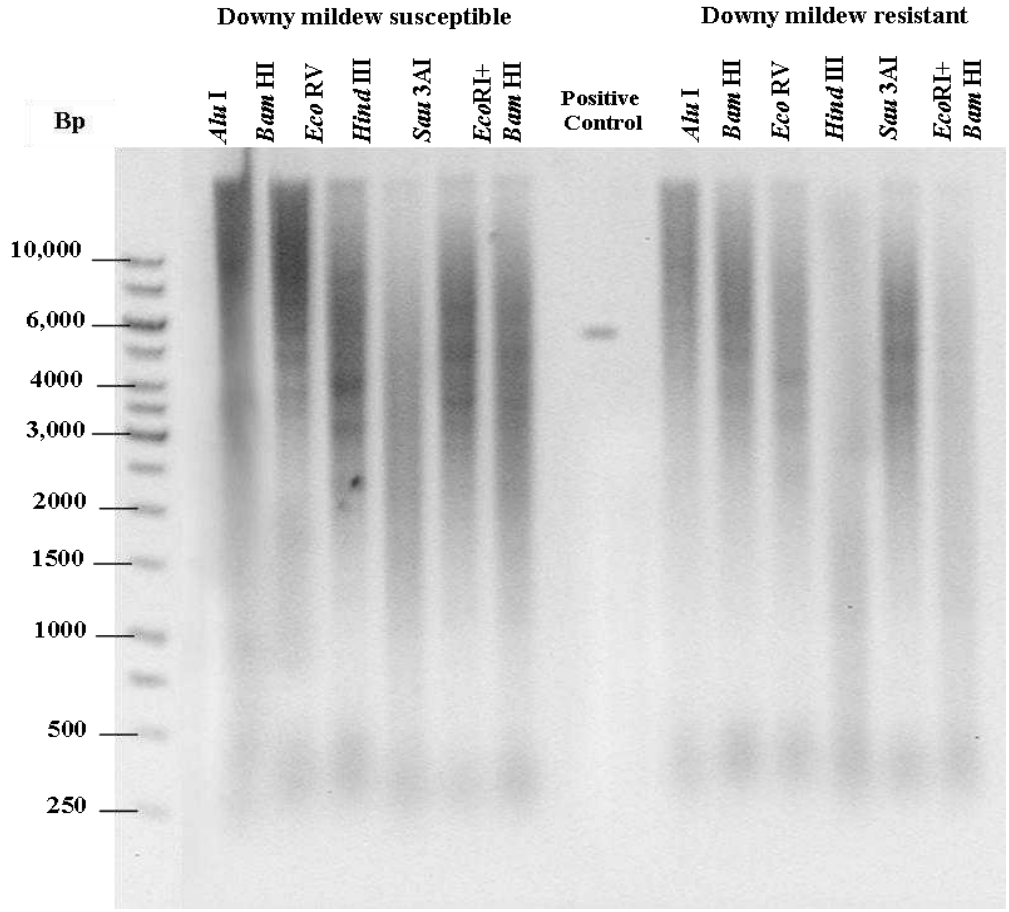


Fig 71. Southern blot analysis with radioactive P32 labeled pearl millet chitinase (482 bp).
First lane is the 1 Kb Gene ruler (Fermentas # SM0311). Radioactive ^{32}P was used for labeling of tlp (fragment) only positive control shows hybridization with the radioactive probe The first 6 lanes are restriction digested genomic DNA samples from downy mildew susceptible pearl millet cultivar and the next six lanes are from downy mildew resistant cultivar (hybridization at 55°C overnight).

The restriction digested genomic DNA was purified by phenol chloroform method and separated on agarose gel in separate lanes. Partial length fragment of pearl millet *chi* (482 bp) used as probe served as the positive control. Radioactive labeling of the *chi* probe and hybridization was carried out as explained under section 2.19 of materials and methods. As seen in the autoradiogram (Fig 71), southern blot analysis could not lead to any conclusive results as there was no specific hybridization of the radio-labeled chitinase probe. Hybridization was carried out at 3 different temperatures namely 55, 58 and 60°C. Only the positive control showed hybridization with the radio labeled probe at 55°C, at higher hybridization temperatures there was no hybridization observed. Therefore it can be concluded that the hybridization temperature for the *chitinase* probe is 55°C; however, nonspecific hybridization was observed at this temperature. Increase in temperature does not lead to any hybridization. The Experiment was repeated three times without any success.

3.15.8 ii) Southern blot analysis using ^{32}P labeled tlp probe.

This was done similar to southern blot analysis with chitinase but partial length pearl millet tlp (422 bp) was used as the probe. Radioactive labeling of tlp probe and hybridization was carried out at 55°C overnight as explained under section 2.19 of materials and methods.

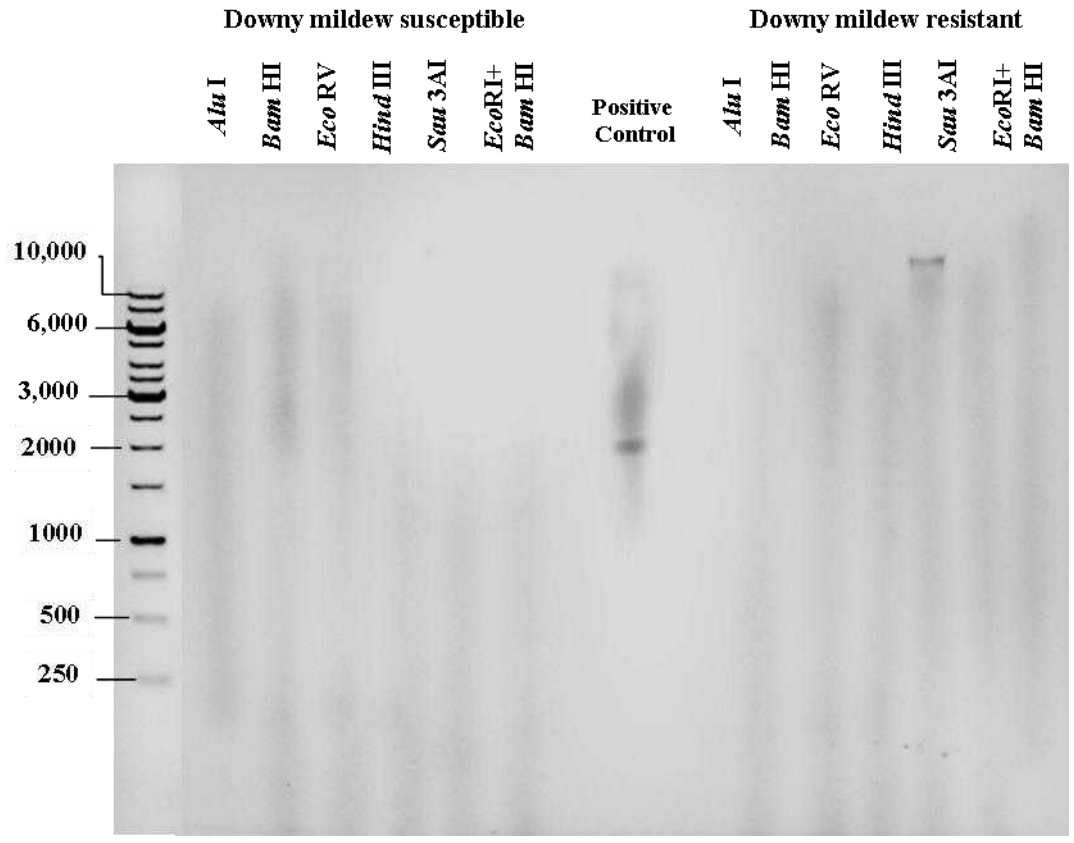


Fig 72. Southern blot analysis of the restriction digested genomic DNA from pearl millet. First lane is the 1 Kb Gene ruler (Fermentas # SM0311). Radioactive ^{32}P was used for labeling of tlp (fragment) only positive control shows hybridization with the radioactive probe. The first 6 lanes are restriction digested genomic DNA samples from downy mildew susceptible pearl millet cultivar and the next six lanes are from downy mildew resistant cultivar (hybridization at 60°C overnight).

Partial length fragment of pearl millet tlp (422 bp) used as probe served as the positive control. After hybridization the blots were washed with wash buffer and sealed between thin transparent plastic sheets and kept facing the autoradiography film overnight and scanned using phosphor image (Raytest autoradiography equipment) the next day. Autoradiogram of southern blot analysis using ^{32}P labeled chitinase DNA probe is shown in the Fig 72.

However, it was not possible to draw conclusion on copy number or tlp gene based on the autoradiogram. Lower hybridization temperature up to 55°C lead to non specific labeling of the bands where all the nucleic acid material showed binding of radioactive ^{32}P. At 58°C hybridization temperature hybridization of only the positive control was observed. The experiment was repeated three times without any success. This could be due to large genome size leading to low copy number of target sequence (gene of interest) in comparison to non target sequence. Therefore there was no hybridization observed at 58°C and above. Lower hybridization decreases the stringency thereby allowing non specific hybridization of the radioactive probe.

Acknowledgment.

Anti-TLP antibody from Douglas fir was kindly provided by Dr. Ekramoddoullah, Canadian Forest Service, Canada). Anti-chitinase antibody from tobacco was kindly provided by M Legrand, IBMP Strasbourg, France). *De novo* sequencing was performed by Dr. Markus Piotrowski Institute of Plant Physiology, Ruhr University, Bochum. Financial assistance was provided by University Grants Commission (UGC) India, Deutscher Akademischer Austausch Dienst (DAAD), Eiselen Foundation Ulm, Germany and Institute for Botany, Leibniz University Hannover. Laboratory Facilities were provided by Institute for Botany Leibniz University Hannover, DOS in Applied Botany and Biotechnology University of Mysore India. Pearl millet seeds were provided from All India Coordinated Pearl Millet Improvement Program (AICPMIP). Sincere thanks to all the above mentioned individuals for providing the antibodies, for scientific support and funding agencies for the scholarship granted.

Declaration.

Individual protein lanes in the figures were arranged using Adobe Photoshop and Microsoft Publisher.

4. Discussion.

4.1 Pathogenesis related proteins in pearl millet downy mildew pathogen interaction.

Role of PR proteins in host defense is an interesting area of study in plant pathology; a number of defense related proteins involved in downy mildew resistance by pearl millet like glucanase, thionin, phenyl alanine ammonia lyase, peroxidase, super oxide dismutase are well studied. However, chitinase and thaumatin like proteins from pearl millet are not characterized. Regalado and Ricardo (1996) reported the constitutive expression of chitinase and TLPs in the intercellular fluid of healthy, unstressed leaves of *Lupinus albus* plants and suggest that perhaps they are part of a preformed defense response. These two PR proteins are constitutively expressed in pearl millet. This study concentrates on characterization of these two defense related proteins.

4.2 Co-expression and synergistic activity of chitinase and TLP in plant defense mechanism.

Plants accumulate a large class of proteins called pathogenesis related proteins (PR) when challenged by plant pathogens (Linhorst, 1991, Bol *et al.,* 1990; Van Loon, 1985). These plant defence proteins are ubiquitous in monocots and dicots and are currently grouped into 17 families based on their structural and functional properties (Van Loon and van Strien, 1999; Christensen *et al.,* 2002). PR-5 proteins exhibit antifungal activity in vitro (Liu *et al.,* 1994; Melchers *et al.,* 1993; Woloshuk *et al.,* 1991) and show enhanced lytic activity when tested in combination with chitinases and/or β-1,3-glucanases (Lorito *et al.,* 1996). Synergistic activity of chitinase, glucanase (enzymes capable of degrading fungal cell wall carbohydrates) and TLP was known to be more effective in fungal disease resistance in different host-pathogen systems. Synergistic activity of chitinase and TLP in transgenic rice showed enhanced fungal disease resistance against sheath blight pathogen *Rhizoctonia solani* and sheath rot pathogen, *Sarocladium oryzae* compared to those expressing the individual genes (Krishnan *et al.,* 2006). Similar observations were made in grapevine infected with powdery mildew (Jacobs *et al.,* 1999) and during infection of wheat spikes by *Fusarium graminearum* (Pritsch *et al.,* 2000) Oilseed rape (*Brassica napus*) transformed with barley TLP showed enhanced resistance to clubroot pathogen (*Plasmodiophora brassicae*). Moreover in banana and grapes the cell wall degrading enzymes and TLP are co expressed during ripening of fruits leading to softening of fruit pulp (Peumans *et al.,* 2002). Because of the substantial evidence on co expression of chitinase and TLP and their synergistic mechanism in host defense both PR proteins were considered for the study.

4.3 Glucan binding activity of pearl millet TLP.

Pearl millet TLP was tested for its ability to bind to curdlan (water insoluble ß-1,3 glucan) and it showed glucan binding at under acidic conditions as observed in case of barley thaumatin-like protein (Grenier *et al.,* 1999). SDS-PAGE analysis of the bound protein fraction showed the presence of minor impurities of apparent molecular weight 24-26 kDa along with a

major protein band having apparent molecular weight of 23 kDa protein. Immuno blot analysis of the bound protein fraction showed a 23 kDa protein cross reacting with anti TLP antibody from Douglas-fir. Considerable amount of research work has been carried out to establish the mode of action PR-5 proteins. However, most of the work has been carried out with osmotin from tobacco and zeamatin from maize which are well characterized; it is established that osmotin and other homologous plant PR-5 proteins are able to permeabilise the plasma membrane (Roberts and Selitrennikoff, 1990). To reach the plasma membrane, the protein must first traverse the cell wall. Presumably, cell-wall recognition and binding by osmotin is needed prior to translocation to the plasma membrane (Ibeas *et al.,* 2000; Yun *et al.,* 1997). The outer layer consists predominantly of mannoproteins which determine most of the surface properties of the cell. Work with phosphomannan deficient yeast strains showed that osmotin binds to phosphomannans in fungal cell walls and absence of phosphomannans on cell wall made the yeast cells resistant to osmotin attack (Ibeas *et al.,* 2000). A simple staining technique using alcian blue, a stain which competes with TLP for its binding site on cell wall confirms the presence of TLP binding site on fungal cell wall. *S. graminicola* sporangia were stained with alcian blue and the sporangia showed positive staining with the dye. This indicated the presence of TLP binding site on *S graminicola* cell walls which makes the downy mildew pathogen susceptible to TLP attack. The same point was proven by another experiment which involved glucan binding by pearl millet TLP where curdlan (water insoluble β-1,3-glucan) was used as the substrate which is a cell wall component of fungi; This is in line with the behavior of TLPs from diverse plant species. However, it has been established that the structural differences between various isoforms of the PR-5 family that are essential for the binding of different targets (Yun *et al.,* 1997). Work on cherry fruit TLP by Fils-Lycaon *et al.,* 1996 showed that there is specificity between structures of the PR-5 proteins and those of target microbe organisms; therefore, their efficacy would depend on a combination of isoforms and pathogenic species. Similar observations made by other researchers showed that each antifungal TLP seems to display specificity (Abad *et al*. 1996; Koiwa *et al*. 1997; Yun *et al*. 1998, Grenier *et al*., 1999).

4.4 Inhibitory effect of pearl millet TLP on downy mildew pathogen *S. graminicola*.

PR-5 proteins exhibit antifungal activity in vitro (Liu *et al.,* 1994; Melchers *et al.,* 1993; Woloshuk *et al.,* 1991). Microscopic observation of *S. graminicola* zoospores released by the lemon shaped sporangia revealed that the zoospores swim freely when they are suspended in water. After treatment of sporangial suspension with TLP, motility of the zoospores was inhibited with more than 90% of zoospores losing their mobility. This shows the toxic effect of TLP on *S. graminicola,* which is in line with the previous findings showed that osmotin and other homologous plant PR-5 proteins are able to permeabilise the plasma membrane leading to leakage of cytoplasmic contents from *Neurospora crassa* and *Candida albicans* after treatment with zeamatin (Abad *et al.,* 1996; Roberts and Selitrennikoff, 1990); inhibition growth and lysis of *Phytophthora infestans* sporangia by tobacco osmotin (Woloshuk *et al.,* 1991) was also studied in detail. *In vitro* analysis of *S. graminicola* membrane permeabilization by pearl millet TLP was carried out according Sytox green staining method (Thevissen *et al.,* 1999). Sytox green is a

nucleic acid dye, which enters the cells only through plasma membrane pores and fluoresces upon binding to nucleic acid. Microscopic observation of *S. graminicola* sporangia treated with pearl millet TLP showed fluorescence of the sporangia; positive staining with Sytox green proved permeabilization of *S. graminicola* plasma membrane by pearl millet TLP which eventually leads to cell lysis. Interestingly *S. graminicola* is an oomycete pathogen and these results provide supporting evidence to inhibitory effect of TLP against oomycete pathogens. The observation is supported by good amount of work on TLPs from different plant species and their inhibitory effect on broad range of pathogens (Vigers *et al.,* 1992), for instance, the presence of the 23-kDa TLP in transgenic rice plants enhanced resistance to *Rhizoctonia solani* causing sheath blight disease (Datta *et al*., 1999). TLP from grapevine displays antifungal effect and inhibited mycelial growth, spore germination, and germ tube growth of *Uncinula necator*, *Phomopsis viticola*, and *Botrytis cinerea* (Monteiro *et al.,* 2003). Abscisic acid- and cold-induced thaumatin-like protein in winter wheat has an antifungal activity against snow mould, *Microdochium nivale* (Kuwabara *et al.,* 2002). TLPs from *Taxus* x *Media Rehder* are a conserved component of conifer ovular secretions and are involved in broad spectrum pathogen defence of ovules (Stephen *et al.,* 2007). Studies on of PR-5d from tobacco showed the presence of pathogen-specific insoluble-carbohydrate binding surface motif (consisting of three tryptophan residues conserved at specific positions -W34/W36/W192) and is highly conserved in all the members of Solanaceae which are commonly attacked by oomycete pathogen *Phytophtora* (Doxey *et al.,* 2010).

4.5 Purification of pearl millet chitinase.

Chitinase from pearl millet shared similarity with other class I chitinases in its chromatographic behavior. The procedure to purify pearl millet chitinase was optimized and involved the use of DEAE-cellulose, hydroxyapatite (HA) and CM-sepharose column chromatography. Most of the contaminant proteins were removed by ion exchange chromatography on DEAE-cellulose and negative chromatography on HA column. The enzyme fraction recovered from DEAE cellulose column was passed through HA column and further purified by chromatography on a CM-Sepharose column. Chitinase like protein from black turtle bean (Chu and Ng, 2005) was not adsorbed to DEAE cellulose column but was adsorbed onto CM sepharose column. Similar to several plant chitinases (Chu and Ng, 2003, 2005) pearl millet chitinase was also not adsorbed by DEAE-cellulose but was adsorbed on CM-cellulose and also showed affinity to colloidal chitin similar to mung bean chitinase and chitinase from black turtle bean (Chu and Ng, 2003, 2005). Similar observations were made with purification of tobacco chitinase (Legrand *et al.,* 1987). A linear NaCl gradient was applied to elute the bound proteins from CM sepharose column and homogenous chitinase (with apparent molecular weight of 24 kDa) was eluted at 300 mm NaCl concentration. Similar chromatographic behaviour was observed in the case of other plant chitinases for instance, spruce (*Picea abies*) chitinase showed binding to CM sepharose column (Salzer *et al.,* 1997). The purified protein showed immunological cross reaction with anti chitinase antibody from tobacco. The homogenous protein band was subjected to in gel trypsin digestion and the obtained peptide

fragments were subjected to de novo sequencing by ESI Q-TOF. Homology search of deduced amino acid sequence revealed that the purified pearl millet chitinase is a class I chitinases belonging to lysozyme super family.

4.6 Partial purification of pearl millet TLP.

Pearl millet TLP (apparent molecular weight - 23 kDa) was partially purified by chromatography which eluted from CM sepharose column (10 mM sodium acetate buffer at pH 5.5) along with two other proteins with apparent molecular weight of 24 and 26 kDa at approximately 200 mM NaCl concentration. Barley thaumatin like proteins also showed similar behavior by not binding to DEAE column and binding to CM sepharose column (Osmond *et al.,* 2001). The procedure to purify pearl millet chitinase was optimized and involved the use of DEAE-cellulose, hydroxyapatite (HA) and CM-sepharose column chromatography. Most of the contaminant proteins were removed by ion exchange chromatography on DEAE-cellulose and negative chromatography on HA column. The enzyme fraction recovered from DEAE cellulose column was passed through HA column and further purified by chromatography on a CM-Sepharose column. TLP purified from pearl millet showed certain resemblances in chromatographic behavior to other plant TLPs; for example, mollisin from chestnuts (Chu and Ng, 2003) and chrysancorin from garland chrysanthemum seeds (Wang *et al.,* 2001), which remained unadsorbed on DEAE-cellulose column. The 23 kDa protein was eluted along with another protein of apparent molecular weight 26 kDa which was later identified to be glucanse. The pearl millet TLP showed cross reaction with anti TLP antibody from douglas-fir (Zamani *et al.,* 2004). The Douglas fir anti TLP antibody was developed using a 29 amino acid long synthetic peptide generated with the amino acid sequence of douglas-fir TLP.

4.7 *De novo* sequencing and partial amino acid sequences of pearl millet PR proteins.

Partial amino acid sequences of pearl millet chitinase, glucanase, peroxidase and TLP are of great importance. Partial sequences of pearl millet chitinase and TLP paved the base for this doctoral work by serving as a reference sequence for primer designing; The partial amino acid sequence of glucanase and peroxidase can be considered for primer designing an amplification of gene of interest. Also it can be of used to develop a synthetic peptide for which antibody can be synthesized. Further work on this aspect makes an interesting area of study. Thus the preliminary data generated in this study is of great value because the sequence data on pearl millet PR proteins in the database is very scarce.

4.8 Sequence analysis of pearl millet chitinase.

Sequence analysis showed significant homology between pearl millet chitinase and class I chitinases from a diverse group of plants including monocotyledonous barley, maize, wheat, sorghum and dicoteyledonous plants like tobacco, common beans and many other species. Despite several efforts it was not possible to amplify the complete gene of pearl millet chitinase; therefore the complete structure could not be elucidated. However, the partial nucleotide was

translated and the deduced amino acid sequence was analysed for the presence of conserved domains which showed that the protein belongs class I chitinases (Lysozyme super family) which belong to Glycoside hydrolase family 19 (GH 19) of glycosyl hydrolase classification system and members of this family are found primarily in plants. Glycosyl hydrolase genes have been previously sequenced for *Oryza sativa* (rice), the model dicotyledonous plant *Arabidopsis thaliana* (Henrissat *et al.,* 2001, Yokoyama and Nishitani, 2004) and the fast-growing tree *Populus trichocarpa* (poplar) (Coutinho *et al.,* 2003 Geisler-Lee *et al.,* 2006). Plant glycosyl hydrolases are known to be involved in cell wall polysaccharide degradation (Minic and Jouanin, 2006; Minic, 2008). Class I chitinases are composed of three domains: a Cys rich chitin-binding domain, a Pro-rich hinge region, and a highly conserved catalytic domain (Collinge, 1993). Of interest are the residues important in catalysis or implicated in maintaining the active site geometry which include active site residues Y of the NYNYG motif, the second N of the NYNY motif, the first T of the SHETTGG motif, the second G and the Q of the GRGPIQL motif. These conserved motifs are present in pearl millet chitinase and this is in line with earlier works on different plant chitinases (Verburg *et al.,* 1992). Therefore the molecular data generated in this study serves as foundation for further work in this area.

4.9 Sequence analysis of TLP.

Sequence analysis showed significant homology between pearl millet TLP and antifungal TLPs isolated from a diverse group of plants including monocotyledonous barley, maize, rice sorghum and dicoteyledonous plants like tobacco, orange, grape wine and many other species. In this group are TLPs shown to have antifungal activity like zeamatin from maize as well as fruit allergens from banana (Leone *et al.,* 2006). The conservation in the 16 cysteine residues responsible for disulfide bridges was evident. These linkages not only help stabilize the molecule and allow for correct folding but also make TLPs resistant to protease degradation and may be essential for antifungal activity (Krebitz *et al.,* 2003; Roberts and Selitrennikoff, 1990). The 3-D crystal structure of PR-5 proteins such as zeamatin from maize and osmotin from tobacco have been used as model templates to determine the occurrence of a negatively charged surface cleft structure on related proteins (Batalia *et al.,* 1996; Hoffmann-Sommergruber, 2002). In TLPs this acidic cleft is comprised of five amino acids (arginine, glutamic acid, and three aspartic acid residues R, E and three Ds) and is believed to be involved in a PR-5-fungal cell interaction that permeabilises the fungal cell membrane, disrupting the osmotic balance inside hyphal cells leading to cell rupture. Though the molecular modeling is not carried out for pearl millet TLP, experimental evidence generated in this study supports the presence of these characteristic features in pearl millet TLP. Despite several efforts it was not possible to amplify the complete gene of pearl millet thaumatin-like protein; therefore the complete structure could not be elucidated. However, the partial nucleotide was translated and the deduced amino acid sequence and analysed for the presence of conserved domains which showed that the protein belongs to thaumatin-like protein super family. This data is of great importance as this is the first report on pearl millet TLP nucleotide and partial amino acid sequence. The nucleotide sequence data generated in this work can be used for further studies;

also the partial amino acid sequence deduced by translating the nucleotide sequence and *de novo* sequencing of trypsin digested peptide fragments will be of great help for further studies.

4.10 Amplification of GC rich pearl millet *chitinase* and *tlp* genes.

PCR-amplification of GC-rich templates is often hampered by the formation of secondary structures like hairpins and higher melting temperatures; also GC-rich regions of template cause formation of stem-loop secondary structures that have been known to promote polymerase jumping during PCR amplification. This jumping of polymerase produces smaller PCR products missing the stem-loop region of the template (Viswanathan *et al.,* 1997). Combination of two additives such as betaine (1-carboxy-N,N,N-trimethyl methanammonium inner salt) and DMSO has been found to enhance amplification of long PCR products (Hengen, 1997 Kang *et al.,* 2005). DMSO disrupts base pairing; betaine, which is an iso-stabilizing agent, equalizes the contribution of GC and AT base pairing to the stability of the DNA duplex (Rees *et al.,* 1993). Pearl millet chitinase gene amplified in this study (482 bp-partial length) is GC rich with 61% GC content and the tlp gene (422 bp partial length) had a high GC content of 69% and this explains the failure of PCR reactions for full length gene amplification. Because GC-rich sequences possess a strong secondary structure that resists denaturation and hampers primer annealing, though different polymerases like KAPA2GRobust polymerase, HiFi Phusion polymerase (from NEB), KAPAHiFi polymerase (from Peqlab) were used in this study, none of them were able to amplify specific products. However, KAPA2GRrobust polymerase was successful in amplification of 482 bp pearl millet chitinase in the presence of enhancer 1 and 2 along with GC buffer (component of KAPA2G Robust PCR kit from Peqlab) at 1X concentration. But the same did not yield any specific products in amplification of full length genes of pearl millet chitinase and TLP. Moreover individual PCR additives in enhancer 1 and 2 and GC buffer and their concentrations are not revealed by the manufacturers and any optimization of concentration of individual components becomes impossible when a commercial kit is used. Different concentrations of PCR additives like DMSO and betaine were used in PCR reactions to amplify the full length gene. But no specific amplification was observed. All these results indicate the difficulty in amplification pearl millet chitinase and tlp genes; unavailability of nucleotide sequence data of these genes poses another challenge in primer designing. If genome size has a negative effect on amplification success at a single locus then, by extension, the success rate of primer sets tested on organisms with large genomes should be lower than the success rate of primer sets tested on organisms with small genomes (Garner, 2002). The primers had to be designed considering the nucleotide sequence of chitinase and tlp from other plant species with which pearl millet genes (partial length nucleotide sequences) shared homology which might lead to poor priming, and success of priming cannot be predicted. Though several difficulties were encountered in amplification of pearl millet chitinase and tlp genes, partial length of both these genes were successfully amplified. This data was useful in classification of these two important PR proteins under respective families. Sequence data on pearl miller PR proteins is very limited and this work contributes to the database. The sequence data available on pearl millet pathogenesis related proteins include

glucanase (NCBI accession number: AF488414) protease (NCBI accession number: ABI95884), peroxidase (NCBI accession number ABX38890), Proton ATPase (NCBI accession number AAV36518) and couple of other partial nucleotide sequences of the PR protein genes.

4.11 Adaptor ligation-mediated genome walking in plants with large genome.

Large genome size and multiploidy in plants is a big challenge in case of PCR-based genome walking in plants. To gain high sensitivity in ligation-mediated –PCR applications, it is important that nearly 100% of all genomic DNA fragments resulting from the initial restriction digest have the adaptor attached to both ends. This enlarges the pool of the desired PCR template. Nevertheless, this cannot be ensured while amplifying unknown flanking regions of a gene because of the lack of nucleotide sequence data that does not allow spotting of restriction site of the enzyme (used to construct genomic DNA libraries) on the gene of interest. This could lead to failure of the technique due to two possible reasons. Firstly, the distance from the primer to the restriction site is greater than the capability of the genome walking system (~6 kb) or the target gene might have restriction site just outside the known sequence separating the unknown flanking region of the gene and thereby not leaving any good length of unknown sequence to be walked by the primer. Or in case of multiploid plants with large genomes, probability of adaptor ligation to the target DNA is highly reduced. Previous studies (Farrelly *et al*., 1995; Hoelzel and Green, 1992) have explained the failure of PCRs in large genomes with two most possible reasons, the first being a reduction in target DNA relative to the total content of template DNA in a single PCR serves to reduce amplification efficiency. As genome sizes increase, this serves to dilute the proportion of available target DNA in a given volume of template DNA (Farrelly *et al*. (1995); because typically, template concentration is considered in terms of total amount of genomic DNA (Hoelzel and Green, 1992). Secondly, as genome size increases, the number of regions complementary to any 20-bp region (the average size of primers) also increases. A larger genome would then increase the amount of nonspecific binding of primers, a concurrent diminishing of the primer pool available for PCR (Garner, 2002). If genome size has a negative effect on amplification success at a single locus then, by extension, the success rate of primer sets tested on organisms with large genomes should be lower than the success rate of primer sets tested on organisms with small genomes (Garner, 2002). Nonetheless, in this study many pairs of primers were designed considering the partial length of the gene and no specific products were observed with any of the primers combinations used. Though increasing the length of primers would improve the specificity, longer primers could not be designed due to high GC content of the genes (482 bp pearl millet chitinase 61% - GC content and 422 bp pearl millet tlp 69% - GC content). Higher GC content of the primers is not recommended for long distance PCRs and high GC content lead to poor results with normal PCRs in amplification of pearl millet chitinase and tlp genes in this study as well. This explains that the available sequence data on pearl millet chitinase and tlp need to be enriched in order to employ techniques like adaptor ligation mediated genome walking successfully.

4.12 Southern and northern blot analysis using radioactive P32 labeled pearl millet *chitinase* and *tlp* probe.

Southern blot analysis of restriction digested genomic DNA to find out the copy number of gene of interest relies on restriction digestion of genomic DNA into small fragments and hybridization of gene of interest with the radioactive labeled probe DNA. Nevertheless, in case of large genomes the fragments generated by restriction enzyme could be too large that would reduce the stringency of the reaction as observed in case of southern blot analysis of pearl millet genomic DNA with radioactive labeled *chitinase* and *tlp*. Similarly total RNA is hybridized with the radioactive labeled probe in case of northern blot analysis and interference of non specific RNA is the major reason for poor results. The amount of target mRNA to rest of the non target RNA could be very low in case of large genomes (because of low ratio of target DNA to non target DNA, Farrelly *et al*., 1995; Hoelzel and Green, 1992). This could be major reason for nonspecific hybridization of the radioactive labeled probes at lowed hybridization temperatures and absence of hybridization at higher hybridization temperature. High GC content of the probe used for labeling might be another reason. Therefore the experiments did not give convincing results.

4.13 Conclusion.

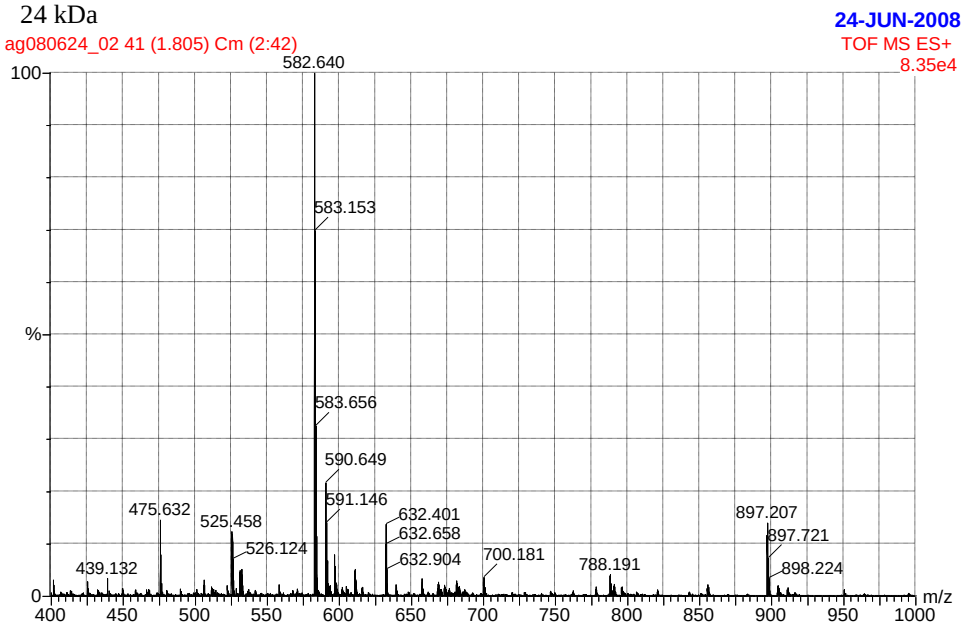
Chitinase is considered to protect plants against fungal pathogens by degrading chitin, a major component of the cell walls of many fungi. For this the enzyme should bind to chitin. Many studies support that the basic class I chitinase had the highest antifungal activity among plant chitinases due to its strong binding ability to fungal cell wall and chitin degrading activity. Unlike fungi a distinguishing feature of oomycetes is that they possess a cell wall containing insoluble cellulose and chitin (Zevenhuizen and Bartnicki-Garcia, 1969, Liu *et al*., 1994, Latijnhouwers *et al*., 2003). Therefore pearl millet chitinase purified in this doctoral study would be of great importance in host defense mechanism and it shows a calculated pI of 8.60. The protein also showed chitin binding and chitin degrading ability *in vitro*. Sequence analysis also showed that the purified peal millet chitinase belongs to class I chitinases. Study of Class I and Class II chitinases from rye (Taira *et al*., 2002) revealed that the class I chitinases bound to hyphal tips, lateral walls and the septa consisting of mature cells walls predominantly by ionic interaction through catalytic domain along with hydrophobic interaction through chitin-binding domain and showed degradation of mature chitin of septa and lateral walls as well as nascent chitin. Conserved tryptophan residues (W) in pearl millet chitinase also provide evidence to this property of class I chitinases. Whereas the basic class II chitinases bound only to the hyphal tip through ionic interaction only was able to degrade only the nascent chitin present in the hyphal tip. Therefore basic class I chitinases are definitely more effective in defense mechanism against invading pathogens than class II chitinases. Moreover GH-19 chitinase is found to be more effective in fungal inhibition than GH-18 chitinases (Taira, 2010), and purified pearl millet chitinase belongs to GH-19 and therefore a major component of plants defense system. In order to understand the mode of action of chitinase the cell wall structure of

the pathogen is equally important. Now that this work shows the presence of class I chitinase in pearl millet and characterization of the same has been carried out, this data would be of great importance in further studies involving *S. graminicola* cell wall and class I chitinase from pearl millet.

There are several reports on thaumatin like proteins conferring resistance against oomycete pathogen in different plants. For instance the latest work by Reiss and coworkers (2009) showed barley TLP enhancing resistance of oilseed rape plants to *Plasmodiophora brassicae*. The present study confirmed that pearl millet TLP inhibited downy mildew pathogen *in vitro*. However, the mechanism of action of TLP is unclear till date. Glucan binding activity of pearl millet TLP has also been established in this study. Membrane pore-formation has been suggested as one possible anti-fungal mechanism (Koiwa *et al.,* 1997). Carbohydrate-binding and hydrolytic functions have also been observed for a number of PR-5 proteins (e.g., ß-1, 3-glucan interactions in thaumatin-like proteins (Grenier *et al.,* 1991, Osmond *et al.,* 2001, Yun *et al.,* 2000 and Ibeas *et al.,* 2000). Structural modeling studies of PR-5 proteins have demonstrated that PR-5 proteins contain highly acidic clefts suitable for carbohydrate hydrolytic function (Osmond *et al.,* 2001, Koiwa *et al.,* 1999). This suggests that the mechanism may involve interactions between PR-5 proteins and pathogen cell wall carbohydrates. Studies on of PR-5d from tobacco showed the presence of pathogen-specific insoluble-carbohydrate binding surface motif (consisting of three tryptophan residues conserved at specific positions -W34/W36/W192) and is highly conserved in all the members of Solanaceae which are commonly attacked by oomycete pathogen *Phytophtora* (Doxey *et al.,* 2010). Study of complete primary structure of pearl millet TLP would confirm the presence of similar conserved motifs in pearl millet TLP can through light upon interaction of pearl millet with oomycete pathogen *S. graminicola*. Experimental evidence in this doctoral study has already shown glucan binding activity of pearl millet TLP and inhibitory effect of pearl millet TLP on *S. graminicola*. Further studies in the same line will be of great interest.

Collectively, this doctoral study has generated vital data that can be of great use for further studies. Experimental evidences on pearl millet chitinase and TLP are in line with the behavior of chitinases and TLPs from other plant species. The partial amino acid sequence and nucleotide sequence of pearl millet chitinase and TLP gens are of immense importance as this thesis is the first report of chitinase and TLP genes from pearl millet.

24 kDa



Peptidspektren

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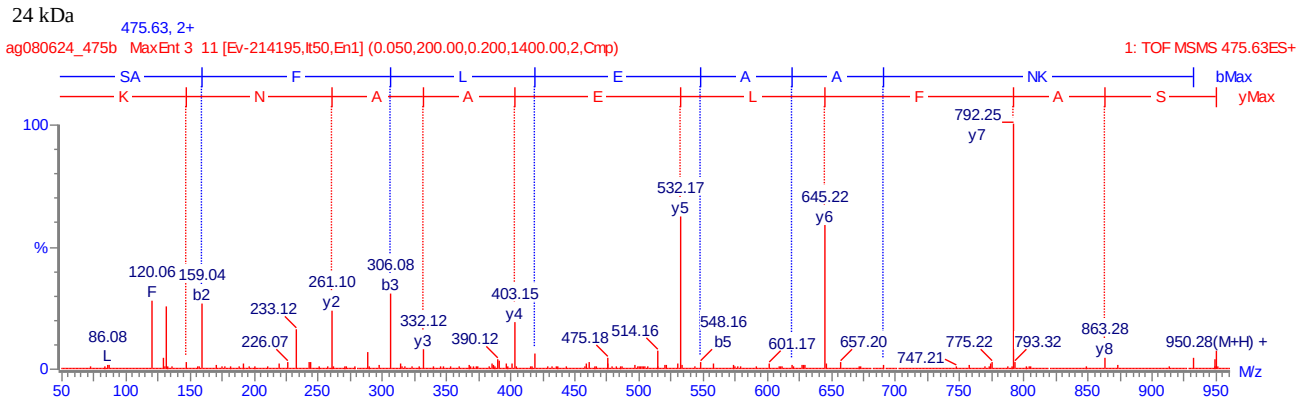

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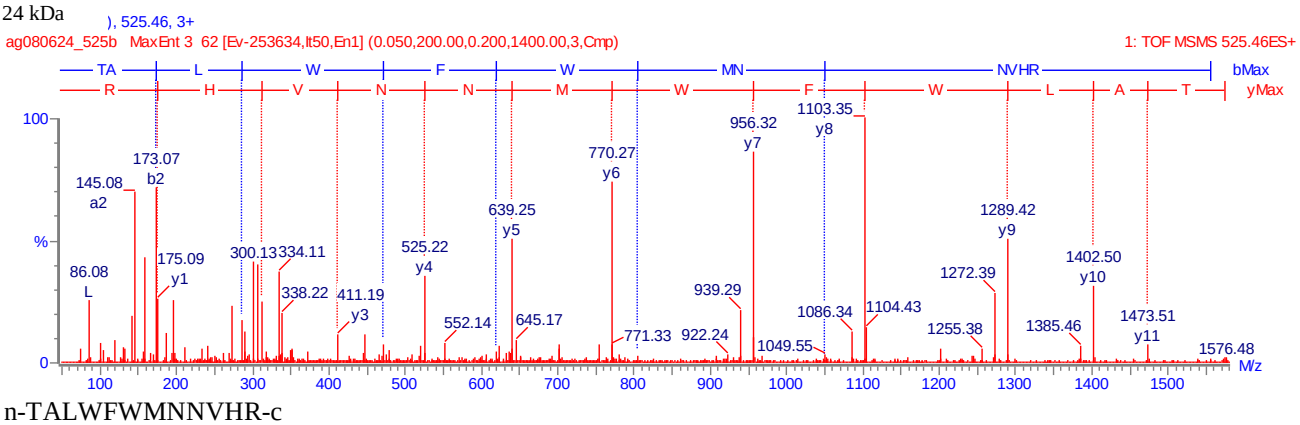

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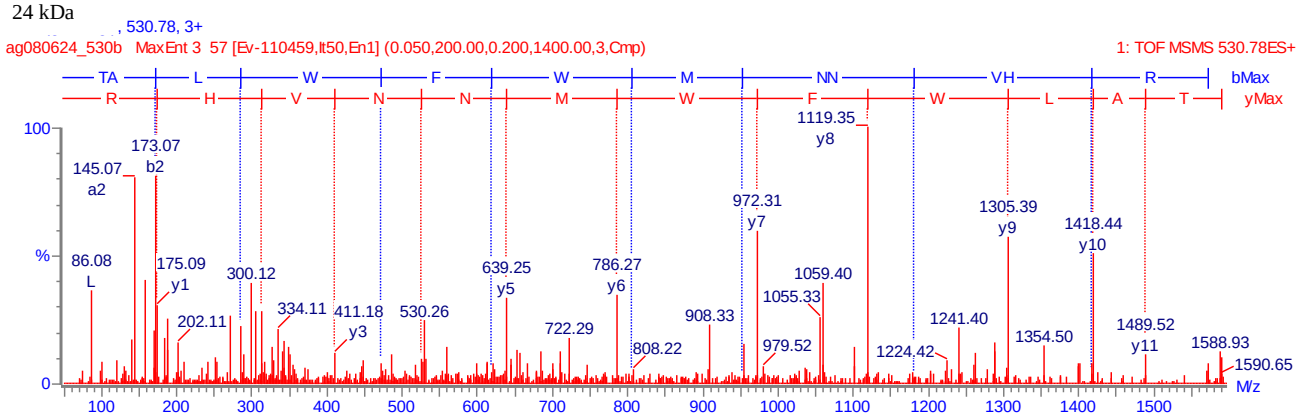

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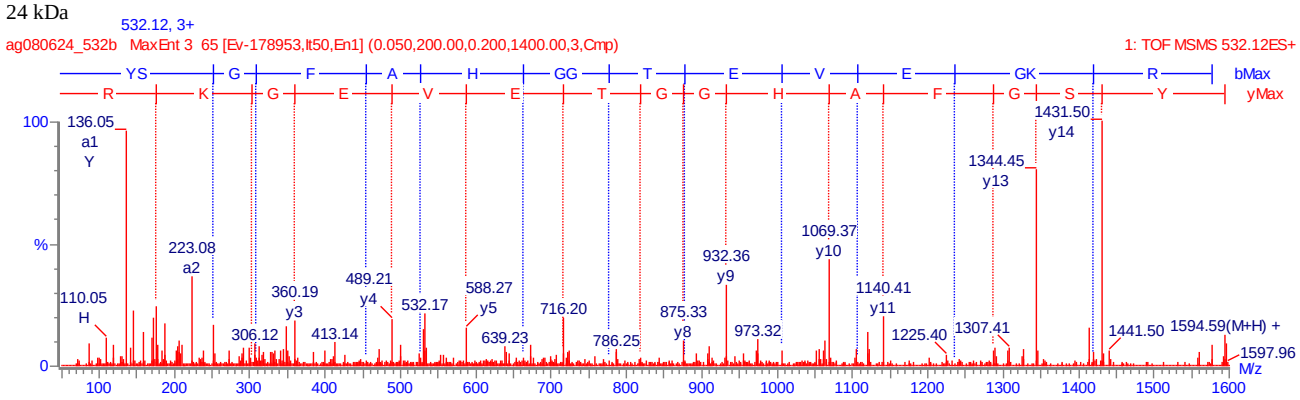

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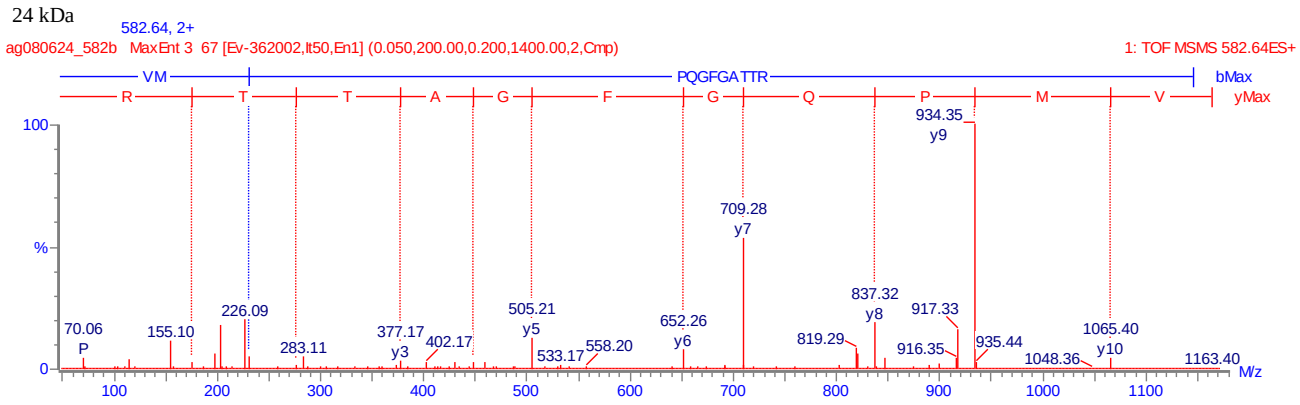

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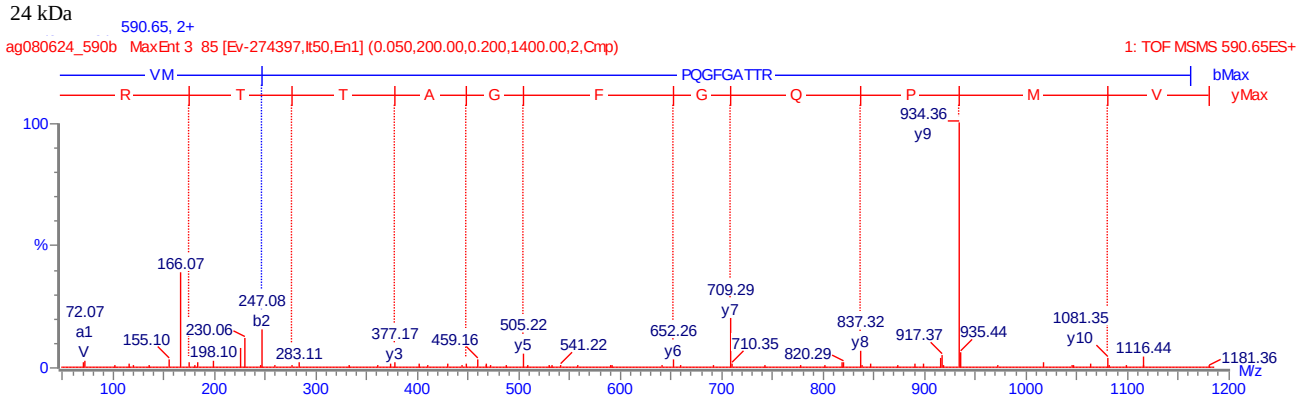

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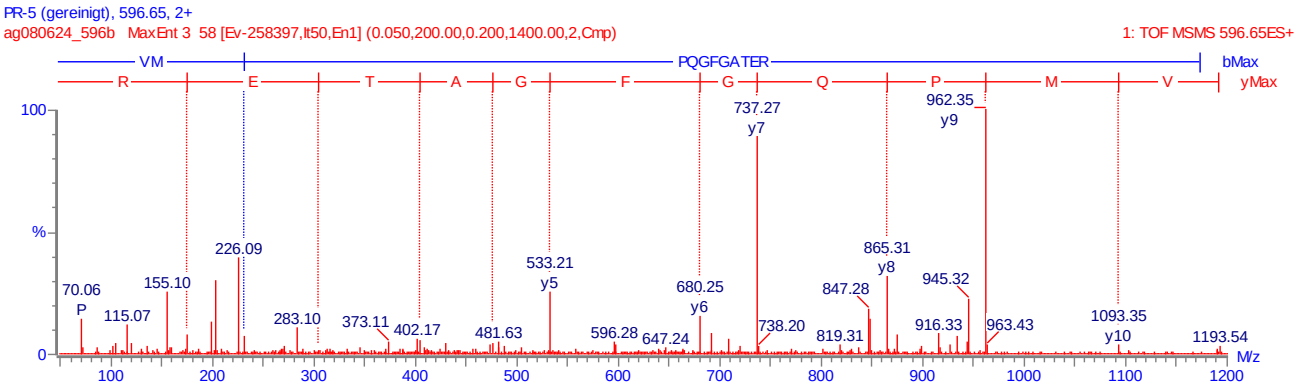

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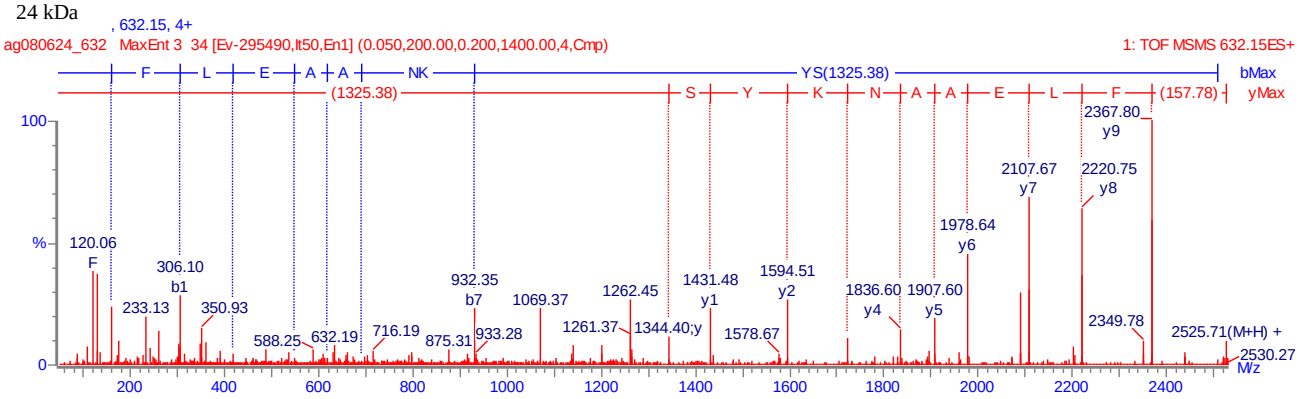
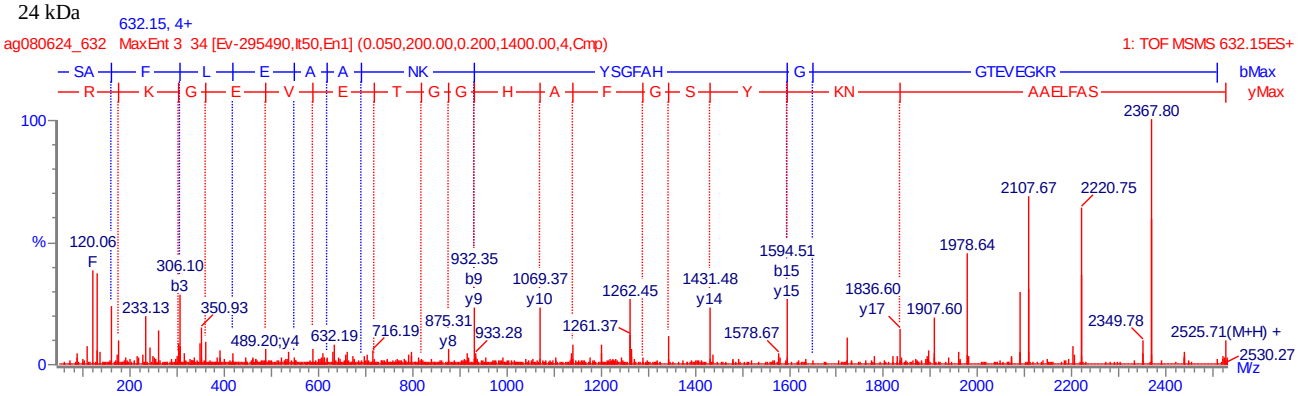
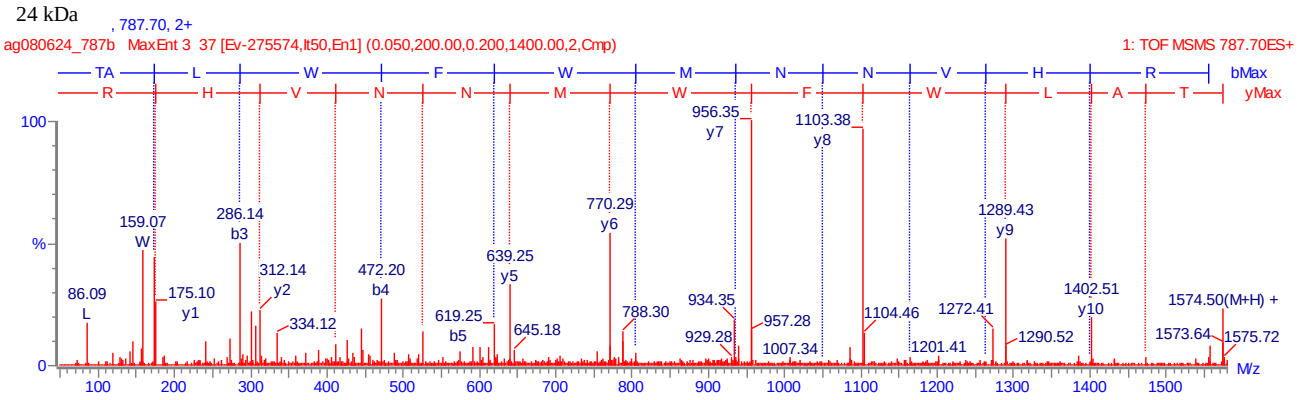


24 kDa



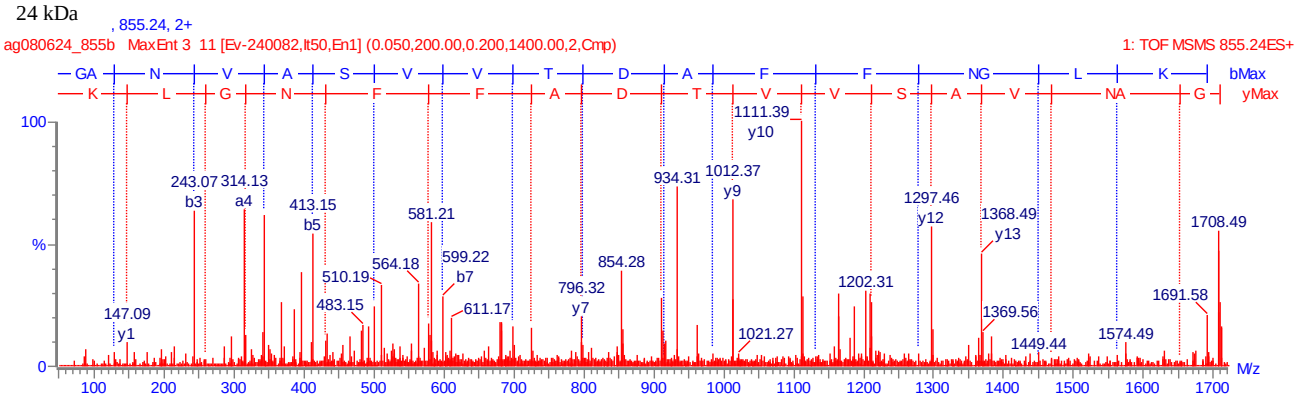
n-(as)FLEAANKYSGFAHGGTEVEGKR-c, 532 (3+) + 475 (2+)

24 kDa



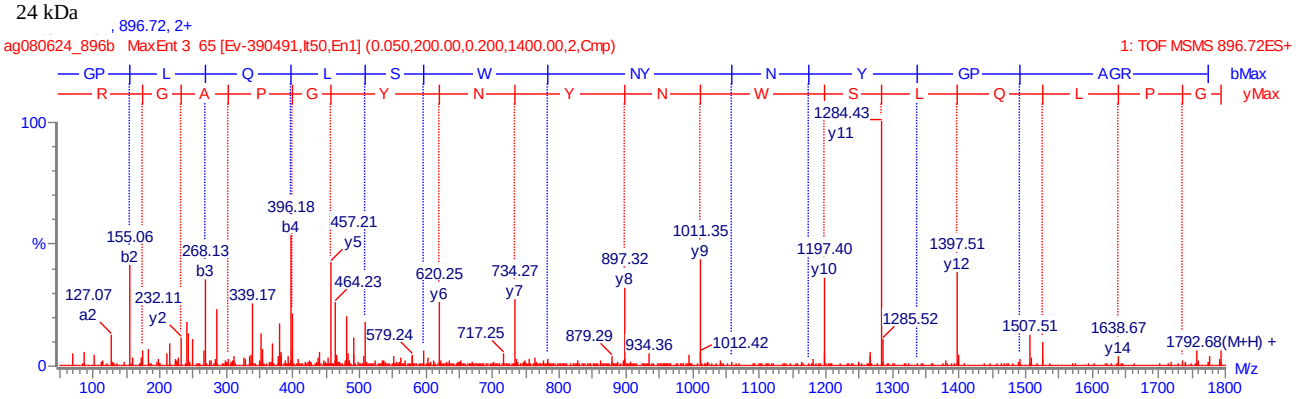
n-TALWFWMNNVHR-c

24 kDa



n-(gan)vASVVTDAFFNgLK-c

24 kDa



n-GPLQLSWNYNYGPAGR-c

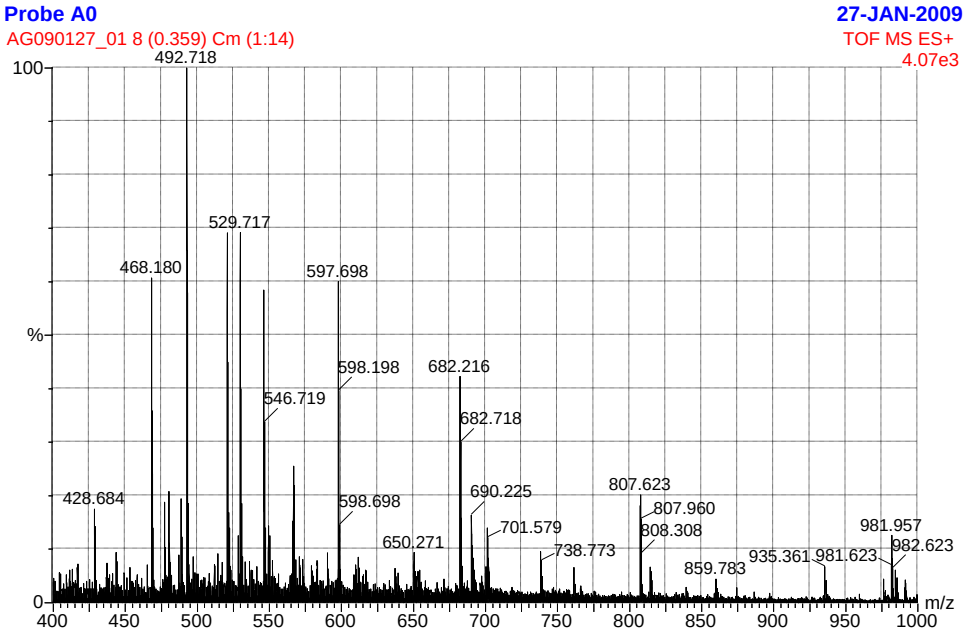
Table 1. Amino acid sequence of the peptide fragments generated by trypsin digestion of purified 24 kDa chitinase from pearl millet.

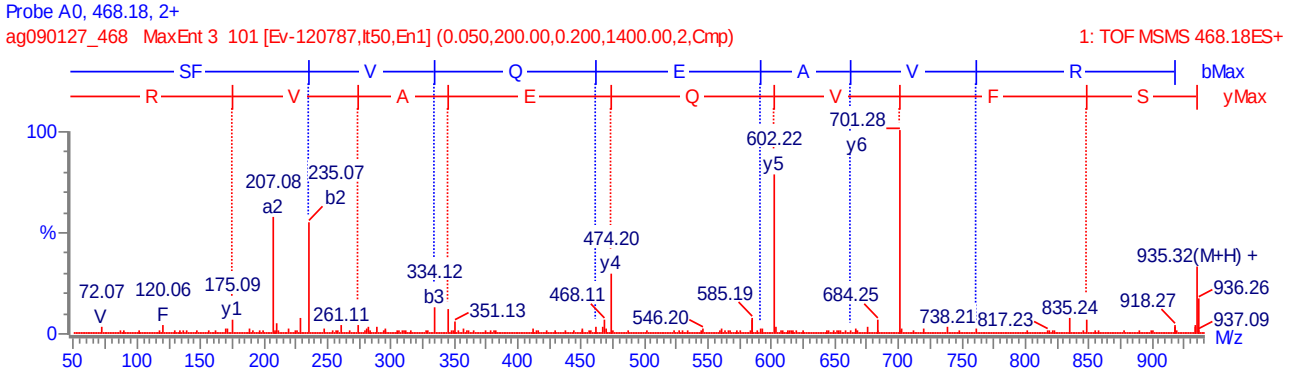
(*indicates oxidised methionine residues, the amino acids in upper case indicate highly accurate sequences generated automatically and the ones in lower case represent the amino acids that are predicted).

Band	Mass	Charge	Rel. Mass	Derived amino acid sequence	Sequence similarity position	Organism	Possible function	Accession number
24kDa	475.63	2+	951.26	SAFLEAANK	SAFLEA 109 S+FLEA 113	*Zea mays*	chitinase A	swissnew|P29022
24kDa	525.46	3+	1050.92	TALWFWMNNVHR	ALWFWMNNVH 219 ALWFWMNNVH 228	*Tripsacum dactyloides*	chitinase	sptrembl|Q6JBL6
24kDa	530.78	3+	1592.34	TALWFWM*NNVHR	ALWFWMNNVH 219 ALWFWMNNVH 228	*Zea diploperennis*	chitinase	sptrembl|Q6JBP3
24kDa	532.12	3+	1596.36	YSGFAHGgTEVEgkR	YSGFAHGGTEVEGKR 117 YPGFAHGGTEVEGKR 132	*Oryza sativa*	chitinase	sptrembl|Q7Y1Z0
24kDa	582.64	2+	1165.28	VMPQGFGATTR	VMPQGFGATTR 230 VMPQGFGATIRA 240	*Oryza sativa*	chitinase	sptrembl|Q7Y1Z0
24kDa	590.65	2+	1181.3	VM*PQGFGATTR	VMPQGFGATTR 230 VMPQGFGATIR 240	*Oryza sativa*	chitinase	sptrembl|Q7Y1Z0
24kDa	632.15	4+	2528.6	asFLEAANKYSGFAHGGTEVEGKR	FLEAANKYSGFAHGGTEVEGKR 109 FLSAVNAYPGFAHGGTEVEGKR 132	*Tripsacum dactyloides*	chitinase	sptrembl|Q6JBL6
24kDa	787.70	2+	1575.40	TALWFWMNNVHR	TALWFWMNNVH 218 TALWFWMNNVH 228	*Zea mays*	chitinase A	swissnew|P29022
24kDa	855.24	2+	1710.48	ganvASVVTDAFFNgLK	GANVASVVTDAFFNGK 77 GANVANVVTDAFFNGIK 93	*Zea mays*	chitinase A	swissnew|P29022
24kDa	896.72	2+	1793.44	GPLQLSWNYNYGPAGR	GPLQLSWNYNYGPAGR 180 GPLQISWNYNYGPAGR 194	*Zea mays*	chitinase A	swissnew|P29022

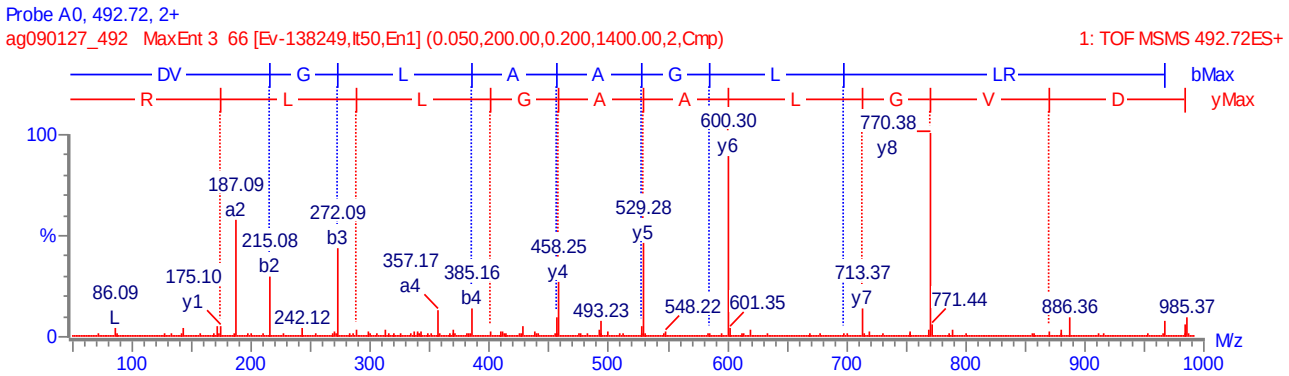
The peptides shared homology to maize endochitinase with Swiss prot accession number sp|P29023.

Probe A_0





n-SFVQEAVR-c



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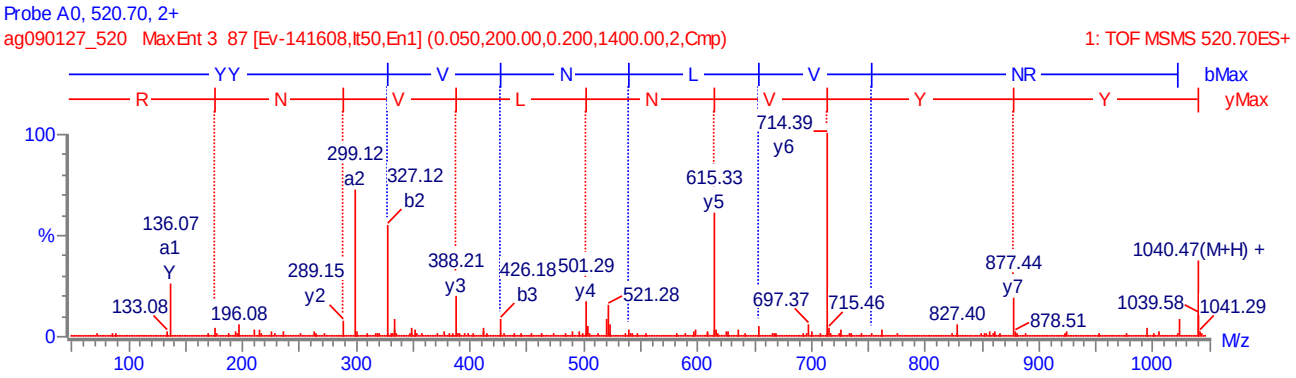
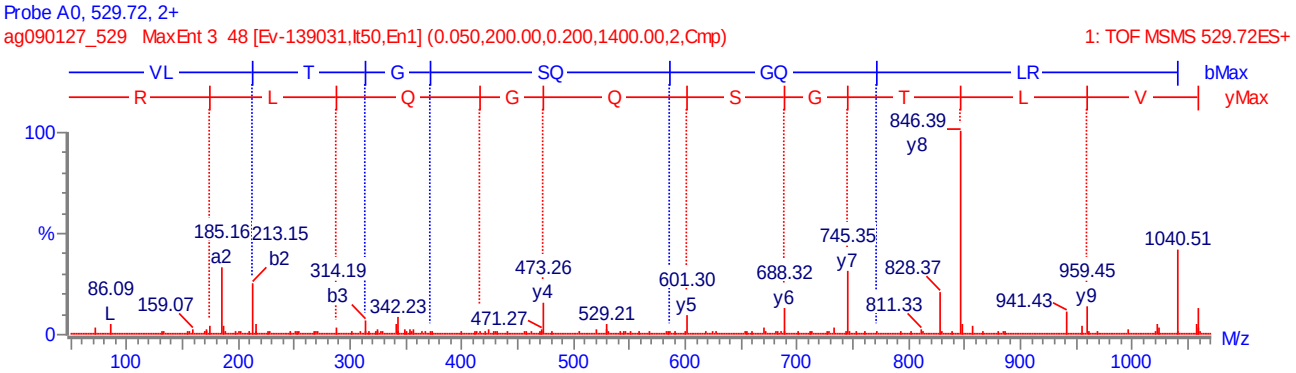
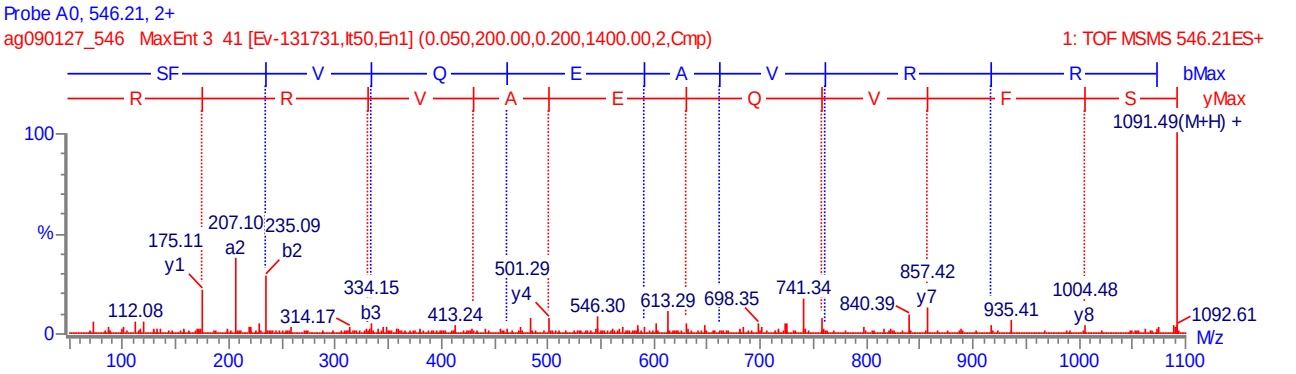


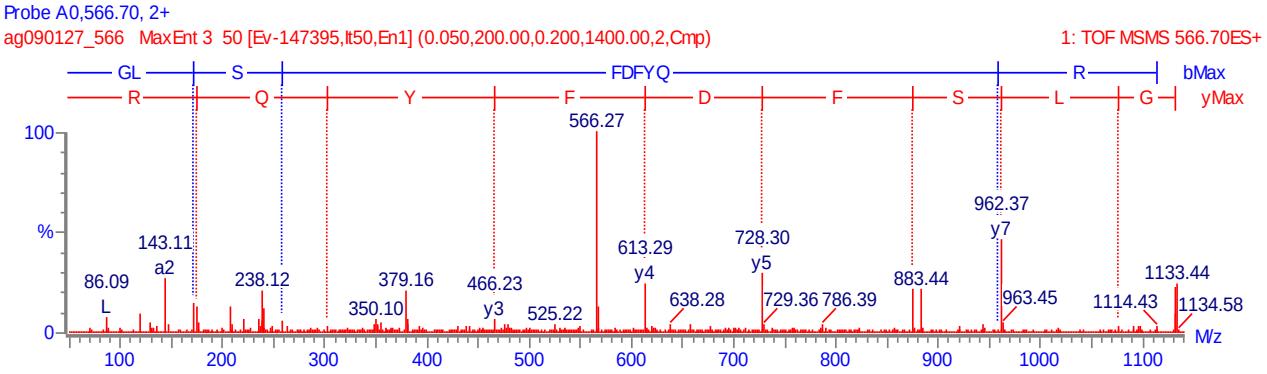
n-YYVNLVNR-c



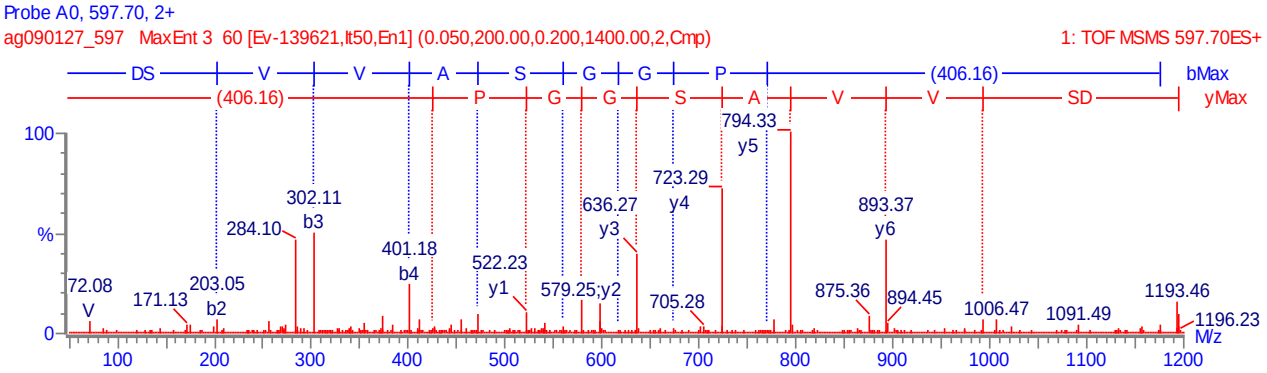
n-VLTGSQGQLR-c



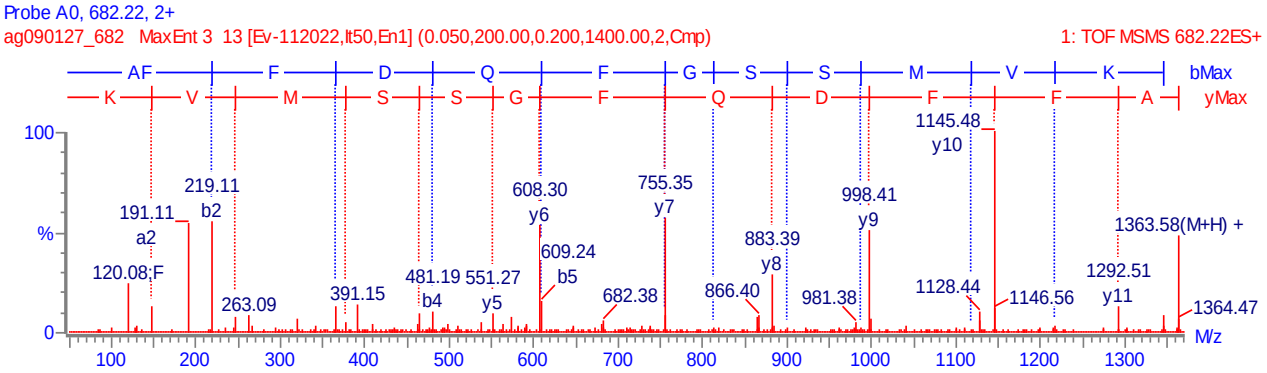
n-SFVQEAVrr-c



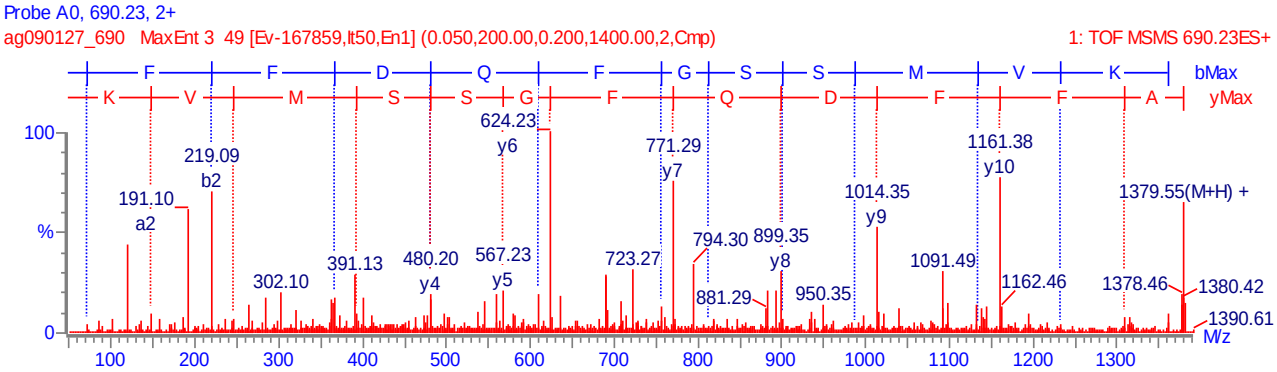
n-GLSFDFYQR-c



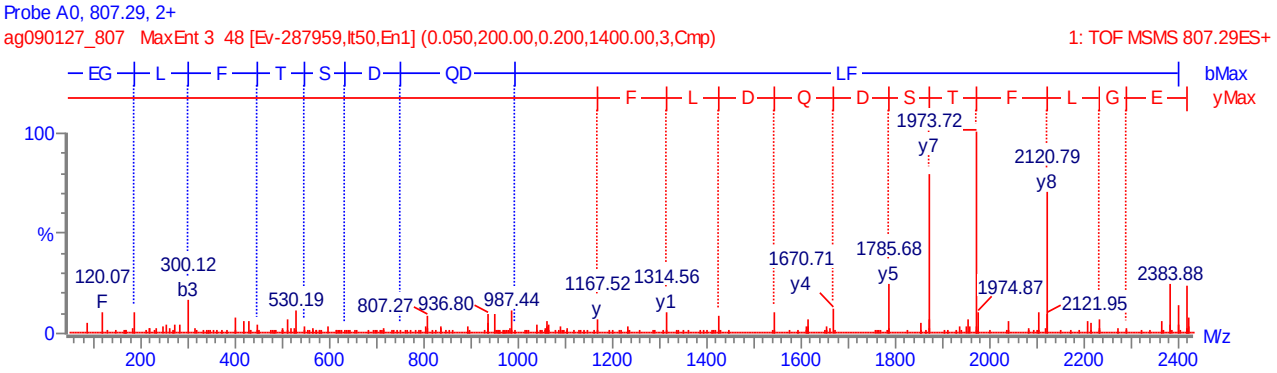
n-(sd)VVASGG…



n-AFFDQFGSSMVK-c



n-AFFDQFGSSM*VK-c, Methionin oxidiert



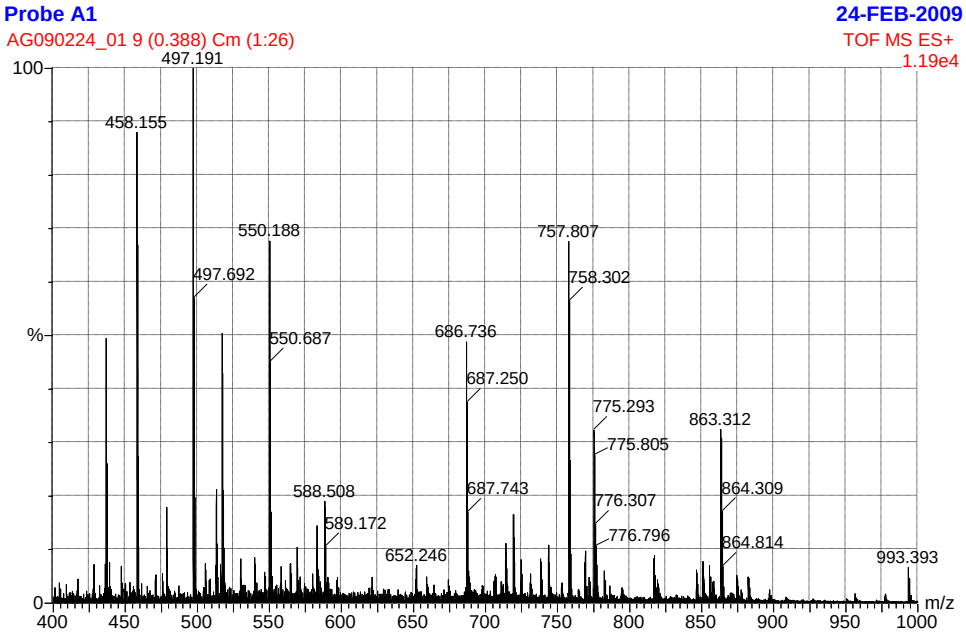
n-EGLFTSDQDLF…

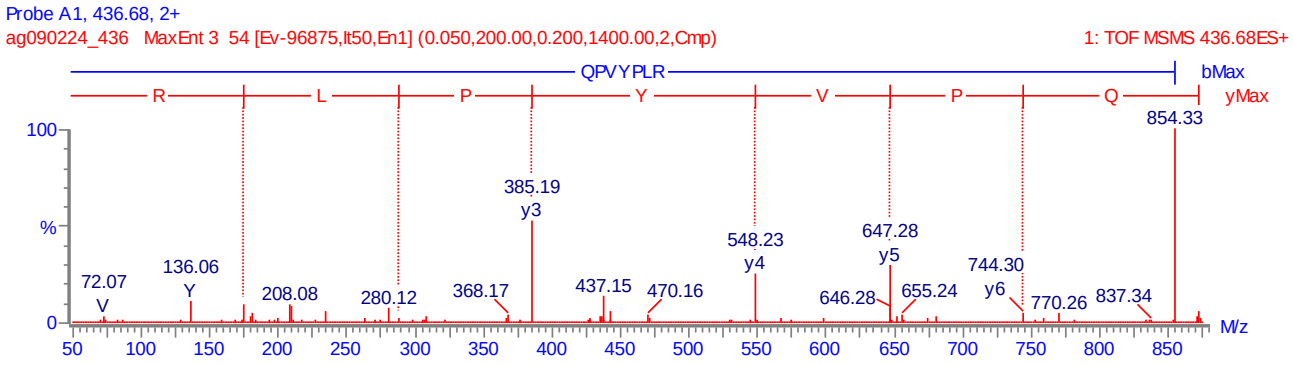
Table 3. Amino acid sequence of the peptide fragments generated by trypsin digestion of protein band A0.

(*indicates oxidised methionine residues, the amino acids in upper case indicate highly accurate sequences generated automatically and the ones in lower case represent the amino acids that are predicted).

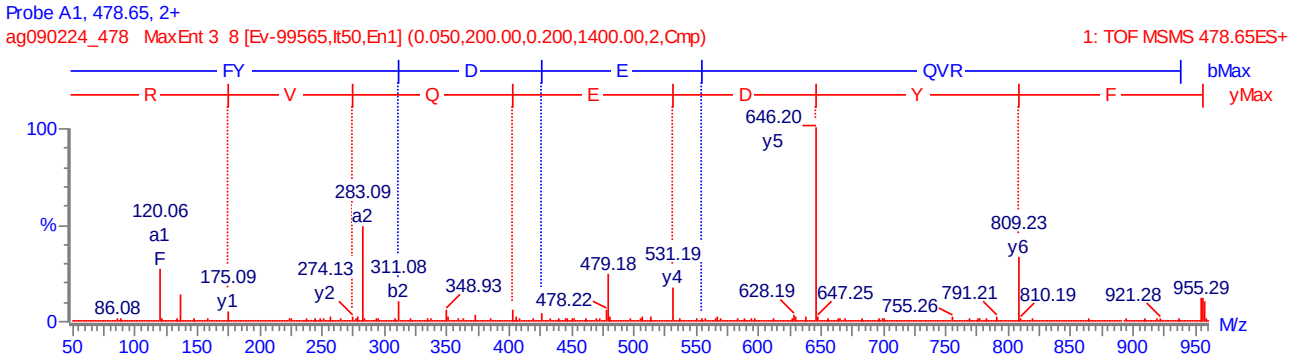
Band	Mass	Charge	Rel. Mass	Derived amino acid sequence	Sequence similarity position	Organism	Possible function	Accession number
A0	468.18	2+	936.36	SFVQEAVR	SFVQEAVR 57 RKFVRDAVR 65	*Oryza sativa*	Peroxidase	sp|Q94DM0
A0	492.72	2+	985.44	DVGLAAGLLR	DVGLAAGLLR 67 DIGLAAGLLR 76	*Hordeum vulgare*	Peroxidase	sp|Q42854
A0	520.70	2+	1041.40	YYVNLVNR	YYVNLVNR 267 YYVNLVNR 274	*Triticum aestivum*	Peroxidase precursor	sp|Q8LK23
A0	529.72	2+	1059.44	VLTGSQGQLR	VLTGSQGQLR 320 VLTGSQGQVR 329	*Hordeum vulgare*	Peroxidase	sp|Q40069
A0	546.21	2+	1092.42	SFVQEAVrr	SFVQEAVR 57 RKFVRDAVR 65	*Zea mays*	Anionic peroxidase	sp|O04710
A0	566.70	2+	1144.40	GSLFDFYQR	FDFY 42 FDFY 45	*Oryza sativa*	Peroxidase	sp|Q94DM0
A0	597.70	2+	1195.40	(sd)VVASGG	VVASGG 149 VVASGG 154	*Oryza sativa*	Peroxidase	sp|Q94DM0
A0	682.22	2+	1364.44	AFFDQFGSSMVK	AFFDQFGSSMVK 303 AFFDQFAVSMVK 314	*Triticum aestivum*	Peroxidase precursor	sp|Q8LK23
A0	690.23	2+	1380.46	AFFDQFGSSM*VK	AFFDQFGSSMVK 303 AFFDQFAVSMVK 314	*Oryza sativa*	Peroxidase	sp|Q94DM0
A0	807.29	2+	1614.58	EGLFTSDQDLF	EGLFTSDQDLF 275 EGLFTSDQDLF 285	*Nicotiana tabacum*	Peroxidase	sp|Q94IQ1

The derived amino acid sequence of peptides recovered from in gel trypsin digestion of protein band A0 showed homology to Peroxidase from *Oryza sativa* with the accession number sptrembl|Q94DM0.

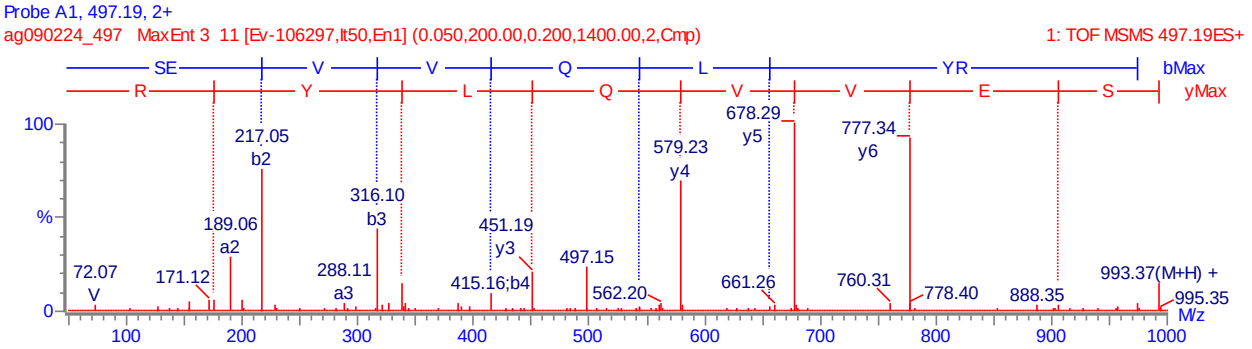




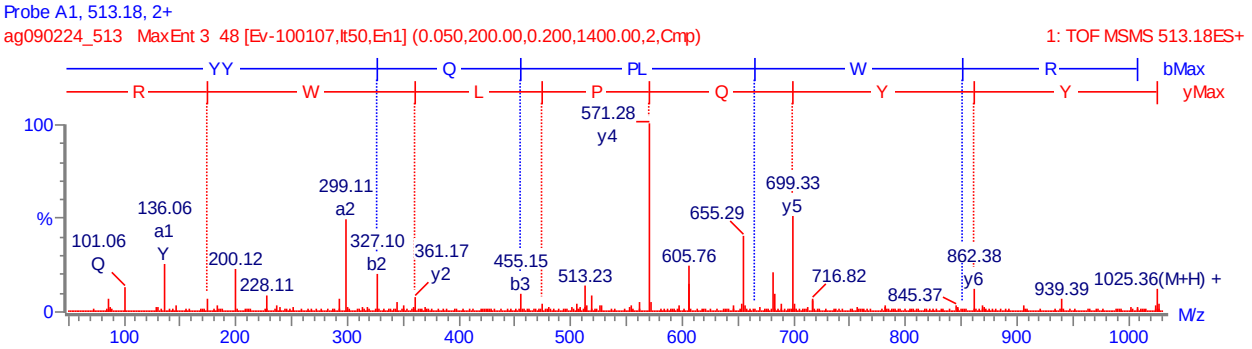
n-QPVYPLR-c



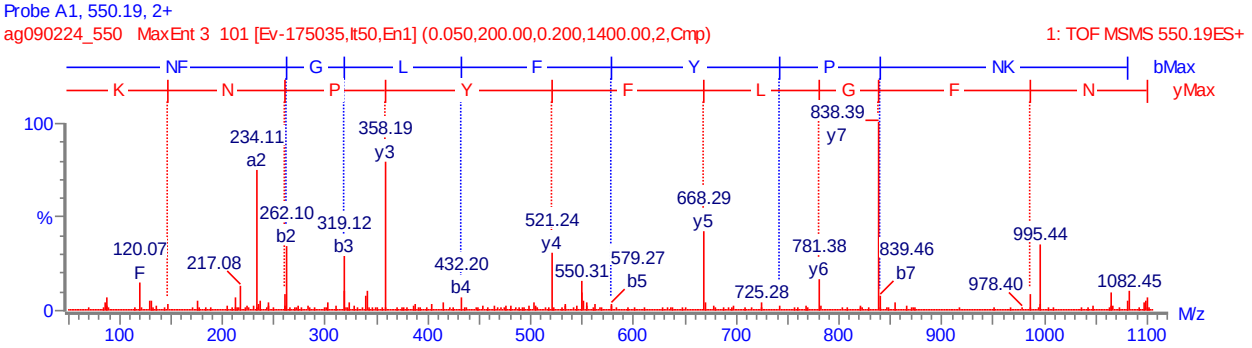
n-FYDEQVR-c



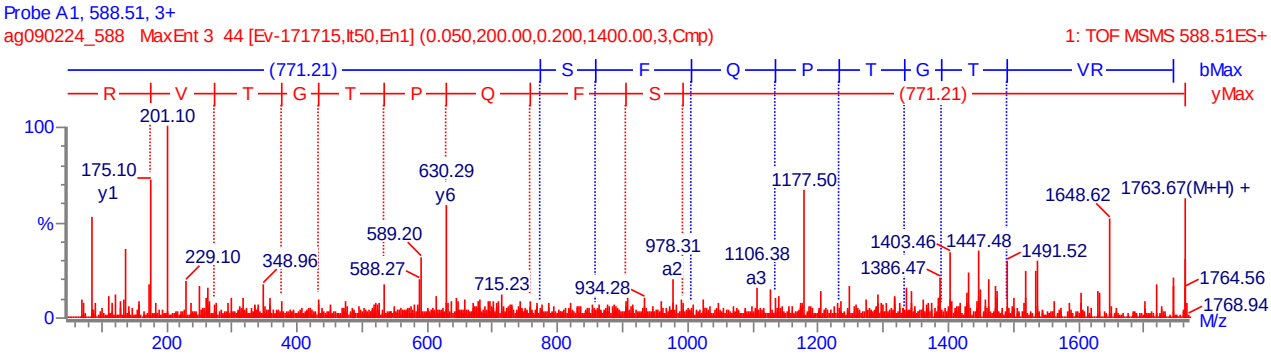
n-SEVVQLYR-c



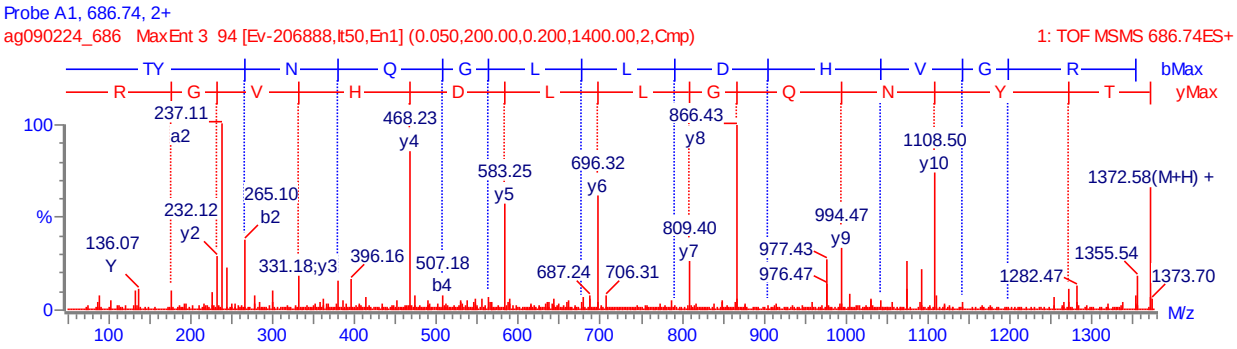
n-YYQPLWR-c



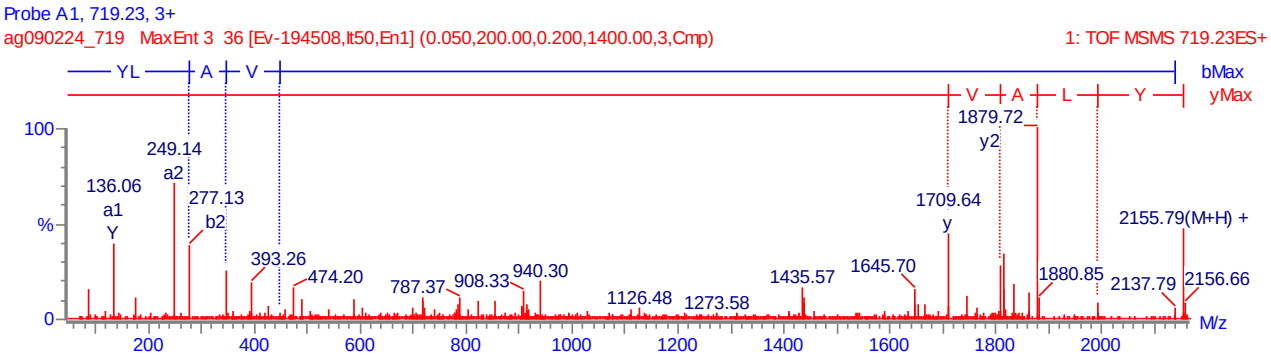
n-NFGLFYPNK-c



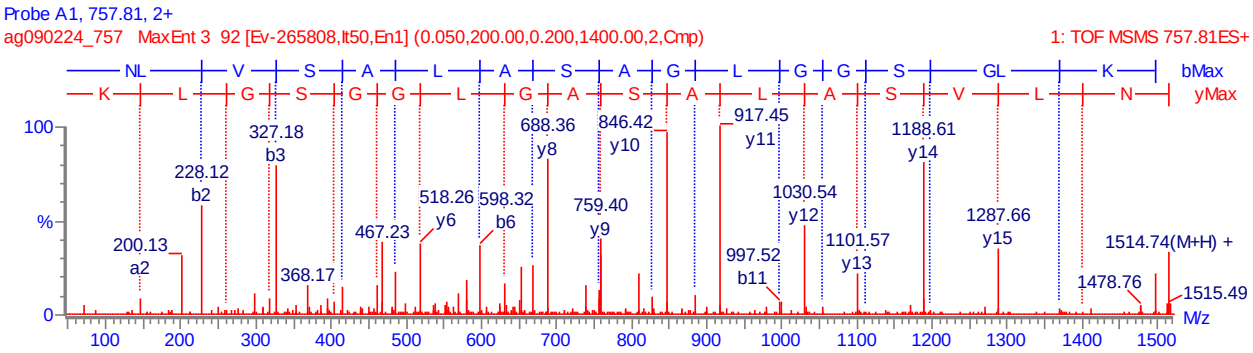
...fqpTGTVR-c



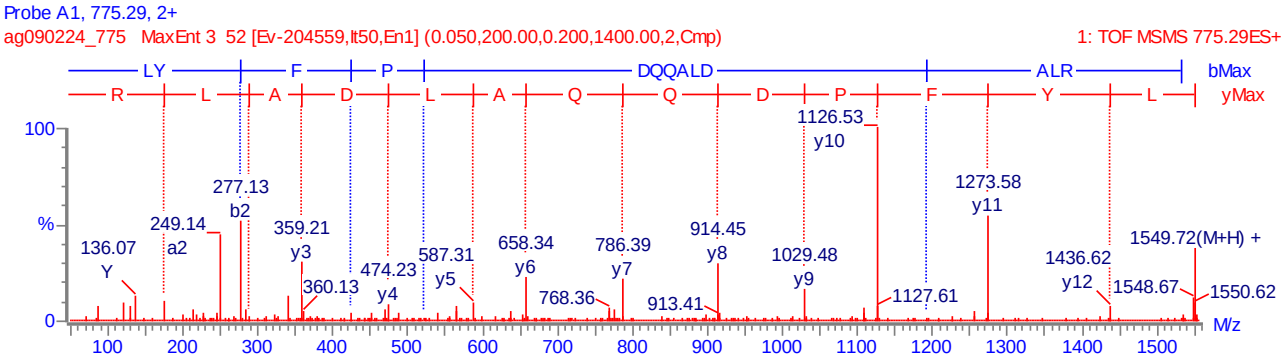
n-TYNQGLLDHVGR-c



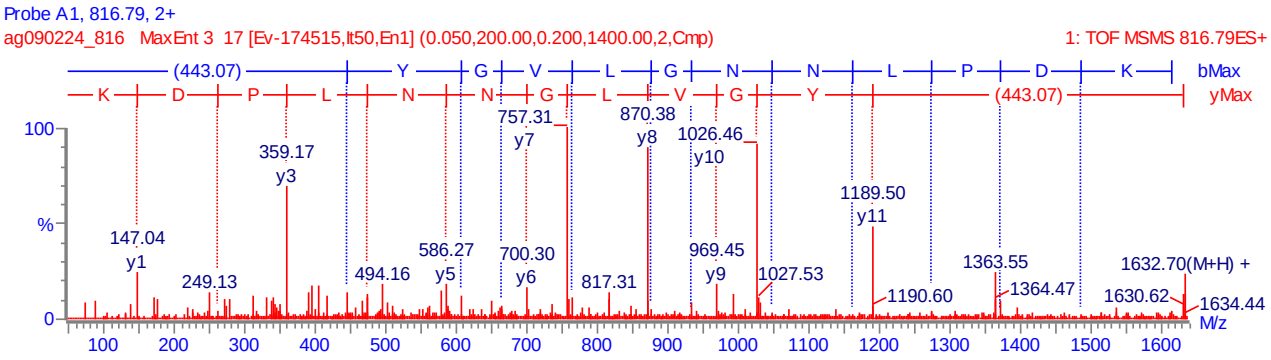
n-YLAV…



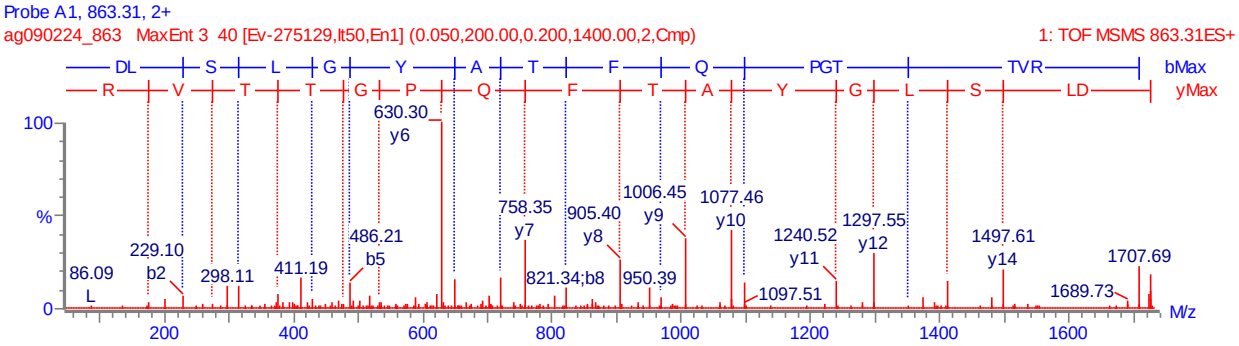
n-NLVSALASAGLGGSGLK-c



n-LYFPDQQALDALR-c



n-(lgv)C*YGVLGNNLPDK-c, C*: Cystein-Arcylamid



n-(dl)SLGYATFQPGTTVR-c

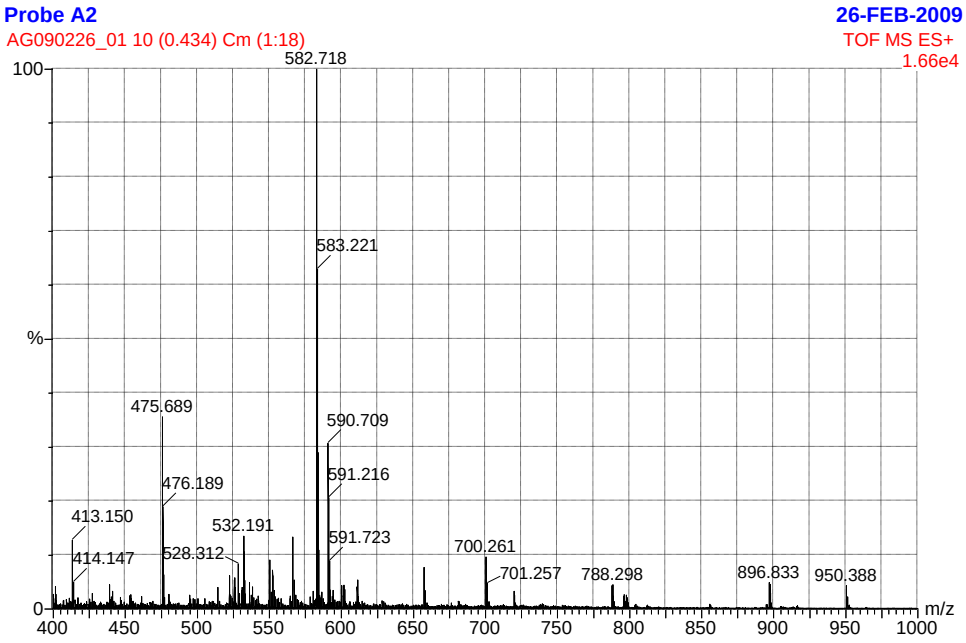
MS blast data is compiled in the table (similar to rice endo-1,3-beta-D-glucosidase)

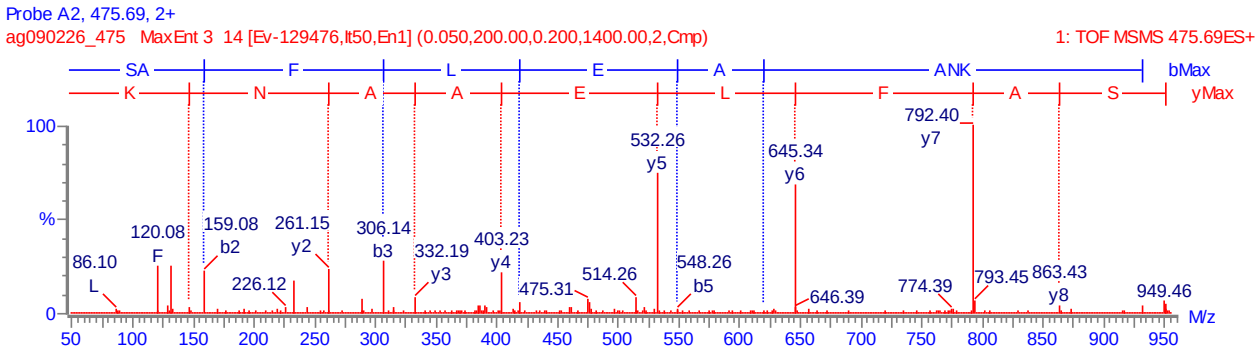
Table 4. Amino acid sequence of the peptide fragments generated by trypsin digestion of protein band A1.

(*indicates oxidised methionine residues, the amino acids in upper case indicate highly accurate sequences generated automatically and the ones in lower case represent the amino acids that are predicted).

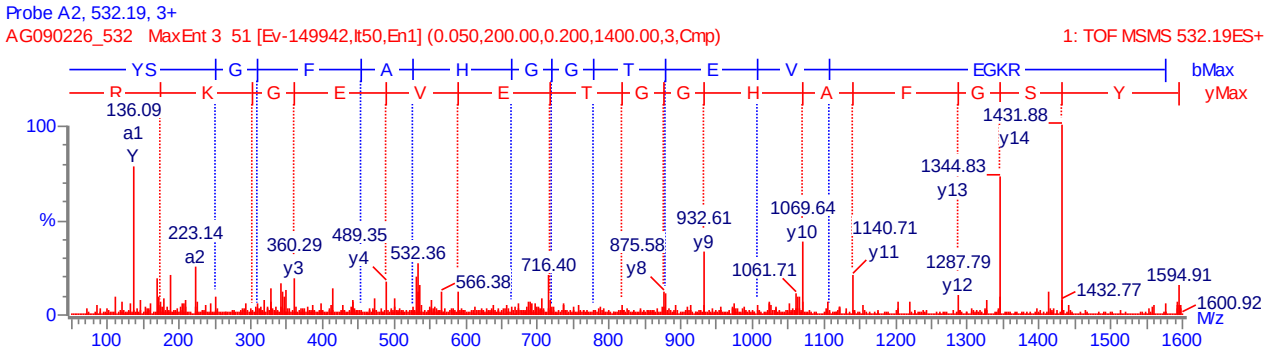
Band	Mass kDa	Charge	Rel. mass kDa	Derived amino acid sequence	Sequence similarity position	Organism	Possible function	Accession number
A1	436.68	2+	873.36	QPVYPLR	PVYPLRFYDE 17 PVATLRYYDE 26	*Oryza sativa*	*Endo-glucanase*	spl|Q8GT15
A1	478.65	2+	957.30	FYDEQVR	FYDEQVR 1 FYDAKVS 6	*Oryza sativa*	*Endo-glucanase*	sp|Q8S9Q6
A1	497.19	2+	994.38	SEVVQLYR	-SEVVQLY 338 RSEVVQMY 345	*Oryza sativa*	*Glucanase*	sp|Q8W4V0
A1	513.18	2+	1026.36	YYQPLWR	-----YYQ 534 VQLYHFYQ 541	Triticum aestivum	*Glucosidase precursor*	sp|Q8GT15
A1	550.19	2+	1100.38	NFGLFYPNK	FGLFYPNK 615 FGLFYPNK 622	Hordeum vulgare	Glucanase 2	sp|O64938
A1	588.51	2+	1177.02	fqpTGTVR	fqpTGTVR 511 FQPGTTVR 518	*Oryza sativa*	*Glucanase*	sp|Q8GT15
A1	686.74	2+	1373.48	TYNQGLLDHVGR	YNQGLLDHVGR 573 RAYNQGLIDHVGR 584	*Oryza sativa*	*Endo-glucanase*	sp|Q8S9Q6
A1	719.23	3+	2157.69	YLAV	YLAV 116 RYIAV 119	*Oryza sativa*	*Endo-glucanase*	sp|Q8S9Q6
A1	757.81	2+	1515.62	NLVSALASAGLGGSGLK	ALASAGLGGS 118 ALVSAGLSSS 127	*Oryza sativa*	*Glucanase*	sp|Q8W4V0
A1	775.29	2+	1550.58	LYFPDQQALDALR	LYFPDQQALDALR 36 IYYPDKEALDALR 48	*Triticum aestivum*	*Glucosidase precursor*	sp|Q8GT15
A1	816.79	2+	1633.58	lgvC*YGVLGNNLPDK	gvCYGVLGNNLP 8 GVCYGVLGDNLP 15	*Oryza sativa*	*Glucanase*	sp|Q8W4V0
A1	863.31	2+	1726.62	dl)SLGYATFQPGTTVR	dlSLGYATFQPGTTVR 188 DISLNYATFQPGTTVR 203	*Oryza sativa*	*Glucanase*	spl|Q8GT15

The derived amino acid sequence of peptides recovered from in gel trypsin digestion of protein band A1 showed homology to endo-1,3-beta-glucanase from *Oryza sativa* with the accession number sptrembl|Q8GT15.

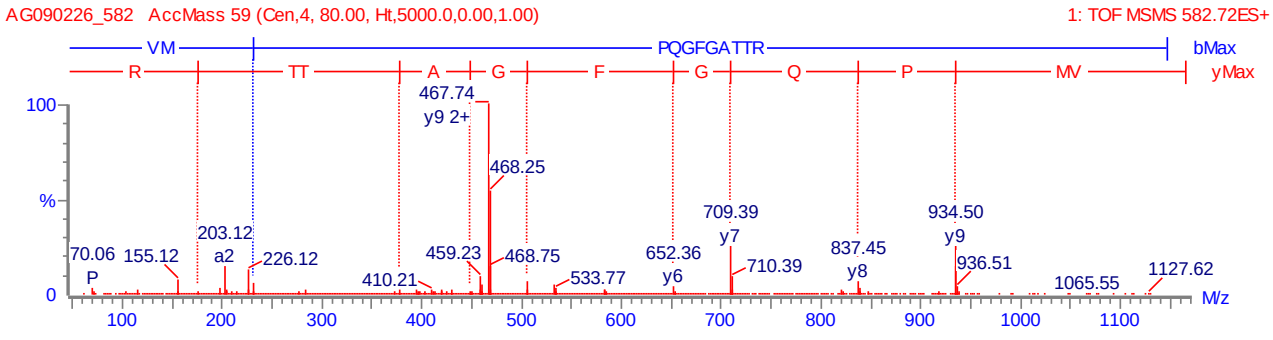


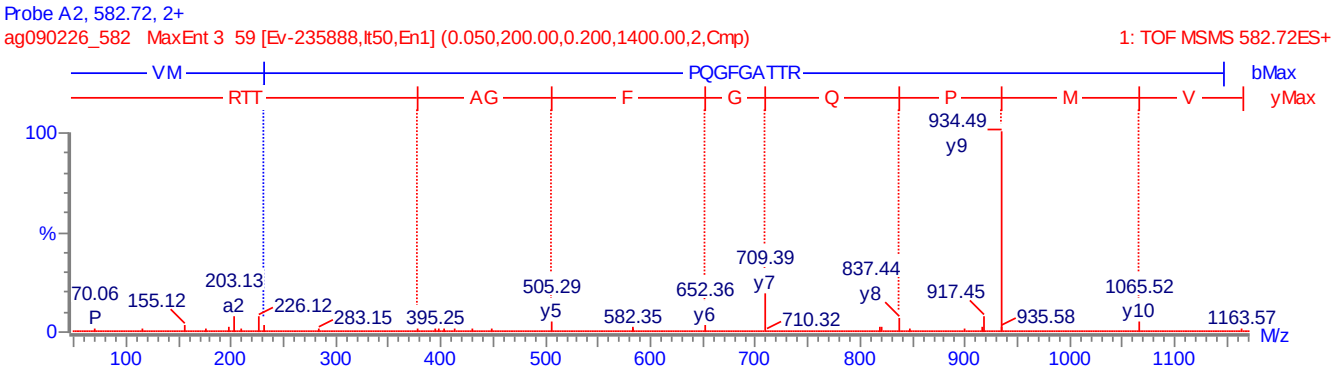


n-SAFLEAANK-c

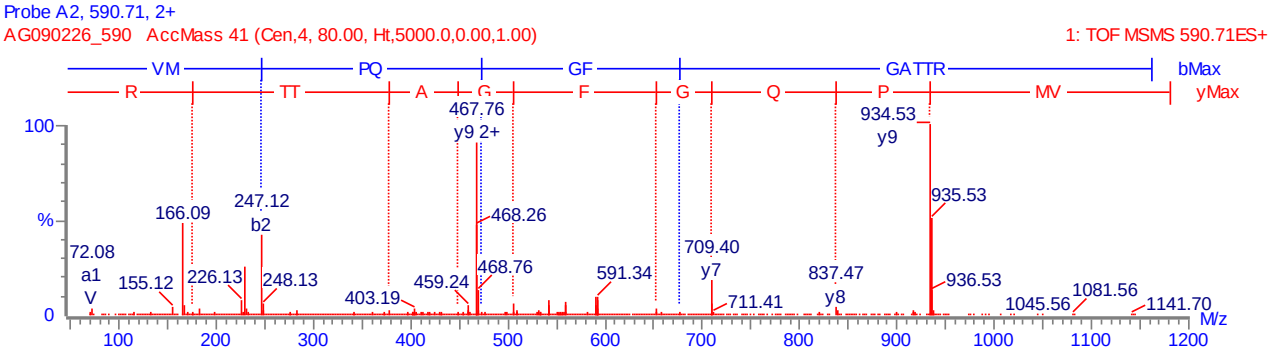


n-YSGFAHGGTEVEGKR-c

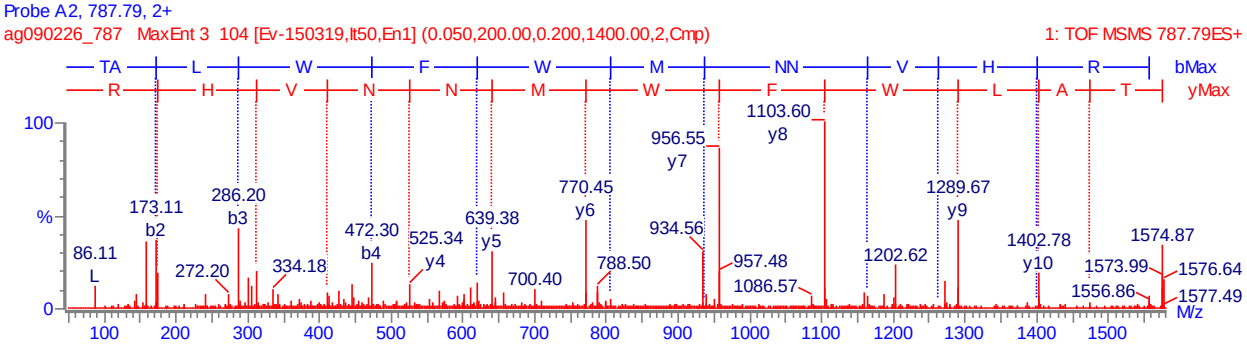




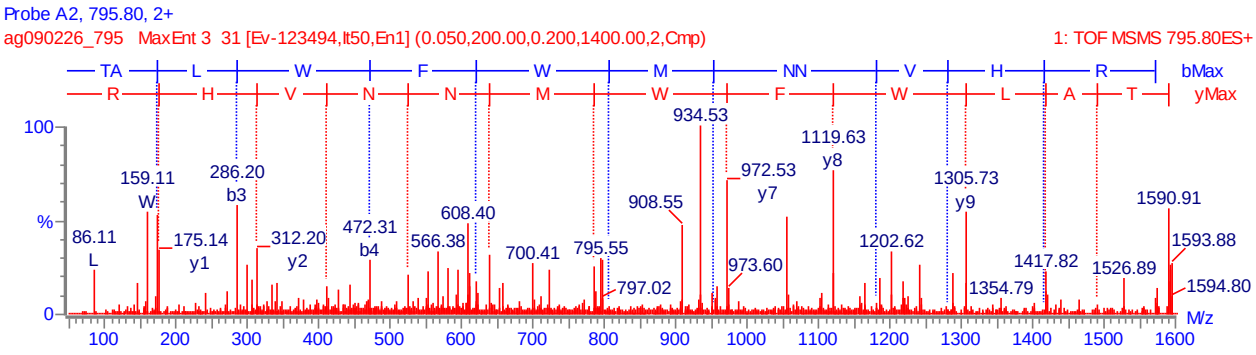
n-VMPQGFGAttR-c



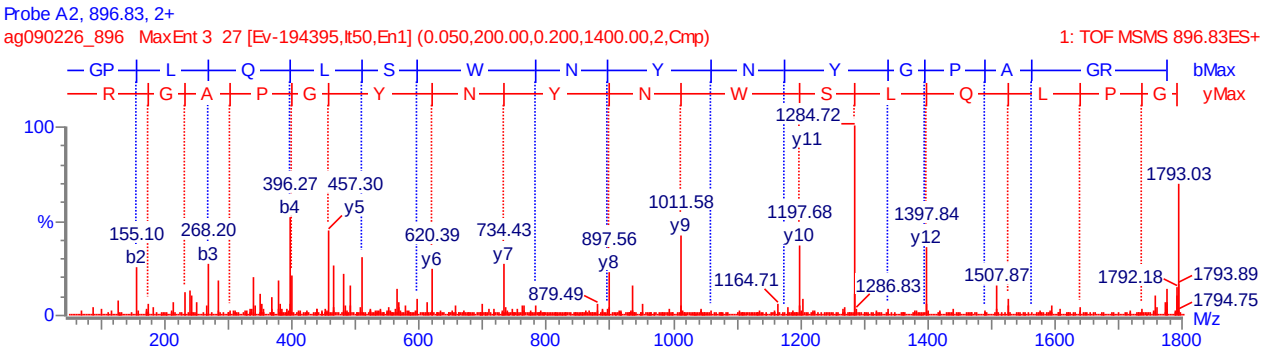
n-VM*PQGFGAttR-c; M* oxidiert



n-TALWFWMNNVHR-c



n-TALWFWM*NNVHR-c, M* oxidiert



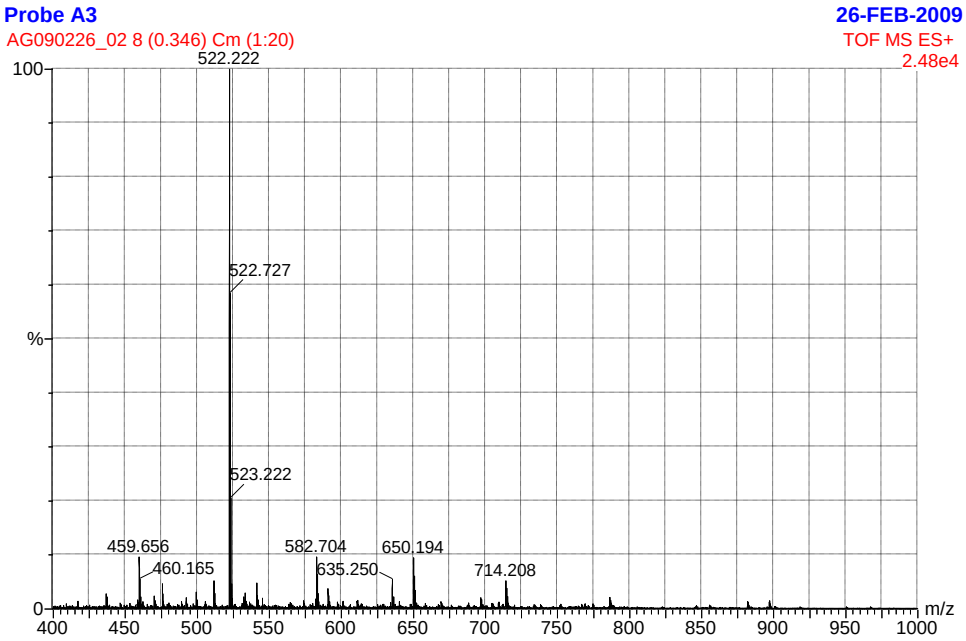
n-GPLQLSWNYNYGPAGR-c

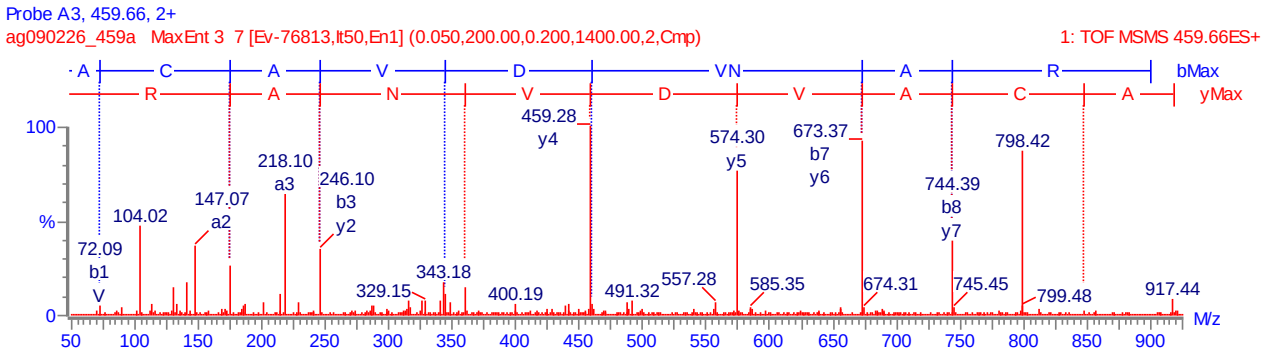
Table 5. Amino acid sequence of the peptide fragments generated by trypsin digestion of protein band A2.

(*indicates oxidised methionine residues, the amino acids in upper case indicate highly accurate sequences generated automatically and the ones in lower case represent the amino acids that are predicted).

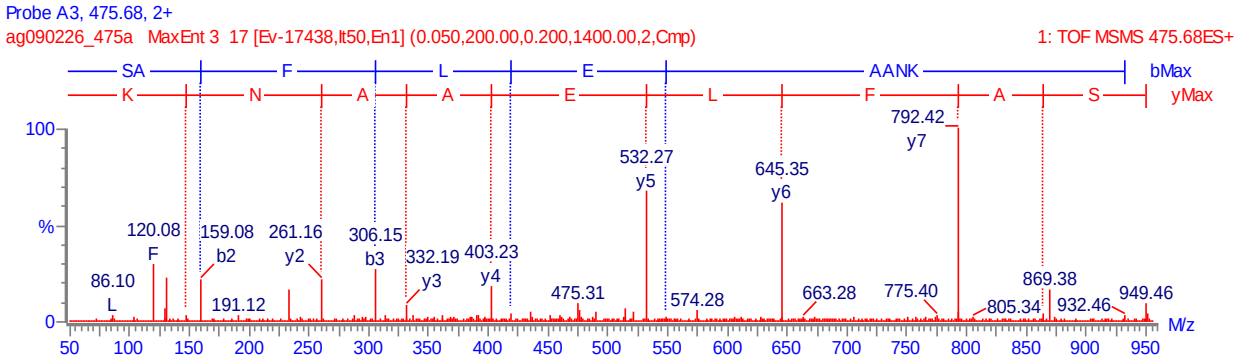
Band	Mass kDa	Charge	Rel. mass kDa	Derived amino acid sequence	Sequence similarity position	Organism	Possible function	Accession number
A2	475.69	2+	951.38	SAFLEAANKYSGFAHGGTEVEGKR	SAFLEAANKYSGFAHGGTEVEGKR 109 SAFLSAVNAYPGFAHGGTEVEGKR 132	*Zea mays*	Chitinase A	sp|P29022.1
A2	582.72	2+	1165.44	Y*GFAHGGTEVEGKR	Y*GFAHGGTEVEGKR 119 YPGFAHGGTEVEGKR 132	*Tripsacum dactyloides*	Chitinase A	sp|Q6JBL6
A2	896.83	2+	1793.66	GPLQLSWNYNYGPAGR	GPLQLSWNYNYGPAGR 179 RGPLQISWNYNYGPAGR 195	*Zea diploperennis*	Chitinase B	sp|Q6JBP3
A2	787.79	2+	1575.58	TALWFWMNNVH	TALWFWMNNVH 218 TALWFWMNNVH 228	*Zea mays*	Chitinase A	sp|P29022.1
A2	590.71	2+	1181.42	VMPQGFGATTR	VMPQGFGATTR 230 VMPQGFGATIR 240	*Zea mays*	Chitinase A	sp|P29022.1

The derived amino acid sequence of peptides recovered from in gel trypsin digestion of protein band A2 showed homology to chitinase from *Zea mays* with swissprot accession number sp|P29022.

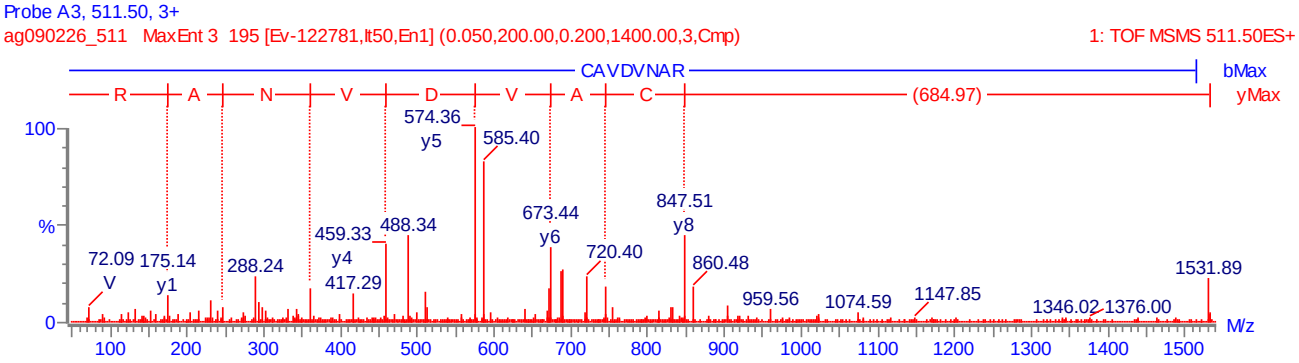




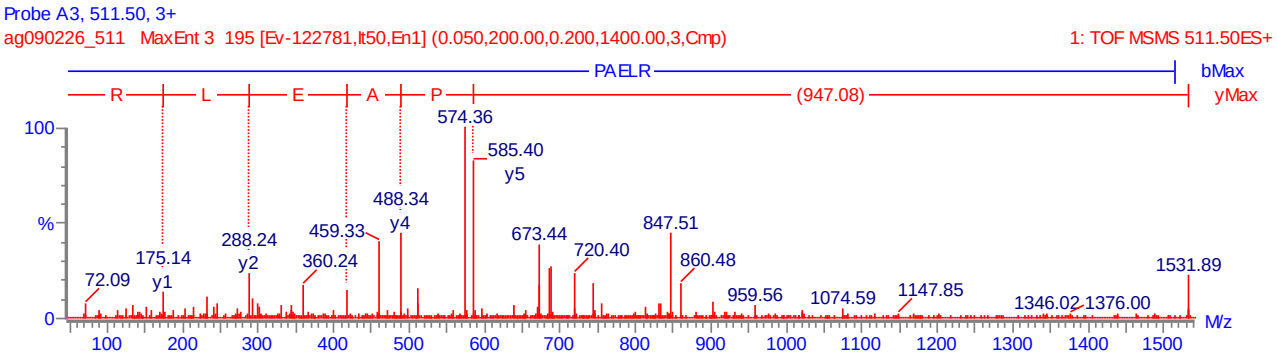
n-C*AVDVNAR-c; AC= C*, Masse von Cystein-Acrylamid



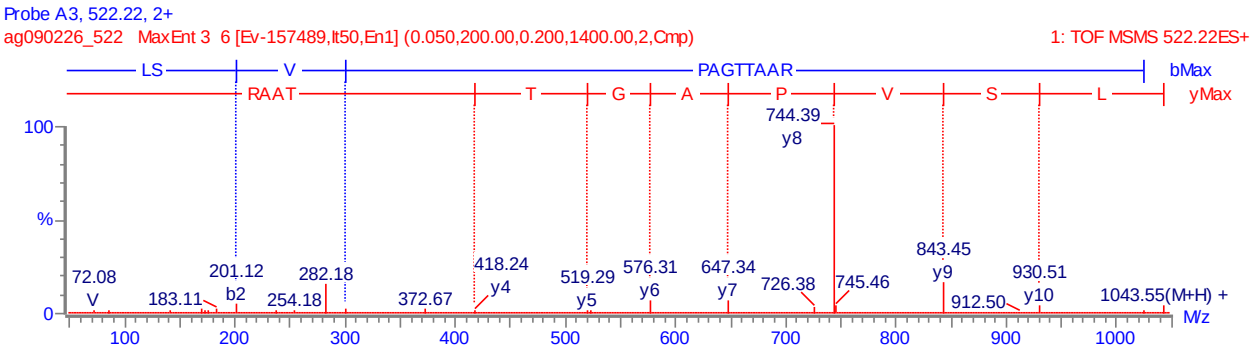
n-SAFLEAANK-c, aus Chitinase



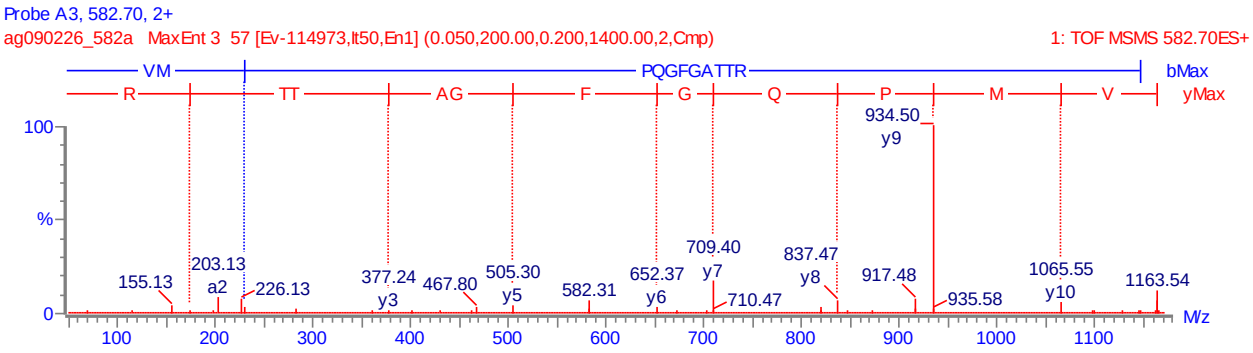
...AVDVNAR-c



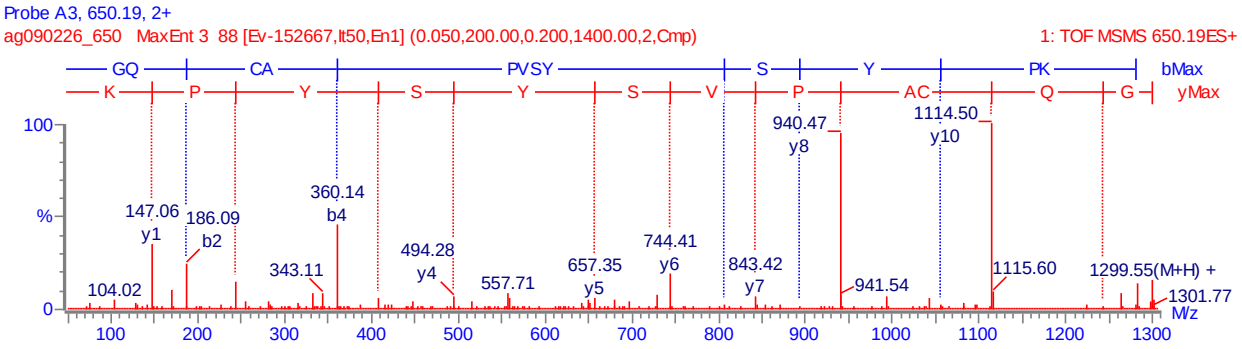
...PAELR-c



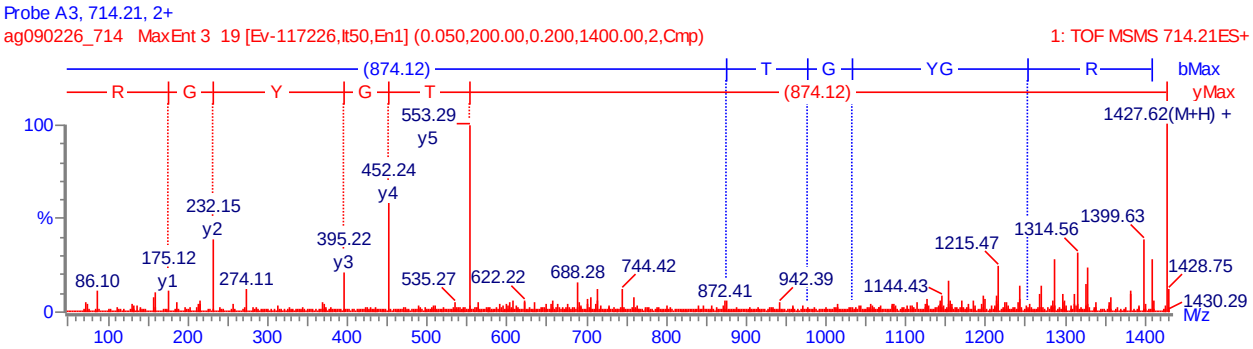
n-LSVPAGT(taar)-c

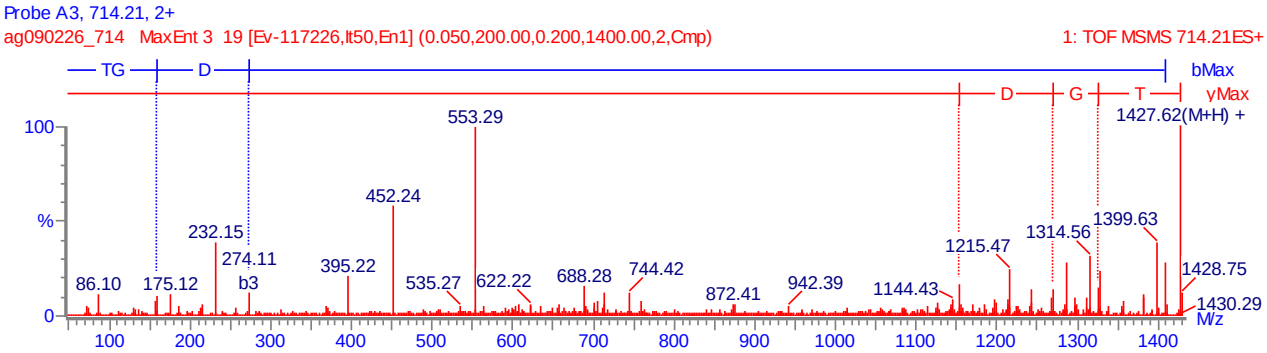


n-VMPQGF(ga)ttR-c, aus Chitinase

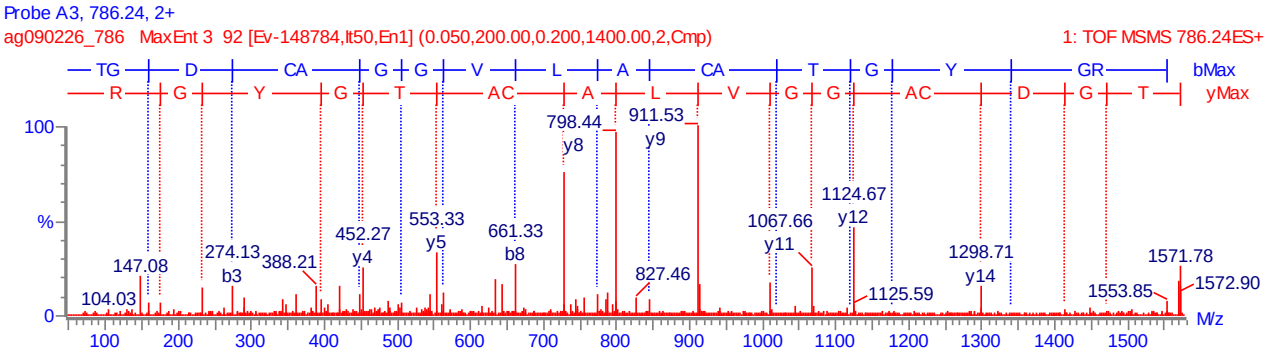


n-GQC*PVSYSYPK-c; AC=C*: s.o.

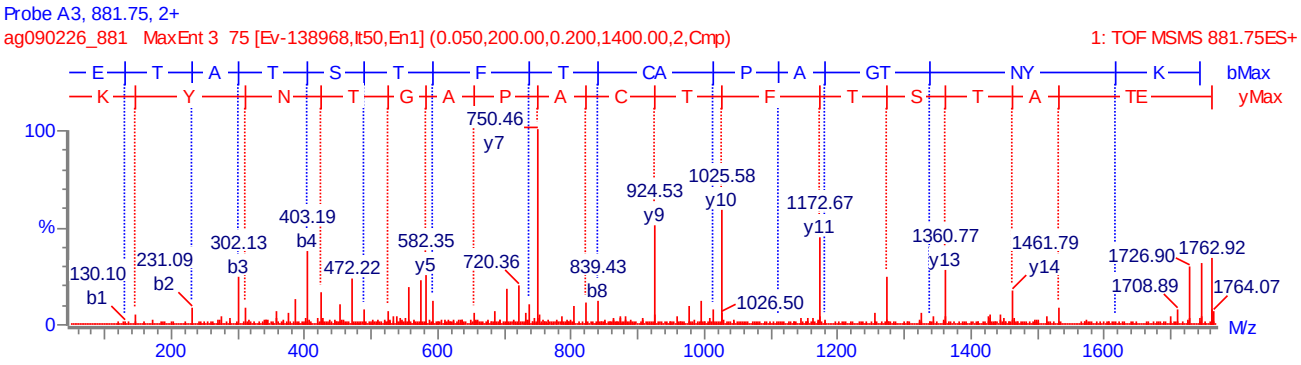




n-TGD...TGYGR-c



n-tgdC*GGVLAC*TGYGR-c, AC=C*



...ATSTFTC*PAGTNYK-c; AC=C*

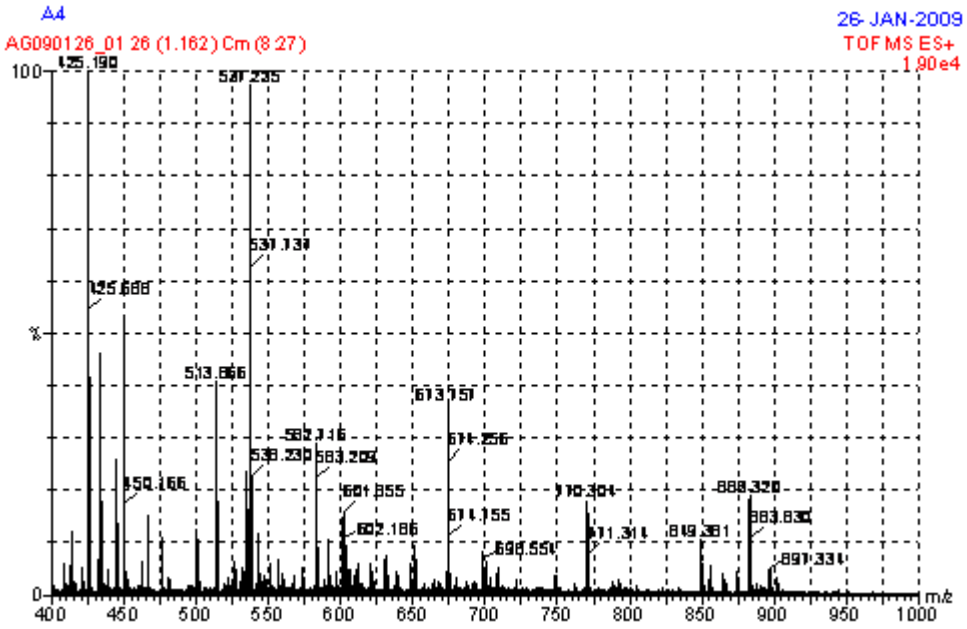
Table 6. Amino acid sequence of the peptide fragments generated by trypsin digestion of protein band A3.

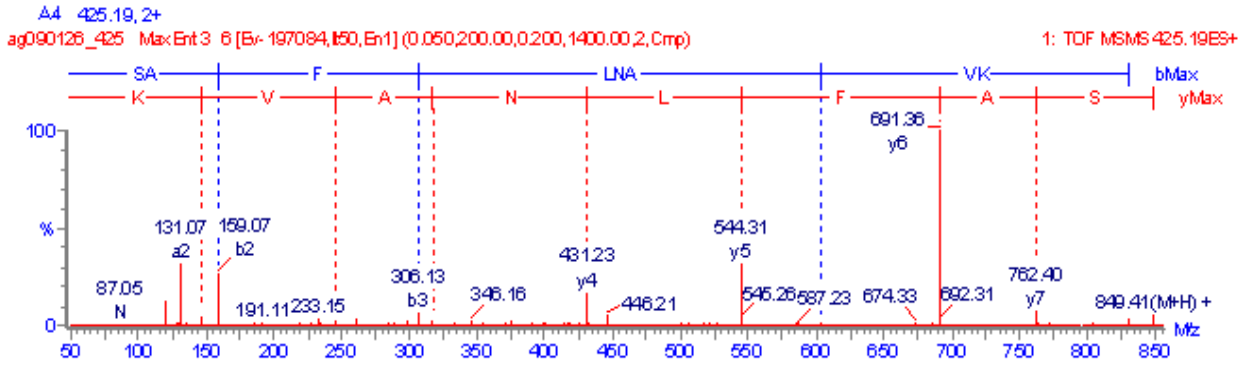
(*indicates oxidised methionine residues, the amino acids in upper case indicate highly accurate sequences generated automatically and the ones in lower case represent the amino acids that are predicted).

Band	Mass kDa	Charge	Rel. mass kDa	Derived amino acid sequence	Sequence similarity position	Organism	Possible function	Accession number
A3	459.66	2+	919.32	C*AVDVNAR	C*AVDVNAR 145 CAVDVNAR 152	*Zea mays*	Thaumatin-like protein	sp| P33679
A3	475.68	3+	1427.04	n-AVDVNAR	AVDVNAR 146 AVDVNAR 152	*Oryza sativa*	Thaumatin-like protein	sp|Q75GX4
A3	511.50	3+	1534.50	PAELR	PAELR 154 PAELR 158	*Hordeum vulgare*	Thaumatin-like protein	sp|Q946Y8
A3	522.22	2+	1044.44	LSVPAGT(taar	LSVPAGTtaar 54 RITAPAGTTAAR 65	*Oryza sativa*	Thaumatin-like protein	sp|O04364
A3	786.24	2+	1572.48	tgdCGGVLAC*TGYGR	tgdCGGVLACTGYGR 84 TGDCGGVLQCTGYGR 98	*Hordeum vulgare*	Thaumatin-like protein	sp|Q946Y9|
A3	881.75	2+	1763.50	ATSTFTC*PAGTNYK	ATSTFTCPAGTNYK 209 ATSTFTCPAGTNYK 222	*Oryza sativa*	Thaumatin-like protein	sp|Q75GX4
A3	650.19	2+	1300.38	GQC*PVSYSYPK	GQCPVSYSYPK 196 GQCPDAYSYPK 206	*Zea mays*	Thaumatin-like protein	sp| P33679
A3	714.21	2+	1428.42	TGDTGYGR	TGDT--------GYGR 83 RTGDCGGVLQCTGYGR 98	*Zea mays*	Thaumatin-like protein	sp| P33679

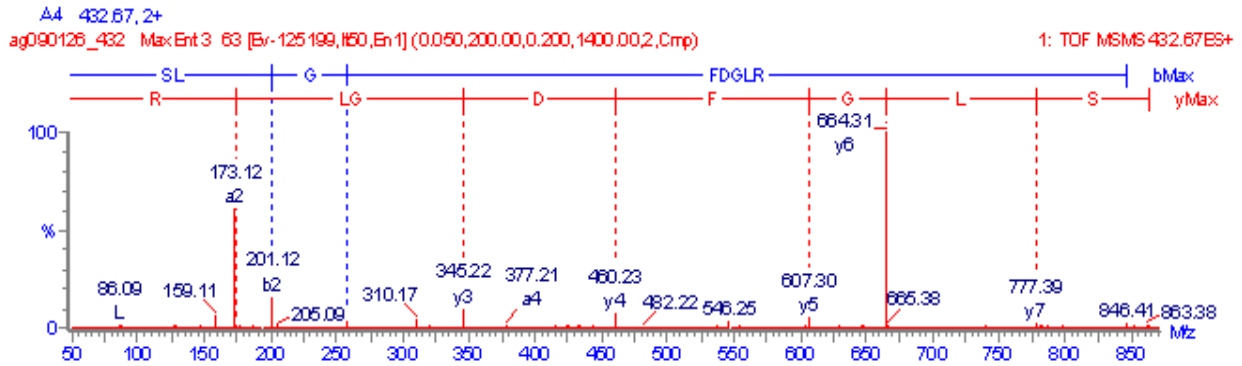
The derived amino acid sequence of peptides recovered from in gel trypsin digestion of protein band A3 showed homology to zeamatin from *Zea mays* with swissprot accession number sp|P33679.

Probe A4

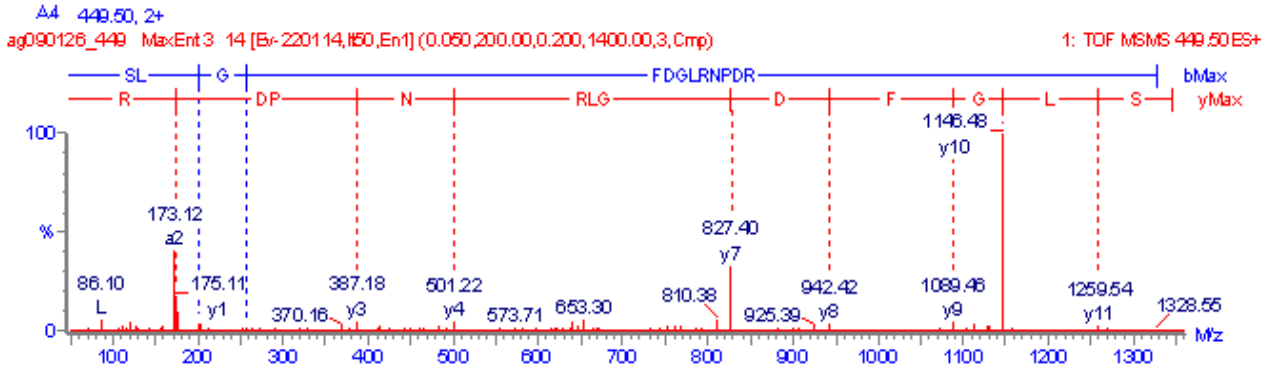




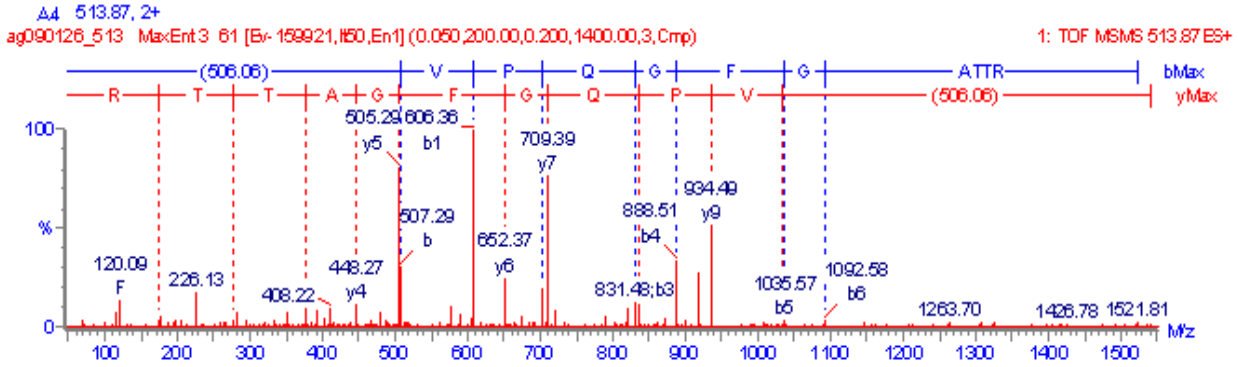
n-SAFLNAVK-c



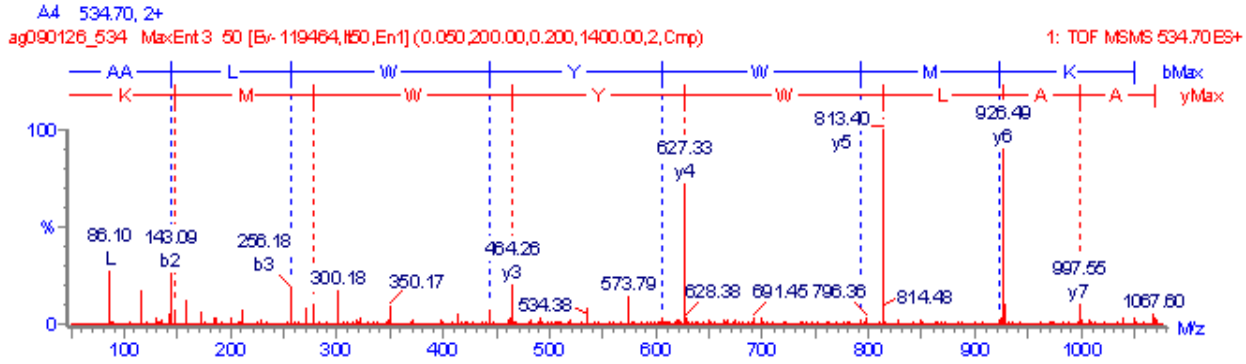
n-SLGFD(gl)R-c



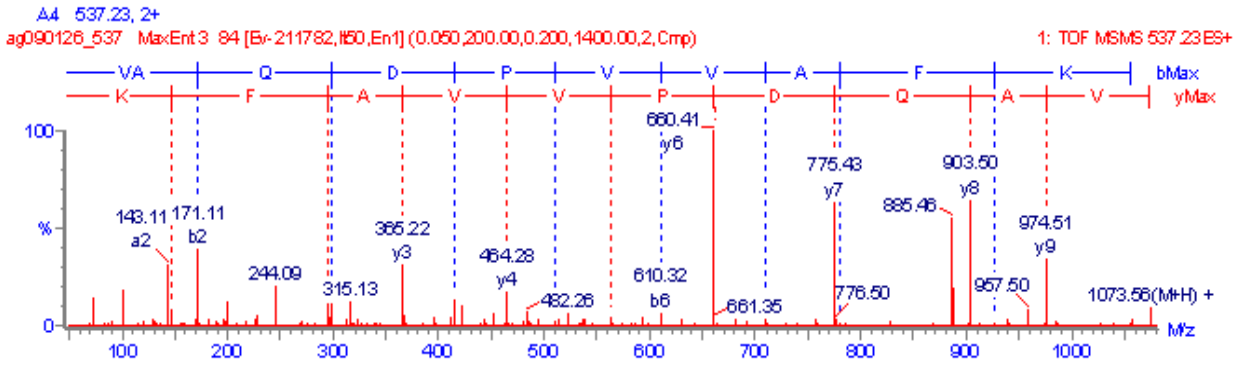
n-SLGFD(glr)n(pd)r-c



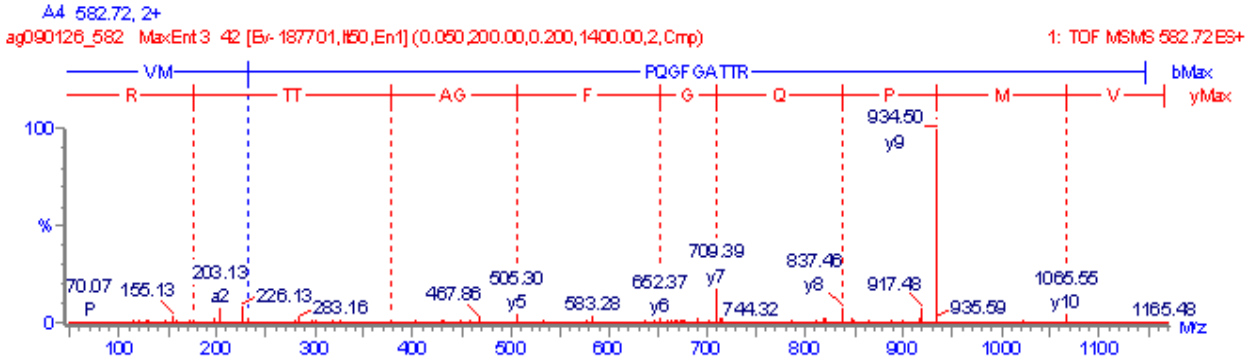
…PQGFGATTR-c



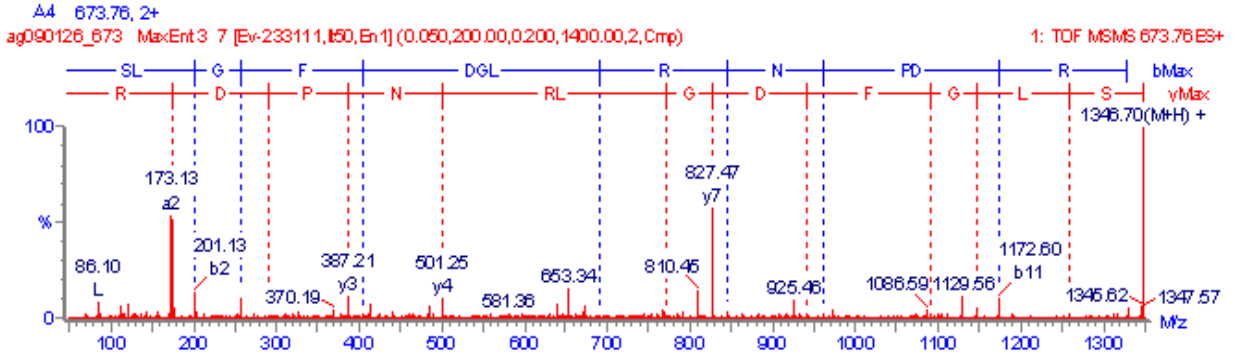
n-AALWYWMK-c



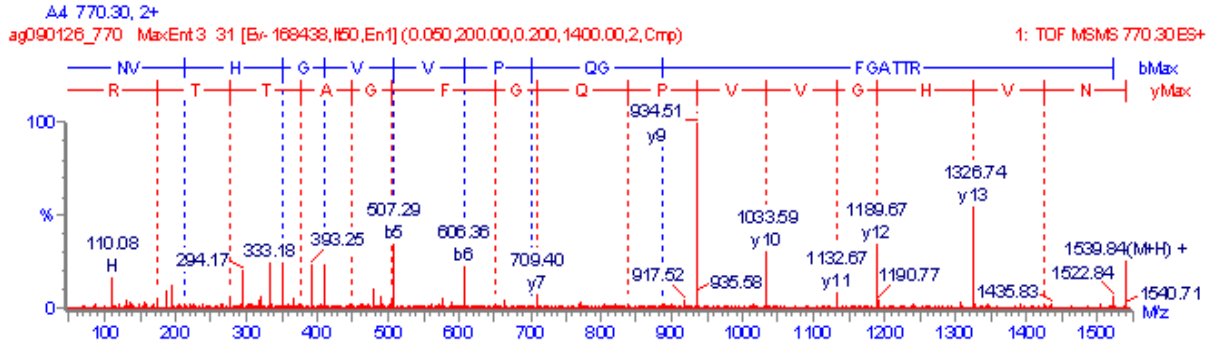
n-VAQDPVVAFK-c



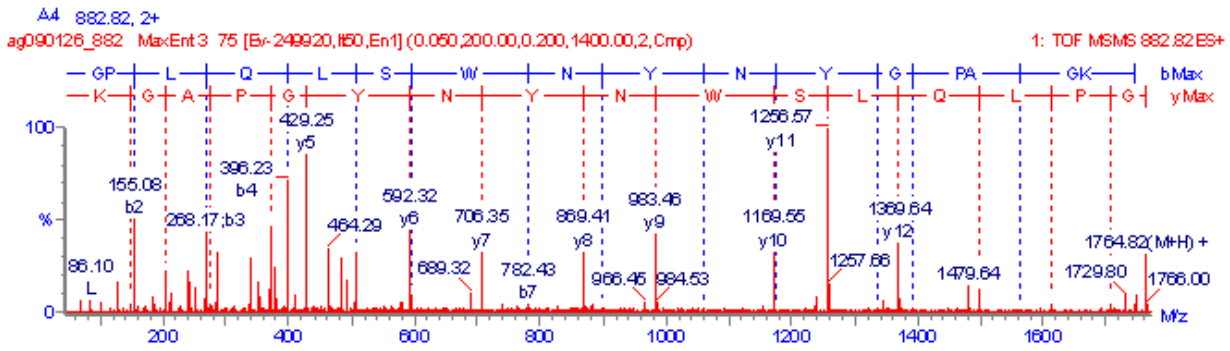
n-VMPQGF(ga)ttr-c



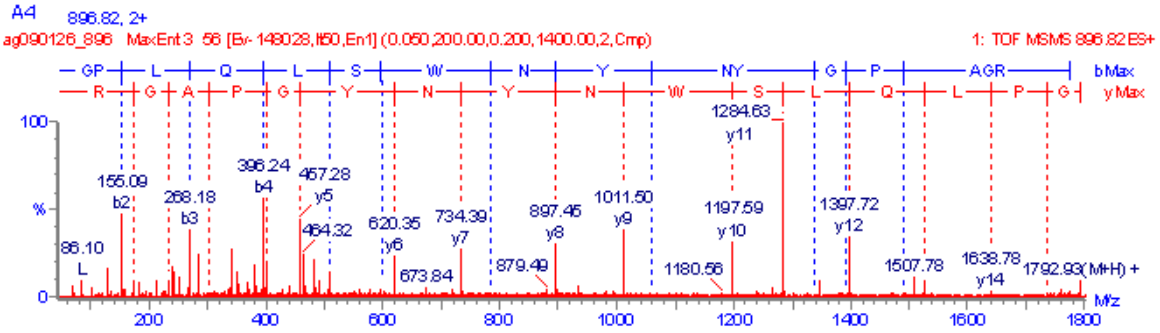
n-SLGFDG(lr)NPDR-c



n-NVHGVVPQGFGATTR-c



n-GPLQLSWNYNYGPAGK-c



n-GPLQLSWNYNYGPAGR-c

Table 7. Amino acid sequence of the peptide fragments generated by trypsin digestion of protein band A4.

(*indicates oxidised methionine residues, the amino acids in upper case indicate highly accurate sequences generated automatically and the ones in lower case represent the amino acids that are predicted).

Band	Mass	Charge	Rel. Mass	Derived amino acid sequence	Sequence similarity position	Possible function	Organism	Accession number
A4	425.29	2+	850.58	SAFLNAVK	SAFLNAVK 115 SAFLSAVK 122	Chitinase	*Zea diploperennis*	UNIPROT. Q6JBP3
A4	432.67	2+	865.34	SLGFD(gl)R	SLGFDgl++++R 202 AIGFDGLGDPGR 213	Chitinase	*Sorghum bicolor*	XP002455593
A4	449.50	2+	899.00	SLGFD(glr)n(pd)r	SLGFDgl+np+r 202 AIGFDGLGDPGR 213	Chitinase	*Zea diploperennis*	UNIPROT. Q6JBP3
A4	513.87	2+	1027.74	PQGFGATTR	PQGFGATTR 238 PQGFGATTR 247	Chitinase	*Zea diploperennis*	UNIPROT. Q6JBP3
A4	534.70	2+	1069.40	AALWYWMK	AALWYWMK 224 AALWFWM 229	chitinase-B1	*Sorghum bicolor*	sp|Q93WT1
A4	537.23	2+	1074.46	VAQDPVVAFK	VA+D+VVAFK 214 VARDAVVAFK 223	class IV chitinase	*Oryza sativa*	sp|O04138
A4	582.72	2+	1165.44	VMPQGF(ga)ttr	V+PQGFgattr 236 VVPQGFGATTR 246	Chitinase	*Zea diploperennis*	UNIPROT. Q6JBP3
A4	673.76	2+	1347.52	SLGFDG(lr)NPDR	SLGFDGlrNP+R 202 AIGFDGLGDPGR 213	Chitinase	*Sorghum bicolor*	XP002455593
A4	770.30	2+	1540.60	NVHGVVPQGFGATTR	NVHGVVPQGFGATTR 233 +VHGVVPQGFGATTR 246	Chitinase	*Zea diploperennis*	UNIPROT. Q6JBP3
A4	882.82	2+	1765.64	GPLQLSWNYNYGPAGK	GPLQLSWNYNYGPAGR 186 GPLQISWNYNYGPAG+ 199	chitinase-B1	*Sorghum bicolor*	sp|Q93WT1
A4	896.82	2+	1793.64	GPLKLSWNYNYGPAGR	GPLKLSWNYNYGPAGR 186 GPLQISWNYNYGPAG+ 199	Chitinase	*Zea diploperennis*	UNIPROT. Q6JBP3

The derived amino acid sequence of peptides recovered from in gel trypsin digestion of protein band A4 showed homology to chitinase B from *Zea diploperennis* with Uniprot accession number Uniprot.Q6JBP3.

2.1 Inverse PCR to amplify of unknown regions flanking the known sequence of pearl millet chitinase.

After isolating partial length gene of pearl millet chitinase and tlp unknown regions of the gene flanking the known sequence can be isolated by primer based genome walking techniques. The simplest and single step method is inverse PCR. To carry out inverse PCR, genomic DNA library was created by restriction digestion of the genomic DNA with non-cutters of the known gene sequence followed by self ligation of restriction digested fragments to form circular double stranded fragments. This library serves as template for PCR. Primers are designed in such way that one primer complimentary to the inner strand of DNA and the other primer complimentary to the outer strand of DNA. The primer pair would not give any product if the gene fragments are not circular as the primers walk in directions away from each other. Only the circular molecules from the library will be amplified. The primers are designed in such a way that the forward primer is complimentary to 3' end of the noncoding strand of the known sequence; reverse primer-complimentary to 5'end of coding strand. The forward primer walks downstream region of the known sequence and the reverse primer-upstream region. Distance amplified depends on the size of circular DNA molecule serving as template. Genomic DNA library was constructed by restriction digestion of genomic DNA with *Alu*I, *Sau*3AI separately. The restriction digested fragments were self ligated overnight by incubating with T4DNA ligase. These self ligated circular DNA molecules were used as template for inverse PCR. Nucleotide sequence of tlp (partial length) and maize zeamatin (ZMU06831) with which it shows close homology were considered as reference sequences for primer designing (Fig 1). The primers designed and their sequence is mentioned in the table 1.

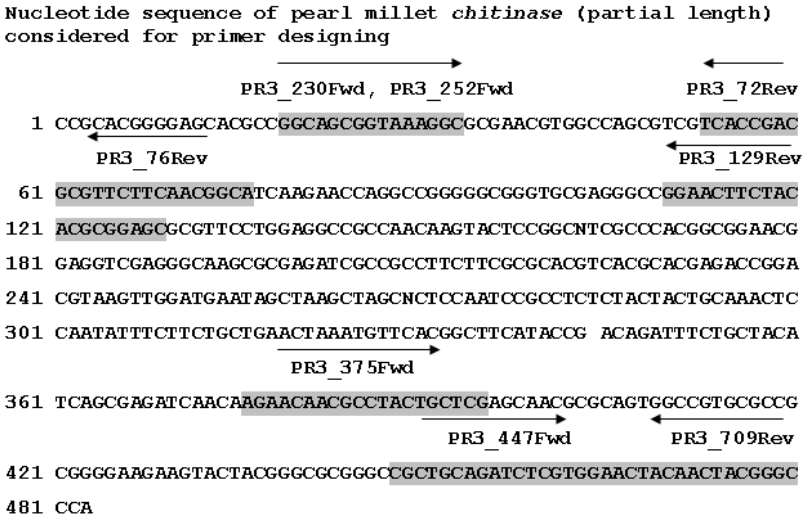


Fig 1. Nucleotide sequence of pearl millet chi showing primers designed. Grey shaded boxes indicate the primers.

Table 1. Primers designed to amplify unknown flanking regions of pearl millet *tlp* by inverse PCR

	Primer	Sequence
1	PR3_72_C	5'-gttgaagaacgcgtcggtga-3'
2	PR3_76_C	5'-tgccgttgaagaacgcgtcg-3'
3	PR3_375	5'-agaacaacgcctactgctcg -3'
4	PR3_ 447	5'-cgcgtcagatctcgtggaac-3'

The products of inverse PCR to amplify flanking regions of pearl millet chitinase gene using different primer combinations with genomic DNA library constructed with *Alu*I digested fragments is seen in Fig 2.

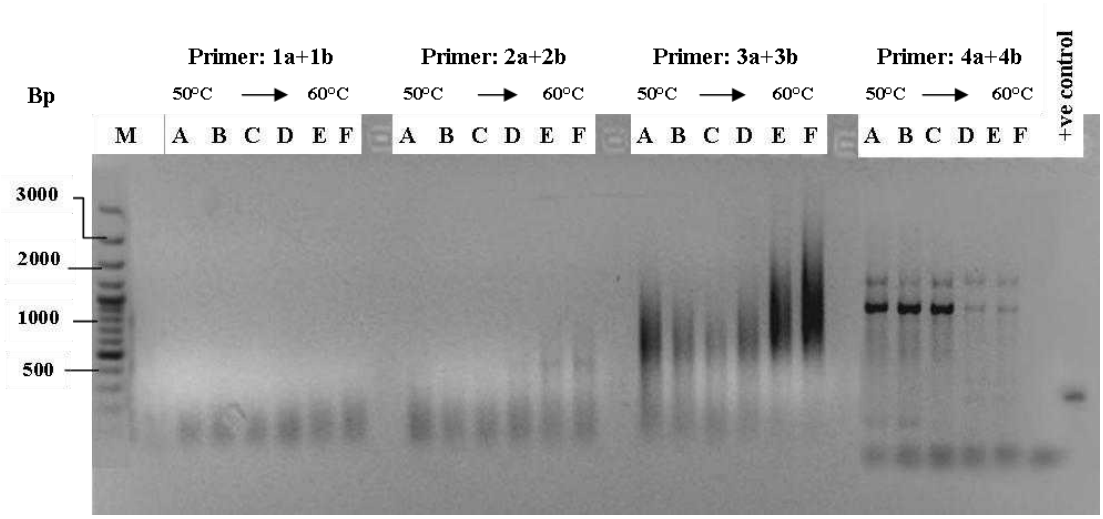


Fig 2. Inverse PCR to amplify unknown flanking regions of pearl millet *chi* with genomic DNA library constructed using restriction enzyme *Alu*I. Lane M: 100 bp DNA ladder plus (Fermentas # SM0321); Lane A-F shows temperature gradient from 50-60°C. The primer pair used in mentioned above the lanes for each set of reaction, last lane is positive control. Primer combinations used are: 1a+1b: (PR3_72 rev + PR3_375 fwd), 2a+2b: (PR3_72 rev+ PR3_447 fwd), 3a+3b: (PR3_76 rev + PR3_375 fwd) and 4a+4b: (PR3_76 rev + PR3_447 fwd).

The primer pair 4a 4b amplified approximately 1 kbp amplicon was purified by gel extraction and cloned into CloneJet cloning vector and *E. coli* competent cells were transformed by heat shock transformation. Transformants were screened by colony PCR using primers provided with CloneJet cloning kit, plasmid was isolated from the positive clone and sequenced. Nucleotide sequence of the approximately 1 kbp product is given in Fig 3.

The primer PR3_76_C TGCCGTTGAAGAACGCGTCGCTG) is repeated in both the strands leading to amplification of this nonspecific product. The amplicon does not show any homology to known chitinase genes. But the fragment shows homology to proteins of unknown function from *Triticum aestivum* (Accession number: CK168143 gi: 39002991).

Nucleotide sequence of the ~1kbp amplified by PR3_76 rev and PR3_447 fwd.

```
  1 CTATAGAATACTCAAGCTATGCATCCAACGCGTTGGGAGCTCTCCCATATGGTCGACCTG
 61 CAGGCGGCCGCACTAGTGATTTGCCGTTGAAGAACGCGTCGCTGTGCAGCCATCCCCGAC
121 GCTGTGCAGGGGTTGTGCGGGATGGCTGGTGTCATTCCTTGGCACTATGTTGCCTGCTCC
181 CGTCCTTCCTCCACACCACACCCCTCGAAAGAGGATAACGACACCCTCTAAGAGCGTACG
241 CCCACGAACTCGGCGCCCATCCAGGACGGTGCCGTGACGCAGGGTGAGCGAAGCACGTCT
301 CCCCCATCACCTCCGACCCTGTGCGAGCGTGCGGCCATCCCCGGCGCTGTGCTGCCATCC
361 CGGGTACTGCAGAGCCATCCCCAACACTGTGGGGACCTTTGCTAACGGGACGCTGCCACG
381 CCAGTCGTTGTGCCGTGTGTGGCCCAGGCTCCCTCGTCAGCTAGACCCTCGAATCGGTGT
421 ATGGATGACGACCAGACGCGAGCACCGACAAGACCACCCCTCGAAGCCGCCTCTAAACGG
481 GCACAGGGGCCACCACGACGCGTTCTTCAACGGCAAATCCCGCGGCCATGGCGGCCGGGA
541 GCATGCGACGTCGGGCCCAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTC
601 GTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCA
661 CATCCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCC
721 AACAGTTGCGCAGCCTGAATGGCGAATGGACGCGCCCTGNAGCGGCGCATTAAGCGCGGC
781 GGGNGTGGGTGGTNCGNGCANCGTGACCGNTCACTTTGCNGCGCCTNGCNCCNTNCNTTC
841 NNTTNTTCCCTTNCCTTCTCGCNCGTTCGCNGNTTTNCCGNNAGCTCTWAATCGGGGGCT
901 CCCTTTAGGGTNCCATTAANNTTTACGGCNCTNNNCCCNAAAACTTGATAGGGGGNNGGT
961 TCACTANGGGCNTCNC
```

Fig 3. Nucleotide sequence of the ~1kbp product amplified by inverse PCR using the primer pair Fwd and PR3_76 Rev+ PR3_447 Fwd. Grey shaded box indicates the primer PR3_76Rev repeated twice leading to non specific amplification.

The products of inverse PCR to amplify flanking regions of pearl millet chitinase gene using different primer combinations with genomic DNA library constructed with *Sau*3AI digested fragments is seen in Fig 4.

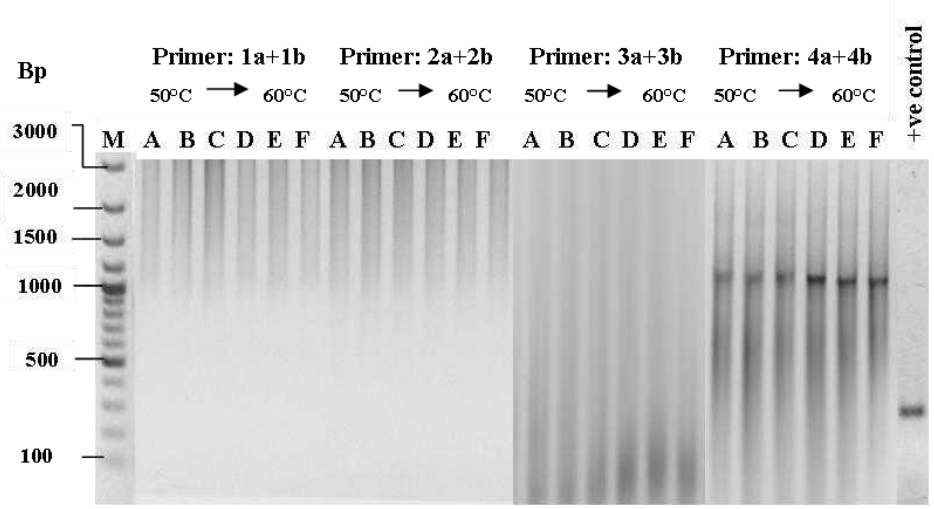


Fig 4. Inverse PCR to amplify unknown flanking regions of pearl millet chi with genomic DNA library constructed using restriction enzyme *Sau*3AI. Lane M: 100 bp DNA ladder plus (Fermentas # SM0321); Lane A-F shows temperature gradient from 50-60°C. The primer pair used in mentioned above the lanes for each set of reaction, last lane is positive control. Primer combinations used are: 1a+1b: (PR3_72 rev + PR3_375 fwd), 2a+2b: (PR3_72 rev+ PR3_447 fwd), 3a+3b: (PR3_76 rev + PR3_375 fwd) and 4a+4b: (PR3_76 rev + PR3_447 fwd).

The primer pair 4a and 4b amplified approximately 1.1 kbp product, which was directly sequenced. Nucleotide sequence of this 1.1 kbp product (a few bases at both the ends are missing as the PCR product was directly sequenced therefore the sequence appears shorter) is given in Fig 5.

Nucleotide sequence of the 954 bp amplified by PR3_76 rev and PR3_447 fwd.

```
  1 CAACANAGGTGCCTCAGTCCACGTCGATCATCATGACCTCCTGNCCAGCTGGTGGATGCG
 61 CTGGGACAGTGGTCGGTGGCGCCGGCTCCGGGATGGATGGCGCGTGAAGCTCNTGGACGA
121 AGACACCCGCTGGTACTTGAGCGCGNGTNGATCCAAGCTTCGANAGGACGTCCGCTGCAA
181 CGTTGTTGTCGCGGACGATGTGATGGAACTCGAGACCATAGAANTTGTTCTCGAGCTTGC
241 GGACTTCTAGGCAGTAGGCGTCCATGTTCTCCTTGTTGCGGTCCCAGGACTTGTTGACTT
301 GGTTGATGACCACTGCGGAGTCGCCGTAGACTAGTAGCCGCTTGATGCCCAGTGAAGCCG
361 CGAGGCGGAGCCCGTGTAGGAGAGCTTCGTACTCGGCTTCGTTGTTTGAGACCTTCCAGA
421 AGATCTGCAGGACGTACTTGAGTTGTTCGCCCGTTGGAGAGATCAGGAGTACCCCTGCAC
481 CTGCGCCCTCGAGGTTGAGTGAGCCGTCAAAGTACATCACCCAATGCTCTGGTCGCNCGG
541 TNGGAGTAGGTAGCTGATTCTCCCGCCACTCTGCCATGAAGTCGACTAGTGCNTGCNACT
601 TGATGGCTGTGCGTGGCTTGAAGTCNATGGTGAGGGCGCCGAGNTCGACNGCCCACTTGG
661 AGATTCTTCCAGTAGCATCTTGGTTCCNCAAGATGTCNCCNAGCGGGTAGTCNGTGACNA
721 CNGAAATCNAGTACTCCTGGAAGTAGTGTCNTANCTTCCNCGANGGTGATGAGTACTGCN
781 TACANNANCTTCTGCNCNGGCTGATAACNAGCCTTTGNATTCTAAAAGAAACCTCNCTNA
841 CAAAATAAAATTGNNCNCTNGACNGNNTAAGNNTGNNCNTNTTNCTGCCNNTCNANAANN
901 ATGGNNNNACTGACCACTTNANTANTCNCANNNAGNTAAACATANNNTTTTNNA
```

Fig 5. Nucleotide sequence of the 954 bp amplified by PR3_76 rev and PR3_447 fwd with genomic DNA library constructed with *Sau*3AI digested fragments.

This 954 fragments shares homology with protein of unknown function from *Zea mays* (NCBI accession number: gi|148990621|gb|EE032011) expressed in seedlings under various stress conditions.

2.2 Inverse PCR to amplify of unknown regions flanking the known sequence of pearl millet chitinase.

To carry out inverse PCR to amplify unknown flanking region, nucleotide sequence of pearl millet tlp (422 bp, partial length) was considered as reference sequences for primer designing.

The primers designed and their sequence is mentioned in the table 2.

Table 2. Primers designed to amplify unknown flanking regions of pearl millet *tlp* by inverse PCR

Primer	Sequence
PR5_59 Rev	5'-CGACTTCTTCGACATCTCGCT-3'
PR5_267 Fwd	5'-CGACTACTCGAGGTACTTCAAGGG-3'
PR5_256 Fwd	5'-GACTGCGGCGGCGTGCT-3'
PR5_208 Fwd	5'-GTGTTCAAGACGGACCAGTA-3'
PR5_271 Fwd	5'-CTGCAGTGCACTGGGTA-3'

Nucleotide sequence of pearl millet tlp (422 bp) with primers designed is shown in Fig 6; grey shaded boxes indicate the primers.

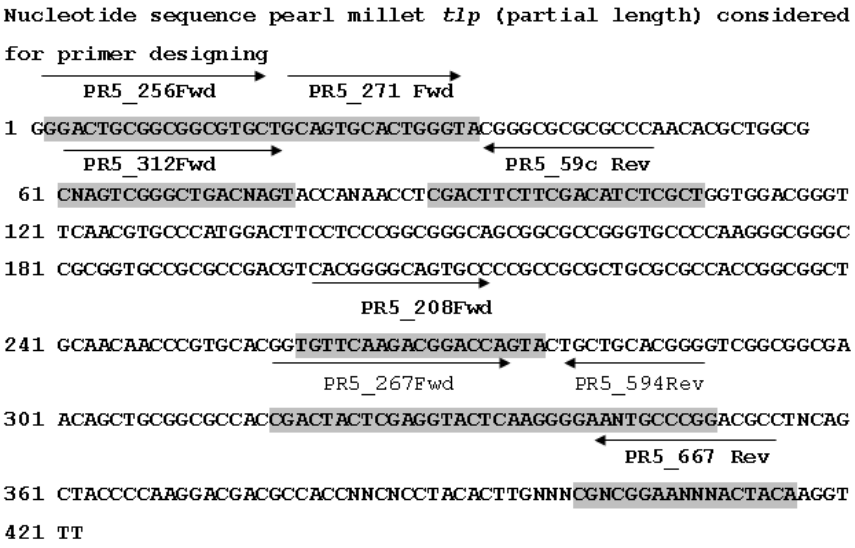


Fig 6. Nucleotide sequence of pearl millet tlp showing primers designed. Grey shaded boxes indicate the primers.

The primer sequence though present in the partial length sequence of known region of pearl millet tlp gene, these primers were unable to amplify the upstream as well as downstream region of the gene. Temperature gradient PCR with annealing temperatures 50-60°C with different primer combinations is illustrated in the Fig 7.

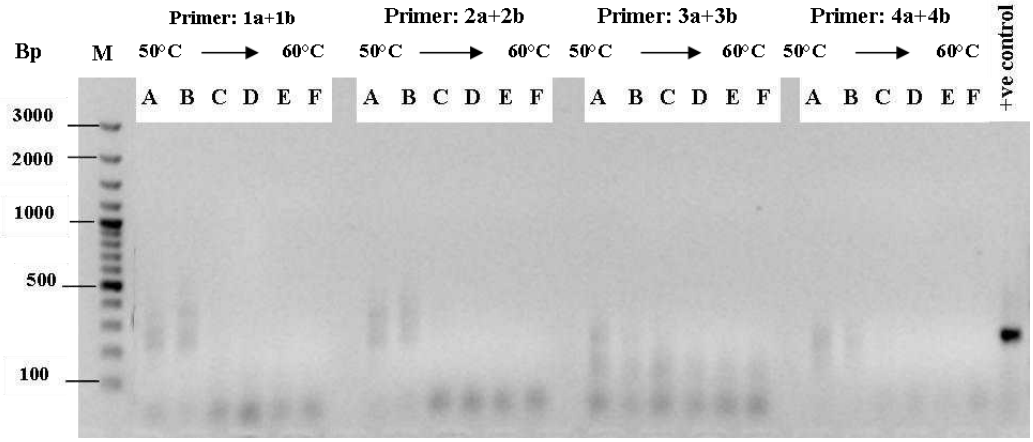


Fig 7. Inverse PCR to amplify unknown flanking regions of pearl millet *tlp* with genomic DNA library constructed using restriction enzyme *Alu*I. Lane M: 100 bp DNA ladder plus (Fermentas # SM0321); Lane A-F shows temperature gradient from 50-60°C. The primer pair used in mentioned above the lanes for each set of reaction, last lane is positive control. Primer combinations used are: 1a+1b: (PR5_59 rev + PR5_267 fwd), 2a+2b: (PR5_59 rev+ PR5_208 fwd), 3a+3b: (PR5_59 rev + PR5_271 fwd) and 4a+4b: (PR5_59 rev + PR5_256 fwd).

As seen in Fig 7 above all the primer combinations were unsuccessful in amplifying the unknown flanking regions of pearl millet tlp gene by inverse PCR using genomic DNA library constructed with *Alu*I digested fragments as template. The reason could be that *Alu*I might have a restriction site just outside the known sequence of pearl millet tlp gene and thereby not cutting the flanking region outside

the known fragment. Therefore one more genomic DNA library was constructed using *Sau*3AI restriction digested genomic DNA fragments. And PCR was carried out with same set of primers. No new primers were designed in this case as the primer sequences were taken from the known region of pearl millet tlp.

Temperature gradient PCR with annealing temperatures 50-60°C with different primer combinations is illustrated in the Fig 8.

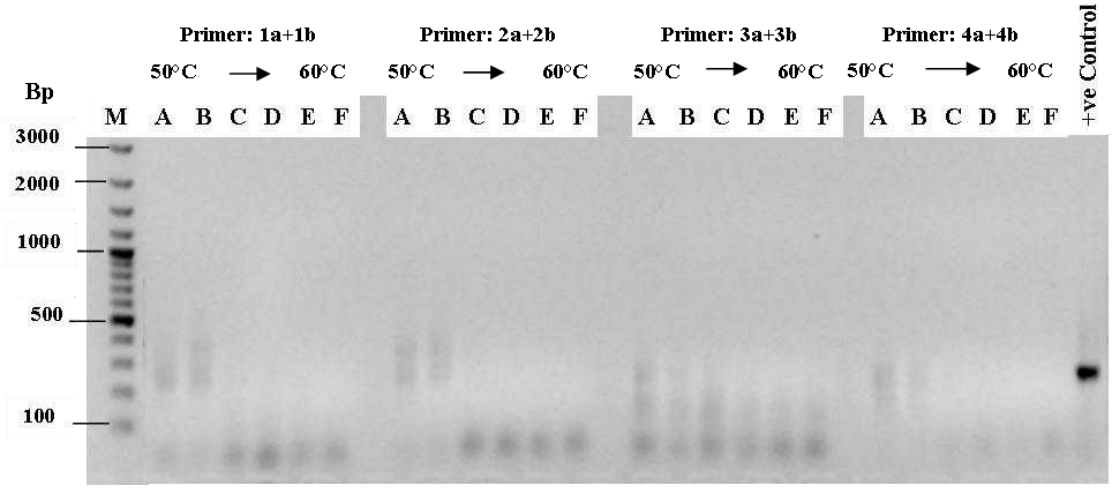


Fig 8. Inverse PCR to amplify unknown flanking regions of pearl millet *tlp* with genomic DNA library constructed using restriction enzyme *Sau*3AI. Lane M: 100 bp DNA ladder plus (Fermentas # SM0321); Lane A-F shows temperature gradient from 50-60°C. The primer pair used in mentioned above the lanes for each set of reaction, last lane is positive control. Primer combinations used are: 1a+1b: (PR5_59 rev + PR5_267 fwd), 2a+2b: (PR5_59 rev+ PR5_208 fwd), 3a+3b: (PR5_59 rev + PR5_271 fwd) and 4a+4b: (PR5_59 rev + PR5_256 fwd).

As seen in Fig 8 above all the primer combinations were unsuccessful in amplifying the unknown flanking regions of pearl millet tlp gene by inverse PCR using genomic DNA library constructed with *Sau*3AI digested fragments as template. No new primers were designed in this case as the primer sequences were taken from the known region of pearl millet tlp.

Despite several efforts no amplification was observed with rest of the primer combinations using different genomic libraries as template. This is accounted to the large genome size of pearl millet. This could be the possible reason for failure of this technique despite using different primer combinations and genomic DNA libraries constructed with various enzymes. To gain high sensitivity in inverse PCR, it is important that genomic DNA is digested into fragments some of which would contain gene of interest with its unknown flanking regions. Nevertheless, this cannot be ensured while amplifying unknown flanking regions of a gene because of the lack of nucleotide sequence data that does not allow spotting of restriction site of the enzyme (used to construct genomic DNA libraries) on the gene of interest. The target gene might have restriction site just outside the known sequence separating the unknown flanking region of the gene and thereby not leaving any good length of unknown sequence to be walked by the primer. Or in case of multiploid plants with large genomes, probability of adaptor ligation to the target DNA is highly reduced. Previous studies (Farrelly *et al*., 1995; Hoelzel and Green, 1992) have explained the failure of PCRs in large genomes with two most possible reasons, the first being a reduction in target DNA relative to the total content of template DNA in a single PCR serves to reduce amplification efficiency.

2.3 Genome walking with universal genome walker kit from Clontech (# 638904).

Genome walking is a simple method for amplifying unknown sequences flanking known gene sequence. The method was developed by Siebert and coworkers in 1995; genome walker universal kit from Clontech (#638904) is designed using this principle by Gwyneth Ingram and Karine Coenen. The method involves construction of genomic DNA libraries, ligation of the restriction digested DNA fragments with Genome walker adaptors (GWAs), designing of gene specific primers and primary and nested PCRs using adaptor primers (APs) and gene specific primers (GSPs). These gene specific primers were designed using the known gene sequence. Two gene specific primers, GSP 1 and GSP 2 were used to perform primary and nested PCR along with adaptor primers 1 and 2 respectively.

Genomic DNA from pearl millet seedlings was extracted by CAB method (Murray and Thompson., 1980) as explained under section 2.11.1 of materials and methods. Genomic DNA libraries were constructed as per manufacturer's instruction using (*Dra*I, *Eco*RV, *Pvu*II and *Stu*I) separately. To check the complete digestion of genomic DNA 5 µL of the digestion mixture run on a 0.8% agarose gel and the pattern was visualised in the presence of ethidium bromide under UV light. Dephosphorylation of the restriction digested purified genomic DNA was carried out as explained under section 2.13.2 of materials and methods and dephosphorylated DNA was again purified by phenol chloroform extraction as explained under section 2.13.2 of materials and methods and resuspended in 10 mM Tris HCl buffer (pH 8.0) to get end concentration of 0.1 µg DNA/µL. To construct each library, 4 µL of digested purified DNA (0.1 µg DNA/µL) was mixed with 1.9 µL Genome Walker adaptor (25 µM), 1.6 µL 10 x ligation buffer and 0.5 µL T4 DNA ligase (6 units/µL). Mixture was incubated at 16°C overnight. The reaction was stopped by incubation at 70 °C for 5 min. 1 µL of this library was used as template for primary PCR with adaptor primer 1 (AP1) and gene specific primer 1 (GSP1). Secondary PCR was carried out using 1:100 diluted primary PCR as the template with adaptor primer 2 (AP2) and gene specific primer 2 (GSP2). The 5' termini of long oligonucleotide fragment of GWA and 3'-OH group of small oligonucleotide fragment of GWA is blocked by a primary amino group to prevent an extension reaction by DNA polymerase.

2.3.1 Amplification of unknown flanking regions of pearl millet chitinase gene.

Genome walker primers, adaptor primers and gene specific primers designed to amplify the upstream and downstream region of pearl millet *chitinase* are listed in the table 3 below. The reference sequence considered for primer designing is the 482 bp pearl millet *chi*tinase (partial length) illustrated in Fig 6.

Table 3. Adaptor primers and gene specific primers to amplify unknown flanking regions of pearl millet chitinase gene (chi).

Primer	Primer Sequence	Purpose
Genome walker Adaptor	5'-GTAATACGACTCACTATAGGGCACG CGTGGTCGACGGCCCGGGCTGGT-3' -3'CCCGACCA-5	Walker adaptor
Adaptor Primer 1	5'-GTAATACGACTCACTATAGGGC-3'	Adaptor primer 1
Adaptor Primer 2	5'-ACTATAGGGCACGCGTGGT-3'	Adaptor primer 2
1a) PR3_129 GSP1	5'-GCTCCGCGTGTAGAAGTTCC-3'	Upstream *chi*
1b) PR3_72 GSP2	5'-GTTGAAGAACGCGTCGGTGA-3'	Upstream *chi*
2a) PR3_129 GSP1	5'-GCTCCGCGTGTAGAAGTTCC-3'	Downstream *chi*
2b) PR3_76 GSP2	5'-TGCCGTTGAAGAACGCGTCG-3'	Upstream *chi*
3a) PR3_230 GSP1	5'-GTGCGAACGTGGCTAATGTG-3'	Downstream *chi*
3b) PR3_447 GSP2	5'-CGCTGCAGATCTCGTGGAAC-3'	Downstream *chi*
4a) PR3_230 GSP1	5'-GCTCCGCGTGTAGAAGTTCC-3'	Downstream *chi*
4b) PR3_375 GSP2	5'-AGAACAACGCCTACTGCTCG-3'	Downstream *chi*

As the template was GC rich, 2% DMSO (higher concentration of DMSO resulted in smearing of DNA) and 0.6 M Betaine (final concentration) was added to the Master Mix after adding rest of the components. The two-step PCR reaction was carried out in thermo cycler PTC 200 (Biozym, Oldendorf, Germany) according to manufacturer's specification. 1 µL of constructed genomic DNA library (0.1 µg DNA/µL) was used as the template for primary (Template DNA used for primary PCR was constructed with the restriction enzymes *Dra*I, *Eco*RV, *Pvu*II and *Stu*I separately as per manufacturer's instruction); 1 µL of 1:100 diluted PCR product of primary PCR was used as the template for secondary PCR. Reaction condition was the same for both primary and secondary PCR, which consisted of 7 cycles of 94°C 25 sec and 72°C 3 min, 32 cycles of 94°C 25 sec and 67°C 3 min. The extremely short incubation time at 94°C was necessary to preserve the integrity of the larger genomic DNA templates required for long distance PCR in the Genome walker protocol. To analyze the primary PCR products, 10 µl were run on 1.5 % agarose gel.

Primer combinations used were AP1 + 1a, 2a, 3a or 4a for primary PCR AP2 + 1b, 2b, 3b or 4b for secondary PCR.

The primer combination AP1+1a (PR3_129) for primary PCR followed by AP2+1b (PR3_72) for secondary PCR amplified approximately 600 bp product using genomic DNA library constructed with *Stu*I digested fragments as seen in the Fig 9 below.

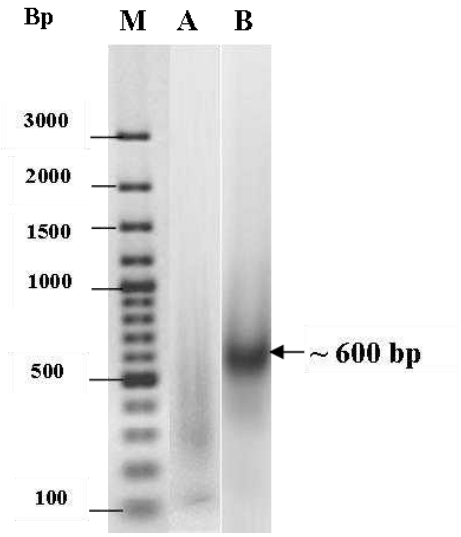


Fig 9. Amplification of approximately 600 bp product. by the primer combination AP1+1a (PR3_129) for primary PCR, followed by AP2+1b (PR3_72) for secondary PCR using genomic DNA library constructed with StuI digested fragments. Lane M is 100 bp ladder plus (Fermentas # SM0321).

Nucleotide sequence of PCR product amplified by the primer combination AP1+1a (PR3_129) for primary PCR followed by AP2+1b (PR3_72) for secondary PCR using genomic DNA library constructed with *Stu*I digested fragments.

```
  1 ATGATTGTGCACGTTGTTCATCCAGAAAGTCGAGTACTCTGTGAGCCTTGGGGCATGTGA
 61 CCGGCTTGACCTCGATCCACTTGGTGAACTTGTCGATGGCCACCAATACTCTGTTGAATC
121 CACCTGGCACAGTTGGCAGTGGCCCAATCATGTCGAGCCCACAGCAAGCAAAGGGCCAGG
181 TCGGCGGTATGGTGACTAGCTTGTAGGCTGGGACGTGTTGCTGCTTGGCAAAGTATTAAC
241 ATCCTTGGCACCGTCTGACGAGTTCTTCAGCGTTGCCCAGGGCTGTGGGCCAGTAGAAGG
301 CTCGCTGGAAGGCCTTGCTGACCAGCGTGCGAGAGGACGCGTGGTTGCCGCAGACGCCCT
361 TGTGGATCTCTTCCAAGATGTCCTTGCCTTCTTGCCTAGGAACGCACTTCATGAGTACTC
421 CAGAAGATGTGCCCCGCTTGTAAAGCTTGTCTCCGACGAGGACGTAGGACTCGGCGCGGC
481 GGATGAGCTGCTCCGCCAAGTTTTTGTCCGCGGGGACCTTCTGCTCGCTGAGGTAGTCAA
541 TGAAGGGTTGCCTCCAGTCCGCGTTNATCATCATGACCTCCT
```

Fig 10. Nucleotide sequence of 582 bp PCR product amplified by the primer combination AP1+1a for primary PCR followed by AP2+1b for secondary PCR using genomic DNA library constructed with *Stu*I digested fragments.

This 582 bp nucleotide sequence shares homology with *Pennisetum glaucum* hypothetical protein (NCBI accession number: gi|32277246 gb|CD726399).

The primer combination AP1+3a (PR3_230) for primary PCR followed by AP2+3b (PR3_447) for secondary PCR amplified approximately 800 bp product using genomic DNA library constructed with *Pvu*II digested fragments as seen in Fig 11.

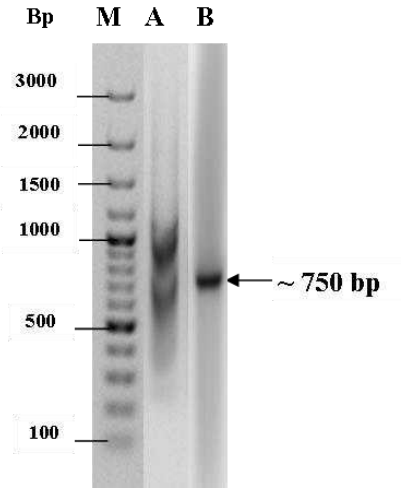


Fig 11. Amplification of approximately 750 bp PCR product by the primer combination AP1+for primary PCR 3a followed by AP2+3b for secondary PCR , using genomic DNA library constructed with *Pvu*II digested fragments. Lane M is 100 bp ladder plus (Fermentas # SM0321).

Nucleotide sequence of PCR product amplified by the primer combination AP1+3a (PR3_230) for primary PCR followed by AP2+3b (PR3_447) for secondary PCR using genomic DNA library constructed with *Pvu*II digested fragments.

```
   1 CCCTCAGCTCCACGGTCGAATCATCATGCACCTCCTGGNCCAGACTGGNTGGGATGACGC
  61 CTGGGTACAGTNGCNNTCGGNTGGCGCTCNGNTCCGGGATGGATNGCGCATGAAGCTTCG
 121 TGGACTNAAGACNNCCGCGTGAGTACTATGAGCNCNTTCTAGATCCAAGNTCTGACAGNG
 181 ANGNCAGCTNCANCTNTNTTGTCGGGACGATGTGTCGTAACTCGAGCACCNTANAACTTG
 241 TTCTCGAGTTTGCGNACTTCTNGGNAGNANNCNTNCATGAACTCCTTGANTGCGGACCCA
 301 NGACTTGATAACTTGGTTGATGACCNCTGNCNAGTCGNCGNAAACTANNAGCCNNTTGAT
 361 NNNNNGTGAAGNCNNCANGCNNAGNCCGTGTAGCAGAGNTTCNAACTCATGCTTCGATTG
 421 TTTNAGACCTTCCAGAAGATCTGNAGGACNTACTTGAGATTGNTCNNCCGTTGTCAGANA
 481 TCAGGAGNACCCACTGCACNTNCGNCCTNGAGNTTNAGNGANNCGTCTAAAANCATCANC
 541 CAATGTTCTNNNCTCTCNNACGGAGAANCCAGCTGATTCTCNCNCTANTCTTCAATGAAG
 601 TTCNACTANTNCNTGNAANTCCAAGGGNNNNTCNTGNNTTGAAANNAATNNTGANNNCNN
 661 CNNAATTTNCAAANNTCACTTCCNAATTNNTNNNANAANCCATATTNTNTNANNAAAATC
 721 CTNNTCNANNNNATNNNNNNNNNANCAT
```

Fig 12. Nucleotide sequence of 748 bp PCR product amplified by the primer combination AP1+3a for primary PCR followed by AP2+3b for secondary PCR using genomic DNA library constructed with *Pvu*II digested fragments.

The 748 bp nucleotide sequence shares homology with *Pennisetum glaucum* hypothetical protein (NCBI accession number: gi|32277246 gb|CD726399). Despite several efforts to optimize the PCR conditions, no amplification was observed with rest of the primer combinations with different genomic DNA library as template.

2.3.2 Amplification of unknown flanking regions of pearl millet thaumatin like protein gene.

PCRs were carried out similar to amplification of flanking regions of chitinase gene explained above, using gene specific primers for amplification of tlp. Gene specific primers designed to amplify the

upstream and downstream region of pearl millet thaumatin-like protein gene are listed in the table 4. The reference sequence considered for primer designing is the 482 bp pearl millet tlp (partial length) illustrated in Fig 6.

Table 4. Adaptor primers and gene specific primers to amplify unknown flanking regions of pearl millet thaumatin like protein gene.

Primer	Primer Sequence	Purpose
1a) PR5_594 GSP1	5′-GCACTGCCCCTTGAAGTA-3′	Upstream *tlp*
1b) PR5_59 GSP2	5'-CGACTTCTTCGACATCTCGCT-3'	Upstream *tlp*
2a) PR5_271 GSP1	5′-CTGCAGTGCACTGGGTA-3′	Downstream *tlp*
2b) PR5_208 GSP2	5'-GTGTTCAAGACGGACCAGTA-3'	Downstream *tlp*
3a) PR5_271 GSP1	5'-CTGCAGTGCACTGGGTA-3′	Downstream *tlp*
3b) PR5_312 GSP2	5'-GGAGTACGCGCTGAAGCAGT-3'	Downstream *tlp*

Primer combinations used were AP1 + 1a, 2a or3a for primary PCR AP2 + 1b, 2b or 3b for secondary PCR.

The primer combination AP1+2a (PR5_271) for primary PCR followed by AP2+2b (PR5_312) for secondary PCR amplified approximately 500 bp product using genomic DNA library constructed with *Eco*RV digested fragments as seen in Fig 13.

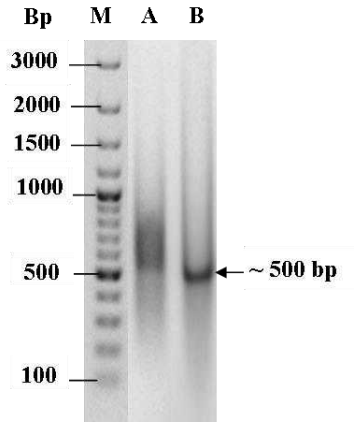


Fig 13. Amplification of 500 bp PCR product amplified by the primer combination AP1+ 2a for primary PCR followed by AP2+2b for secondary PCR using genomic DNA library constructed with *Eco*RV digested fragments. Lane M is 100 bp ladder plus (Fermentas # SM0321).

Nucleotide sequence of PCR product amplified by the primer combination AP1+2a for primary PCR followed by AP2+2b for secondary PCR using genomic DNA library constructed with *Eco*RV digested fragments.

```
  1 CTAATNCAGGACGGTCCTCATNNTAATGTCTNATGAGNTTATCTTGGNATTTGGAATCGC
 61 ATNAAAAANTCTGTATTATGCTCACTCGAGGAAAGTAGATGTGGATAGGGGCTTATACCC
121 TCAAAAAAAATACACTAGCTTCTTAAATTAAAACGTAAAAAAANGATTTTTTTTATTACG
181 TCGGGAAATAATTTTCCAGAAGAACCAATGTCCGTTAGGCACCTAATCCTTATGTCATAA
241 TAGATCCGAACACTTGCCTCGGATTGACTTCAATATATAATTGCTCCAGTAAATAACTAA
301 AAAAAAAAAAAATAGAAGGACGGNAGATAGTAAAGAAAAGGACTAATCNTAATNTCTNTC
361 TTTCAAAGATTCAATAAAAAGACAGTTGGCGGGTCNCTTTGTATGTCTTGNCCGGAAAGA
421 GGAGGACTTAATGATTNTTCGTTCNCCGGAACCAACTACAAGGN
```

Fig 14. Nucleotide sequence of 464 bp PCR product amplified by the primer combination AP1+2a for primary PCR followed by AP2+2b for secondary PCR using genomic DNA library constructed with *Eco*RV digested fragments.

This 464 bp nucleotide sequence shares homology with *Pennisetum ciliare* (Syn. *Cenchrus ciliaris*) (NCBI accession number: gi|27532828 gb|BM084919).

The primer pair AP1+3a for primary PCR followed by AP2+3b for secondary PCR amplified a 654 bp PCR product using genomic DNA library constructed with *Stu*I digested fragments as seen in Fig 15.

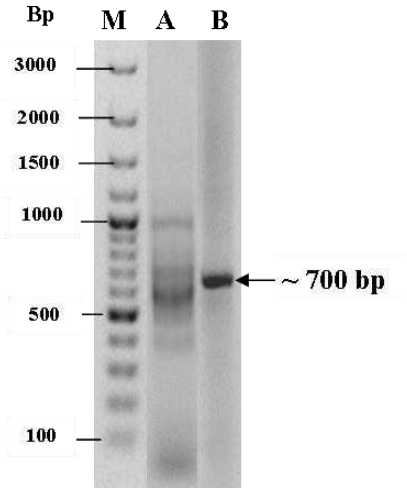


Fig 15. Amplification of 700 bp PCR product amplified by the primer combination AP1+ 3a for primary PCR followed by AP2+3b for secondary PCR using genomic DNA library constructed with *Stu*I digested fragments. Lane M is 100 bp ladder plus (Fermentas # SM0321).

Nucleotide sequence of PCR product amplified by the primer combination AP1+3a for primary PCR followed by AP2+3b for secondary PCR using genomic DNA library constructed with *Stu*I digested fragments.

```
  1 CGACANAAGNTTGCCTCCAGNTCACGATCAATCATCATGAACCTCCTGCCCAGCTGGNGG
 61 ATGNGCTGGNTACAGTGGTCGGAGGCGCCGGCTCCGGGATGGATGGCGCGTGAAGCCTCG
121 TGGACGAAGACACCCGCTTGNTACTTGAGCGCGAGTGGATCCAATCCTTCNAGAGGACGT
181 CCGCTGCAACGATTGTTGTCGCGGACGATGTGATGGAACTCGAGACCNTAGAANTTGTTC
241 TCGAGCTTGCGGACTTCTAGGCAGTAGGCGTCCATGTNCTCCTTGTTGCGGTCCCANGAC
301 TTGTTGACTTGGTTGATGACCACTGCNGAGTCGCCGTANACTAGTAGCCGCTTGATGCCC
361 AGTGAAGCCGCGAGGCGGANCCCGTGTAGGAGAGCTTCNNTACTCGGCTTCNTTGTTTGA
421 GACCTTCCANAAGATCTGCANGACGTACTTGAGTTGTTCGCCCGTTGGAGAGATCAGGAG
481 TACCCCTGCNCTTTGCNCCCTCGAGGTTTGAGTGAGCCGTCGAAGTACANCACCCANTGC
541 TCTNGCNGCTCGNTTNGGAGNNNGTNNCTGAATTCTCCCGCCACTCTNNCATGAANTCNA
601 CTANAGNTTGCAAANNTTGNTGCNCNNTGCCTGCNTTNGAAATNNATGTCTTAC
```

Fig 16. Nucleotide sequence of 654 bp PCR product amplified by the primer combination AP1+3a for primary PCR followed by AP2+3b for secondary PCR using genomic DNA library constructed with *Stu*I digested fragments.

This 654 bp nucleotide sequence shares homology with *Pennisetum glaucum* hypothetical protein (NCBI accession number: gi|32277246 gb|CD726399). Despite several efforts no amplification was observed with rest of the primer combinations using different genomic libraries as template. This is accounted to the large genome size of pearl millet which on restriction digestion generates numerous smaller DNA fragments which will be ligated to the walker adaptor. The chances of target DNA – adaptor ligation is crucial for successful amplification of unknown flanking regions of gene of interest. However in this case because of large genome size, ratio of adaptor ligated target DNA to non target DNA is very low. This could be the possible reason for failure of this technique despite using different primer combinations and genomic DNA libraries constructed with various enzymes.

2.4 Straight walk: A ligation-mediated method of genome walking (Tsuchiya *et al.,* 2009).

An important step in plant genome research is the isolation of unknown genomic regions flanking known sequences. Several polymerase chain reaction (PCR) (Abd-Elsalam, 2003) based techniques have been developed for this purpose. Although these methods have been successfully used for plants with relatively small genomes (e.g., *Arabidopsis thaliana* and *Oryza sativa*), they often fail for plants with large genomes because the genome size has a negative effect on PCR amplification (Garner, 2002). The problem of PCR-based genome walking in plants with large genomes is likely caused by the extremely low ratio of the number of target DNA molecules to other genomic sequences in the reaction mixes. For this reason, the amplification of the desired product can completely fail or be obscured due to the excessive accumulation of nonspecific products. Therefore, a genome walking method that allows both high sensitivity and high specificity is needed to obtain satisfactory results in plants with large genomes. This is an improved ligation-mediated PCR (LM–PCR) procedure that is especially effective for plants with large genomes.

For straight walking to amplify unknown genomic regions flanking known sequences of *chi* and *tlp* gene from pearl millet, adaptor primers were designed as explained by Tsuchiya and coworkers (2009). These adaptor primers include straight walk adaptors (SWA - forward and reverse), Specific adaptor primers SAP - 1 and 2) which work regardless of the genome used (Tsuchiya *et al*., 2009). The 5' termini of SWA-R1 and SWA-R2 are phosphorylated for ligation, and 3'-OH is blocked by a primary amino group to prevent an extension reaction by DNA polymerase. SWA is composed of a combination of SWA-F and either SWA-R1 or SWA-R2. The 5'-end sequences of SWA-R1 and SWA-R2 are compatible with the restriction enzyme sites of *Bam*HI, *Bgl*II, *Bcl*I, or *Sau*3AI and *Spe*I, *Nhe*I, or *Xba*I, respectively. SAP2 has a *Not*I restriction enzyme site to ease cloning.

In order to construct the genomic library, Genomic DNA from pearl millet seedlings was extracted by CAB method (Murray and Thompson., 1980) as explained under section 2.11.1 of materials and methods. Genomic DNA libraries were constructed by restriction digestion of 500 ng of genomic DNA was digested separately with 10 units of BamHI, BglII, BclI, Sau3AI, SpeI, NheI, and XbaI. All enzymatic reactions were carried out in a total volume of 10 µL at 37°C except for BclI which was incubated at 55°C. The four base 5' overhangs were partially filled by adding 1 µL of 1 mM deoxyguanosine triphosphate (dGTP) for the *Bam*HI, *Bgl*II, *Bcl*I, and Sau3AI digestions. For the *Spe*I, *Nhe*I, and *Xba*I digested DNAs 1µL of 1 mM deoxycytidine triphosphate (dCTP) was added. The end fill reactions were performed with 1 unit of Klenow enzyme (3' → 5' exonuclease activity, Klenow Fragment, Fermentas # EP0051) as explained under section XX of materials and methods. After the end fill in reaction, the Klenow enzyme was heat-inactivated by 75°C for 20 min. The partially end-filled DNA fragments generated by *Bgl*II, *Bcl*I, or *Sau*3AI were ligated to the adaptor composed of 1 µL of 100 µM SWA-F and 1 µL of 100 µM SWA-R1. The DNA fragments generated by *Spe*I, *Nhe*I, and *Xba*I were ligated to the adaptor composed of 1 µL of 100 µM SWA-F and 1 µL of 100 µM SWA-R2. Ligation was carried out at 16°C overnight. To check the complete digestion of genomic DNA 5 µL of the digestion mixture run on a 0.8% agarose gel and the pattern was visualised in the presence of ethidium bromide under UV light. Dephosphorylation of the restriction digested purified genomic DNA was carried out as explained under section 2.13.2 of materials and methods and dephosphorylated DNA was again purified by phenol chloroform extraction as explained under section 2.13.2 of materials and methods and resuspended in 10 mM Tris HCl buffer (pH 8.0) to get end concentration of 0.1 µg DNA/µL.

PCR conditions

PCR conditions for primary PCR were as follows: hot-start denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 65°C for 30 s, and 68°C for 5 min. The PCR conditions for secondary PCR were the same as for the first PCR except that only 30 thermal cycles were performed and 1µl of 100-fold diluted primary PCR products was used as template of secondary PCR.

2.4.1 Amplification of the unknown flanking regions of pearl millet chitinase gene.

Known sequence-specific primers (KSPs) designed to amplify the unknown flanking regions of pearl millet chitinase gene (chi) are compiled in the table 5.

Table 5. Straight Walk adaptor (SWA) primers and specific primers for the amplification of unknown flanking regions of chitinase gene.

Primer	Primer Sequence	Purpose
SWA-F	5'-CGCAGGCTGGCAGTCTCTTTA GGGTTACACGATTGCTT-3'	Straight walking forward primer
SWA-R1	5'-ATCAAGCAATCGTGT-3'	Straight walking reverse primer 1
SWA-R2	5'-TAGAAGCAATCGTGT-3'	Straight walking reverse primer 2
SAP1	5'-CGCAGGCTGGCAGTCTCTTTAG-3'	Specific adaptor primer 1
SAP2	5'-ATGCGGCCGCTCTCTTTAGGGTT ACACGATTGCTT-3'	Specific adaptor primer 2
1a)PR3_129 KSP1	5'-GCTCCGCGTGTAGAAGTTCC-3'	Upstream *chi*
1b)PR3_72 KSP2	5'-GTTGAAGAACGCGTCGGTGA-3'	Upstream *chi*
2a)PR3_129 KSP1	5'-GCTCCGCGTGTAGAAGTTCC-3'	Downstream *chi*
2b)PR3_76 KSP2	5'-TGCCGTTGAAGAACGCGTCG-3'	Upstream *chi*
3a)PR3_230 KSP1	5'-GTGCGAACGTGGCTAATGTG-3'	Downstream *chi*
3b)PR3_447 KSP2	5'-CGCTGCAGATCTCGTGGAAC-3'	Downstream *chi*
4a)PR3_230 KSP1	5'-GCTCCGCGTGTAGAAGTTCC-3'	Downstream *chi*
4b)PR3_375 KSP2	5'-AGAACAACGCCTACTGCTCG-3'	Downstream *chi*

Primer combinations used were SAP1 + 1a, 2a,3a or 4a for primary PCR SAP2 + 1b, 2b, 3b or 4b for secondary PCR. Despite several efforts no amplification was observed with any of these primer combinations mentioned above with all the different genomic libraries as template. This is accounted to the large genome size of pearl millet which on restriction digestion generates numerous smaller DNA fragments which will be ligated to the walker adaptor. The chances of target DNA – adaptor ligation is crucial for successful amplification of unknown flanking regions of gene of interest. However in this case because of large genome size, ratio of adaptor ligated target DNA to non target DNA is very low. This could be the possible reason for failure of this technique despite using different primer combinations and genomic DNA libraries constructed with various enzymes.

2.4.2 Amplification of the unknown flanking regions of pearl millet tlp gene.

Known sequence-specific primers (KSPs) designed to amplify the unknown flanking regions of pearl millet thaumatin like protein gene (tlp) are compiled in the table 6.

Table 6. Adaptor primers and gene specific primers to amplify unknown flanking regions of pearl millet thaumatin like protein gene (tlp).

Primer	Primer Sequence	Purpose
1a)PR5_594 KSP1	5'-GCACTGCCCCTTGAAGTA-3'	Upstream *tlp*
1b)PR5_59 KSP2	5'-CGACTTCTTCGACATCTCGCT-3'	Upstream *tlp*
2a)PR5_271 KSP1	5'-CTGCAGTGCACTGGGTA-3'	Downstream *tlp*
2b)PR5_208 KSP2	5'-GTGTTCAAGACGGACCAGTA-3'	Downstream *tlp*
3a)PR5_271 KSP1	5'-CTGCAGTGCACTGGGTA-3'	Downstream *tlp*
3b)PR5_312 KSP2	5'-GGAGTACGCGCTGAAGCAGT-3'	Downstream *tlp*

PCR conditions for primary PCR were as follows: hot-start denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 65°C for 30 s, and 68°C for 5 min. The PCR conditions for secondary PCR were the same as for the first PCR except that only 30 thermal cycles were performed and 1µl of 100-fold diluted primary PCR products was used as template of secondary PCR. Primer combinations used were SAP1 + 1a, 2a or 3a for primary PCR SAP2 + 1b, 2b or 3b for secondary PCR. The primer pair AP1+1a for primary PCR followed by AP2+1b for secondary PCR amplified approximately 1 kbp PCR product using genomic DNA library constructed with BglII digested fragments as seen in Fig 17.

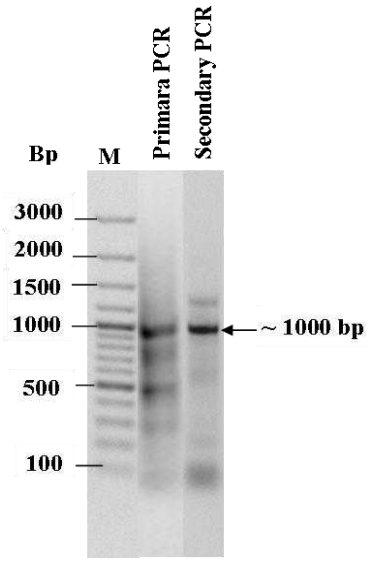


Fig 17. Amplification of approximately 1 kbp PCR product amplified by the primer combination AP1+ 1a for primary PCR followed by AP2+1b for secondary PCR using genomic DNA library constructed with BglII digested fragments. Lane M is 100 bp ladder plus (Fermentas # SM0321).

Nucleotide sequence of 957 bp PCR product amplified by the primer combination AP1+1a for primary PCR followed by AP2+1b for secondary PCR using genomic DNA library constructed with *Bgl*II digested fragments.

```
  1 AGAGGTNCTCAGTCCACGTCGATCATCATGACCTCCTGCCCAGCTGGTGGATGNGCTGGG
 61 ACAGTGGTCGGTGGCGCCGGCTCCGGGATGGATGGCGCGTGAAGCTCNTGGACGAAGACA
121 CCCGCTGGTACTTGAGCGCGNGTNGATCCAAGCTTCGAGAGGACGTCCGCTGCAACGTTG
181 TTGTCGCGGACGATGTGATGGAACTCGAGACCATAGAANTTGTTCTCGAGCTTGCGGACT
241 TCTAGGCAGTAGGCGTCCATGTTCTCCTTGTTGCGGTCCCAGGACTTGTTGACTTGGTTG
301 ATGACCACTGCGGAGTCGCCGTAGACTAGTAGCCGCTTGATGCCCAGTGAAGCCGCGAGG
361 CGGAGCCCGTGTAGGAGAGCTTCGTACTCGGCTTCGTTGTTTGAGACCTTCCAGAAGATC
421 TGCAGGACGTACTTGAGTTGTTCGCCCGTTGGAGAGATCAGGAGTACCCCTGCNCCTGCG
481 CCCTCGAGGTTGAGTGAGCCGTCAAAGTACATCACCCAATGCTCTGGTCGCACGGTGGGA
541 GTAGGTAGCTGNTTCTCCCGCCACTCTGCCATGAAGTCGACTAGTGCNTGCGACTTGATG
601 GCTGTGCGTGGCTTGAAGTCNATGGTGAGGGCGCCGAGNTCGACNGCCCACTTGGAGATT
661 CTTCCAGTNGCATCTTGGTTCCTCAGGATGTCGCCGAGCGGNTAGTCNGTGACAACGGAA
721 ACCNANTACTCCTGGAAGTAGTGTCGTANCTTCCNCGAGGTGATGAGNACTGCNNTACAG
781 NANCTTCTGCACNGGNTGANANCNAGCCTTGNANTCTAANAGAACTCNNTTGANNAAANN
841 AANTGGNNNTTGACNGNNANGNNTGNCNTNTTNTTCCTTCCAANACANTGGANTANNNNC
901 CNCTGATTATNCCNACNAGTAAANCANTATNCTTTTTTTAANNNNNNNNNNNNNNNNN
```

F8ig R. Nucleotide sequence of 957 bp PCR product amplified by the primer combination AP1+1a for primary PCR followed by AP2+1b for secondary PCR using genomic DNA library constructed with *Bgl*II digested fragments.

This 757 bp nucleotide sequence shares homology with *Pennisetum glaucum* hypothetical protein (NCBI accession number: gi|32277246 gb|CD726399).

The primer pair AP1+2a for primary PCR followed by AP2+2b for secondary PCR amplified approximately 1 kbp PCR product using genomic DNA library constructed with Sau3AI digested fragments as seen in Fig 19.

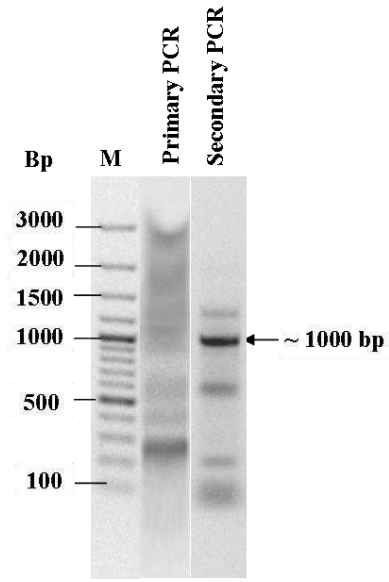


Fig 19. Amplification of 968 bp PCR product amplified by the primer combination AP1+ 2a for primary PCR followed by AP2+2b for secondary PCR using genomic DNA library constructed with *Sau*3AI digested fragments. Lane M is 100 bp ladder plus (Fermentas # SM0321).

Nucleotide sequence of 968 bp PCR product amplified by the primer combination AP1+2a for primary PCR followed by AP2+2b for secondary PCR using genomic DNA library constructed with *Sau*3AI digested fragments.

```
  1 CAACANAGGTGCCTCAGTCCACGTCGATCATCATGACCTCCTGNCCAGCTGGTGGATGCG
 61 CTGGGACAGTGGTCGGTGGCGCCGGCTCCGGGATGGATGGCGCGTGAAGCTCNTGGACGA
121 AGACACCCGCTGGTACTTGAGCGCGNGTNGATCCAAGCTTCGANAGGACGTCCGCTGCAA
181 CGTTGTTGTCGCGGACGATGTGATGGAACTCGAGACCATAGAANTTGTTCTCGAGCTTGC
241 GGACTTCTAGGCAGTAGGCGTCCATGTTCTCCTTGTTGCGGTCCCAGGACTTGTTGACTT
301 GGTTGATGACCACTGCGGAGTCGCCGTAGACTAGTAGCCGCTTGATGCCCAGTGAAGCCG
361 CGAGGCGGAGCCCGTGTAGGAGAGCTTCGTACTCGGCTTCGTTGTTTGAGACCTTCCAGA
421 AGATCTGCAGGACGTACTTGAGTTGTTCGCCCGTTGGAGAGATCAGGAGTACCCCTGCAC
481 CTGCGCCCTCGAGGTTGAGTGAGCCGTCAAAGTACATCACCCAATGCTCTGGTCGCNCGG
541 TNGGAGTAGGTAGCTGATTCTCCCGCCACTCTGCCATGAAGTCGACTAGTGCNTGCNACT
601 TGATGGCTGTGCGTGGCTTGAAGTCNATGGTGAGGGCGCCGAGNTCGACNGCCCACTTGG
661 AGATTCTTCCAGTAGCATCTTGGTTCCNCAAGATGTCNCCNAGCGGGTAGTCNGTGACNA
721 CNGAAATCNAGTACTCCTGGAAGTAGTGTCNTANCTTCCNCGANGGTGATGAGTACTGCN
781 TACANNANCTTCTGCNCNGGCTGATAACNAGCCTTTGNATTCTAAAAGAAACCTCNCTNA
841 CAAAATAAAATTGNNCNCTNGACNGNNTAAGNNTGNNCNTNTTNCTGCCNNTCNANAANN
901 ATGGNNNNACTGACCACTTNANTANTCNCANNNAGNTAAACATANNNTTTTNNNNNNNTA
961 NNNNNAAN
```

Fig 20. Nucleotide sequence of 968 bp PCR product amplified by the primer combination AP1+2a for primary PCR followed by AP2+2b for secondary PCR using genomic DNA library constructed with *Sau*3AI digested fragments.

This 968 bp nucleotide sequence shares homology with *Pennisetum glaucum* hypothetical protein (NCBI accession number: gi|32277246 gb|CD726399).

Conclusion

Despite several efforts no amplification was observed with rest of the primer combinations using different genomic libraries as template. This is accounted to the large genome size of pearl millet which on restriction digestion generates numerous smaller DNA fragments which will be ligated to the walker adaptor. The chances of target DNA - adaptor ligation is crucial for successful amplification of unknown flanking regions of gene of interest. However in this case because of large genome size, ratio of adaptor ligated target DNA to non target DNA is very low. This could be the possible reason for failure of this technique despite using different primer combinations and genomic DNA libraries constructed with various enzymes.

Pennisetum glaucum hypothetical protein (NCBI accession number: gi|32277246 gb|CD726399) with which the amplicons share homology shares strong homology with retrotransposon from *Sorghum bicolor* (NCBI Reference Sequence: XP_002447610). Retrotransposons are known to integrate to genome and therefore it was difficult to clone the several PCR products mentioned in this doctoral study. It is estimated that retrotransposons contribute to approximately 50% of whole genome in case of plats with large genome. Because of this the ratio of target DNA to non target DNA gets highly reduced leading to failure of PCRs. The success of primer based genome walking techniques also depends on the presence of restriction site for the restriction enzyme used. The farther the restriction site longer is the distance walked by the primer. However the presence of restriction site cannot be predicted for the unknown flanking sequence of gene of interest the success rate is limited.

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Erklärung zur Dissertation

Hierdurch erkläre ich, dass ich meine Dissertation mit dem Titel A study of pathogenesis-related (PR) proteins from pearl millet (*Pennisetum glaucum*) after infection with the downy mildew pathogen (*Sclerospora graminicola*). selbständig verfasst und die benutzten Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfeleistungen herangezogenen Institutionen vollständig angegeben habe. Die Dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere Prüfungsarbeit verwendet.

Hannover, den 30.09.2011 Manjula Mundakana

Curriculum Vitae

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Doctoral work:

Completed doctoral study titled 'Study of pathogenesis related proteins in Pearl millet (*Pennisetum glaucum*) after infection with the downy mildew pathogen (*Sclerospora graminicola*)' to the faculty of natural sciences, Leibniz University of Hannover, Germany. Doctoral work involved purification of pearl millet chitinase and thaumatin -like protein. Characterization of the purified proteins was carried out along with amplification, cloning and sequencing of genes encoding these PR proteins.

Work experience in detail

08/2011 till date	Working as Guest faculty at DOS in Biotechnology University of Mysore.
02/2011 to 04/2011	Worked as a guest faculty and taught Post graduate students at Pondicherry Central University, Pondicherry, India.
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06/2007 to 09/2009	**Worked as PhD student /DAAD scholarship holder** at Institute for Botany, **Leibniz University of Hannover.**
02/2004 to 02/2007	Worked as a **Junior Research Fellow** at 'DOS in Biotechnology' University of Mysore, India, with **UGC fellowship (NET, JRF).**
02/2007 to 05/2007	Worked as a **Senior Research Fellow** (UGC) at 'DOS in Biotechnology' University of Mysore, India.

Education

Sl.No	Name of Examination	Name of Institute	Name of University	Year	Marks Scored
2.	Master of Science	Dept of Applied Botany	Mangalore University	2002	73%
3.	Bachelor of Science	SDM College Ujire, D.K.	Mangalore University	2000	84%

Research Interest

- Purification and characterization of enzymes.
- Interested in life science research with special interest on molecular plant pathology, namely physiological changes and related gene expression in diseased condition.
- Gene cloning, overexpression of recombinant proteins and characterization.
- Working with knock out mutants to study the gene function.

Techniques known

- Protein purification and characterization; experienced in performing electrophoresis, Chromatographic separation of proteins, FPLC, and immunological studies.
- Purification of nucleic acids and genomic DNA library construction PCR, Gene isolation and cloning, Southern and Northern Blotting with radioactive probes.
- Sequence analysis using different molecular biology tools and able to work with different molecular biology databases.
- Biochemical (spectroscopic) assys, standardization of protocols.
- Skilled in Microscopy, identification, isolation and pure culture of Fungi.
- Carrying out plant histological and anatomical studies, plant tissue culture.

Languages known

German	**Fluent** (Completed 'Mittelstufe -1')
English	**Excellent**
Hindi and Kannada	**Excellent** (Kannada - Mother tongue).

Computer knowledge

MS- Word und Internet	Excellent
MS- Excel	Excellent
MS-PowerPoint	Excellent
Molecular biology databases	Very good

Nucleotide sequence submitted to NCBI:

Pearl millet chitinase sequence generated in the doctoral study has been submitted to **NCBI** and the accession number (gi|296011301 gb|ADG65345) was obtained.