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Molecular cloning and expression of chitinase cDNA of tea plant [*Camellia sinensis*] for its functionality in plant defense system

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Abstract

Tea is one of the important beverages of the world. Darjeeling tea is considered as 'champagne of tea' due to its flavour and aroma. Tea drinkers prefer quality tea but due to some disease infestation quality cannot be maintained at per. Tea plants have own defense mechanisms to combat diseases which are caused by either bacteria or fungal pathogens. Defense mechanisms are excited/induced in response to pathogenic attack and initiate to accumulate some pathogenesis related (PR) proteins like β-glucanase, chitinases and others. Tea chitinase1b gene (909 bp) was constructed *in vitro* after codon optimization and cloned into pPIC9K vector for multicopy insertion and efficient expression at the histidine locus to generate Mut^+ phenotype. Recombinant plasmid vector pPIC9K carrying chitinase1b insert was introduced into host cell *Pichia pastoris* strain GS115 through electroporation. Transformed host cells were placed on minimal dextrose (MD) and minimal methanol (MM) agar plates for selection of Mut^+ transformants. Transformed colonies were screened for multicopy integration into the *Pichia* genome using YPD agar plates with different concentration of geneticin (0.25 mg/ml to 4 mg/ml). Colony PCR was performed to confirm the insert integration into the genome of *P. pastoris* GS115 using AOX 5′ forward and AOX 3′ reverse primer to amplify chitinase1b from *Pichia* GS115 genome. The expected size of the PCR product was ~1400 bp. Positive clones of chitinase1b and negative control (vector backbone control) was inoculated in 25 ml BMG medium and incubated 30˚C with 250 rpm agitation. Cells were pelleted at OD_{600} ~2.0 and re-suspended in 100 ml of BMM and MM media for gene expression induction at different conditions (25˚ C or 30˚ C). Methanol was added to media every 24 h to a final concentration of 0.5%. Supernatant was collected at different time intervals and analyzed for chitinase1b protein expression on 12% SDS-PAGE gel. In comparison to the negative control sample (no band

observed), two prominent bands were visible at~ 32 kDa and ~40 kDa in the gel. Chitinase1b protein band ~ 32 kDa was observed during 48 h-72 h of expression on MM media at 30˚C temperature, which is the expected chitinase protein without PTM. Other band in the molecular size of 40 kDa was observed during 60 h of expression on BMM media at 25˚ C. The higher molecular weight may be due to posttranslational modification of chitinase during controlled expression. Chitinase protein sequence was confirmed by Mass-spectrometry. The knowledge gained in the present investigation will help to design the tea defense system in future.

Keywords: Tea, chitinase gene cloning, gene expression in *Pichia pastoris*, SDS-PAGE.

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Introduction

Tea is the oldest and non-alcoholic caffeine containing beverage producing from the young shoot tips (two and a bud) of tea plant [*Camellia sinensis* (L.) O. Kuntze]. The genus *Camellia* belongs to the family Theaceae. There are several major diseases of tea plant that threatens production such as red rot, blister blight, root rot, caused by several pathogens. The pathogen infects only the economically important, tender shoots which lead to enormous crop loss, estimated up to 50% depending on the severity of infection. Plant has its own defense strategies they produce pathogenesis-related protein (PR-proteins)/enzymes and elicited other mechanisms to combat the pathogenic infection. Among these PR proteins, chitinases are the enzymes belong to a family of pathogenesis-related (PR) proteins, which are over-expressed by plants in response to a pathogen attack (Giazinazzi, 1987; Boller *et al.*, 1983; Collinge *et al.*, 1993; Legrand *et al*, 1987). Chitinases (PR-3) catalyze the hydrolysis of the β-1,4 linked N-acetylglucosamine polymers that form chitin chains, a major component of fungal cell walls. Researchers have tried to isolate different chitinase gene from different plant species to use it in crop improvement program in order to increase the plant's own immune system (Sekeli *et al*., 2003; Eilenberg *et al.*, 2006; Metraux *et al*., 1989; Roby *et al*., 1991; Samac *et al.*, 1990; Datta *et al.*,1999; Broglie *et al*., 1991; Kumar *et al*., 2004; Schlumbaum *et al.*, 1986; Huynh *et al.*, 1992; Wu *et al.*, 1994; Roy and Chakraborty 2009; 2012). Members of the chitinase gene family are found in all plants, which express inducibly as PR-3 proteins

and constitutively in tissues vulnerable to pathogen attack (Samac *et al.,* 1990; Collinge *et al*., 1993). Pritsh *et al.,* (2000) studied the pattern of transcripts accumulation of six-typical defense response genes, POX (peroxidase), PR-1, PR-2 (β-1, 3-glucanase), PR-3 (chitinase), PR-4 & PR-5 (thaumatin like protein) in spray-inoculated panicles of both the susceptible cv. Wheatson and the resistant cv. Sumai3 of wheat. Chitinase gene specific transcript accumulation was studied in tea after induction with methyl jasmonate (Roy and Chakraborty, 2012). The acidic and basic chitinase genes have isolated and characterized fully in *Arabidopsis thaliana* (Samac *et al*., 1990). Genes for chitinases have been analyzed at the molecular evolutionary level in maize and family poaceae (Wu *et al.,* 1994; Peter Tiffin, 2004). Plant-pathogen co-evolution is analyzed in *Arabis* sp, in relation to class-I chitinases (Bishop *et al*., 2000). Sekeli *et al.* (2003) have isolated and cloned chitinase-I gene from winged bean seed and characterized its structure in relation to defense system. Samac *et al.* (1990) have critically demonstrated the isolation and cloning of chitinase gene in model plant *Arabidopsis thaliana*. Datta *et al.* (1999) has shown that transgenic rice plant over expressing PR-5 gene can be more resistance against sheath blight disease pathogen *Rhizoctonia solani.*

So, there is no report on chitinase gene isolation and cloning from tea plants and no initiative has been taken for the improvement of Indian tea crop by manipulating the defense protein like chitinase. The present investigation is carried out to clone the chitanase1b gene in appropriate vector and small scale trial for expression in *Pichia pastoris.*

Material and Methods

The Chitinase 1b gene was codon optimized and synthesized The construct was sequenced using vector specific primers. The sequencing result was found to be positive while Blast with the NCBI database. Total length of the Chitinase1b gene construct was 909 bp (given below).

FASTA >caagagcaatgtggtaagcaagctggaggaaaactatgcccaggagggctgtgttgtagccaatttgggttttg
tggcagcactccagactactgctctaataactgccagagccaatgtgggggatcccccgccactccgtcaacgccaacaccca
ctcctagcggtggtggcggtgacattagctctcttatcagcagagatctcttcaatcagatgttgaagcaccgggacgatgctagt
tgccctggaaaagggttctacacttatgatgcttttgtagctgctgccaagtcttttgggggttttggtaccaccggggatactgaca
ctcgtaagagagagattgccgcttttctagctcaaacttcgcatgaaactactggcgggtggccaagtgcgccagatggaccata
tgcatggggatattgccatgtacgggaacaaaaccctgctggggactattgtagtgcaagtcaagaatggccctgtgctcctggt
aaacaatactatggtcgtggtcccatccaaatttcacacaactacaactacggtccagctgggaaagctatagggtctgatctgtt
gggcaaccctgacttagtcgcaactgacacaaccatatctatcaagacagcattctggttctggatgacaccgcaatccccaaaa

ccctcgtgccacgatgtcaacacgggcggttggacaccatctagtgcggacacctcagcgggtcgggtccctggctacggtgt
gattacaaacatcatcaatggcggacttgaatgtggcaaaggctctaatgcacaggcagaggaccggatcggattctataaaag
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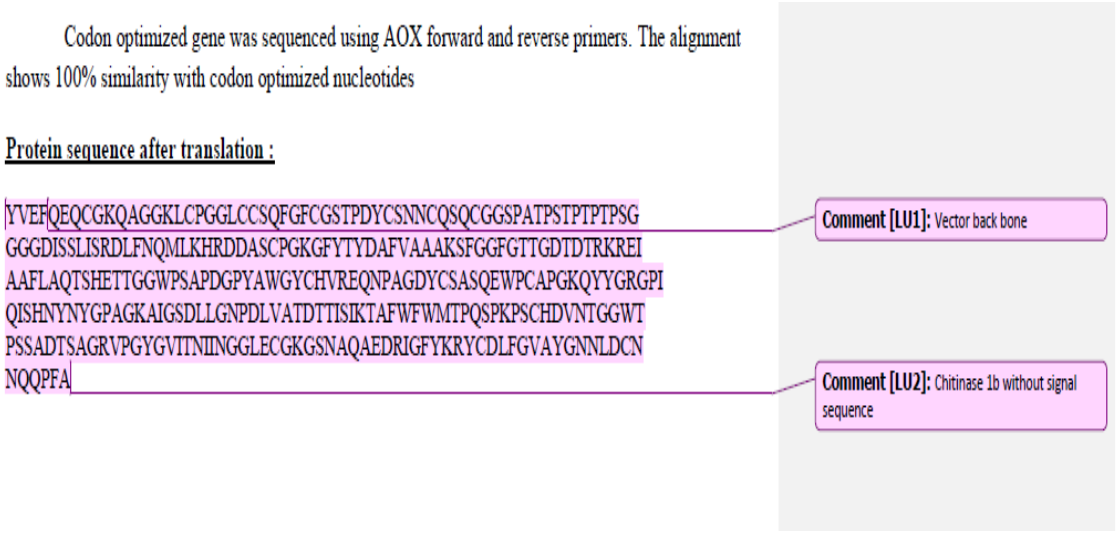

Gene cloning in vector pPIC9K

The plasmid vector pPIC9K was chosen for the present work because it is functional in foreign gene expression in eukaryotic system such as *Pichia pastoris* strain GS115. Main features of the pPIC9K vector (Fig. 1) includes: 9276 bp fusion vector, four unique restriction sites for cloning in frame with the α-factor secretion signal: *Sna*B I, *Eco*R I, *Avr* II, *Not* I. Secreted expression of the gene using the α-factor secretion signal has been attached with this vector. Dominant histidine marker *HIS4* is another criterion for selection in *Pichia.* Multiple copy integration of recombinant genes in *Pichia* has been demonstrated to increase expression of the desired cloned gene. Selection *in vivo* method utilizes resistance to geneticin (G418 sulfate) to screen for possible multicopy inserts. The chitinase1b gene was cloned in pPIC9K vector (Invitrogen, USA) which is a *Pichia pastoris* based vector for multicopy integration and secreted protein expression. Chitinase 1b gene has been inserted at *HIS4* region to make it mut^+ and before that it was cut and linearized with *Sal* I, which destroy the HIS4 function but keeping methanol utilization gene AOX intact (which generates Mut^+ in GS115).

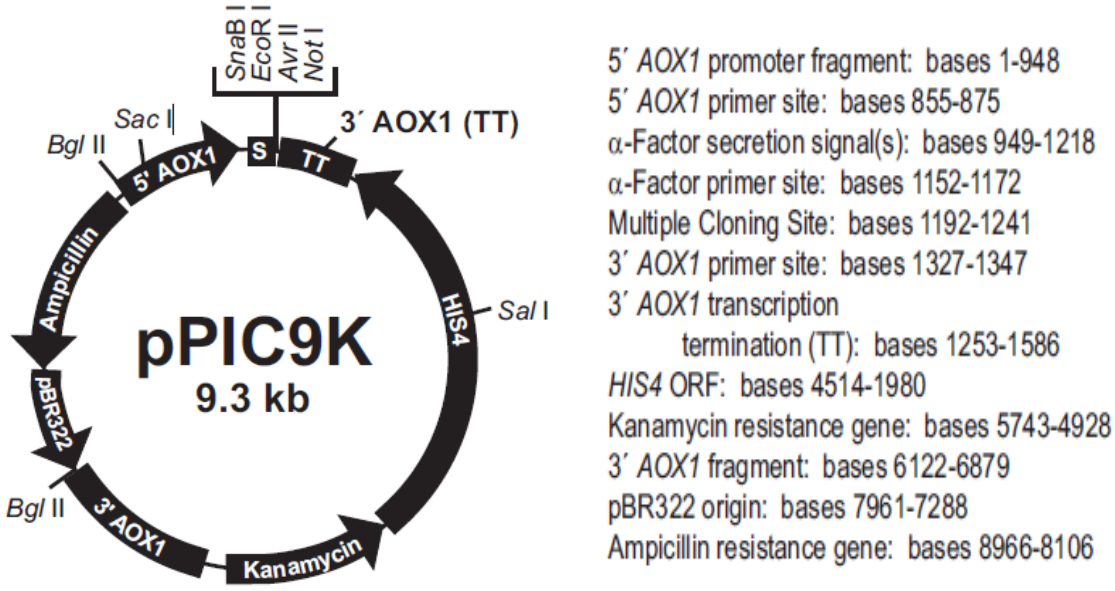

Figure 1. Main genetic features of the cloning vector pPIC9K

pPIC9K contains the bacterial kanamycin gene (*kan* from Tn*903*) that confers resistance to geneticin in *Pichia*. The level of geneticin resistance roughly depends on the number of kanamycin genes integrated. A single copy of pPIC9K integrated into the *Pichia* genome confers resistance to geneticin to a level of ~0.25 mg/ml. Multiple integrated copies of pPIC9K can increase the geneticin resistance level from 0.5 mg/ml (1–2 copies) up to 4 mg/ml (7–12 copies). Because of the genetic linkage between the kanamycin gene and the "expression cassette" (P*AOX1* and inserted gene of interest), it can infer that geneticin resistant clones contain multiple copies of the gene. Secreted protein expression may increase because of a gene dosage effect. Thus, the presence of the *kan* gene on pPIC9K can be used as a tool to detect pPIC9K transformants that harbour multiple copies of foreign (chitinase 1b) gene (Fig.2).

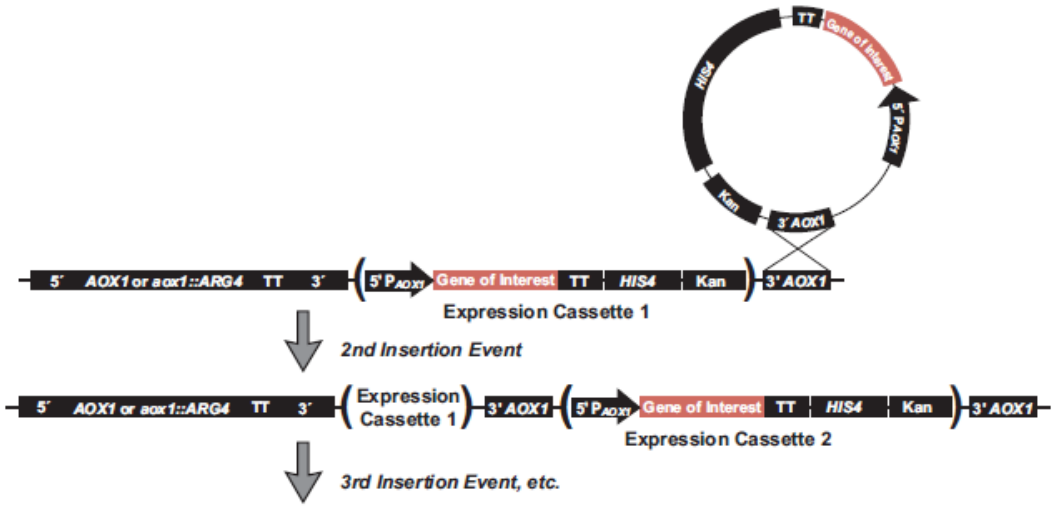

Figure 2. Multiple gene insertion events through recombination and integration in *Pichia* genome.

Media for Pichia pastoris

YPD or YEPD (Yeast Extract Peptone Dextrose Medium, 1 liter)

1% yeast extract, 2% peptone, 2% dextrose (glucose). Dissolved 10 g yeast extract and 20 g of peptone in 900 mL of water. Added 20 g of agar and 100 mL of 10 X D solutions for making YPD plates. Autoclaved for 20 minutes at 121°C. Stored the YPD plates at 4°C.

10X D (20% Dextrose)
Dissolved 200 g of D-glucose in 1,000 mL of water. Autoclave for 15 minutes or filter sterilize.

10X M (5% Methanol)
Mixed 5 mL of methanol with 95 mL of water. Filter sterilized and stored at 4°C.
10X GY (10% Glycerol)
Mixed 100 mL of glycerol with 900 mL of water. Sterilize by autoclaving and stored at room temperature.

Transformation into Pichia pastoris (GS115) by electroporation

Genotype of Pichia pastoris

The *Pichia pastoris* host strain GS115 has a mutation in the histidinol dehydrogenase gene (*his4*) that prevents it from synthesizing histidine. All expression plasmids pPIC9K carry the *HIS4* gene that complements *his4* in the host, so transformants can be selected for their ability to grow on histidine-deficient medium.

Preparing host cell for Electroporation

Grow host cell, *Pichia pastoris* in YPD medium in a 50 mL conical flask at 30°C overnight. Inoculate 500 mL of fresh medium in a 2 liter flask with 0.1–0.5 mL of the overnight culture. Grow the culture overnight again to an OD_{600} ~1.3-1.5. Centrifuge the cells at 1,500 g for 5 minutes at 4°C. Re-suspend the pellet with 500 mL of ice-cold, sterile water. Centrifuge the cells as above and re-suspend the pellet with 250 mL of ice-cold, sterile water. Centrifuge the cells and re-suspend the pellet in 20 mL of ice-cold 1 M sorbitol. Centrifuge the cells and re-suspend the pellet in 1 mL of ice-cold 1 M sorbitol for a final volume of approximately 1.5 mL (according to manufacturer protocol, Invitrogen, USA).

Transformation through electroporation

Above re-suspended pellet cells about 80 µL (with 5-20 µg of linearized DNA) was mixed with 10 µL TE Buffer and transferred them to an ice-cold 0.2 cm electroporation cuvette. Incubated the cuvette with the cells on ice for 5 minutes. Then Pulse the cells according to the parameters for yeast (*Saccharomyces cerevisiae*) suggested by the manufacturer of the specific electroporation device (BioRad GenePulser). Immediately add 1 mL of ice-cold 1 M sorbitol to the cuvette. Transferred the cuvette contents to a sterile microcentrifuge tube. Spread 200–600 µL of aliquots on minimal dextrose (MD) plates. Incubated at 30°C until colonies appeared. Colonies were screened for multicopy integration using (Yeast Extract Peptone) YPD agar plates with different concentration of geneticin.

Screening for Mut^+/Mut^S transformants

Individual colonies were platted on minimal dextrose (MD) plates and minimal methanol (MM) plates for screening Mut^+ (methanol fast utilizer) and Mut^s (methanol slow utilizer) transformants for various time durations (48 h and 80 h).

Colony PCR for confirming insert in the selected clones

Colony PCR was performed to confirm the insert integration into the genome of *Pichia pastoris* GS115. Cells were lysed by treating with zymolase to release genomic DNA. This was used as template for PCR. AOX 5′ forward primer and AOX 3′ reverse primers were used to amplify the Chiti1b from the *Pichia pastoris* GS115 genome. The forward primer and reverse primer bind at 379 bp upstream and 115 bp downstream of the 909 bp Chiti1b gene, respectively. The expected size of PCR amplification product is~1400bp.
Set up a 50 µL PCR for a hot start:

10X Reaction Buffer	5 µL
25 mM MgCl2	5 µL
25 mM dNTPs	1 µL
5′ *AOX1* primer (10 pmol/µL)	1 µL
3′ *AOX1* primer (10 pmol/µL)	1 µL
Sterile water	27 µL
Cell lysate	5 µL
Total Volume	45 µL

Placed the solution in the thermocycler (BioRad Mini MJ) and incubated at 95°C for 5 minutes. Added 5 µL of a 0.16 U/µL solution of *Taq* polymerase (0.8 units). Cycle 30 times using the following parameters:

Denaturation	95°C 1 minute
Annealing	54°C 1 minute
Extension	72°C 1 minute

Included a final extension of 7 minutes at 72°C. Analyzed a 10 µL aliquot by 1% agarose gel electrophoresis (Fig.7).

Small scale trials for optimization of Chi1b gene expression conditions

Positive clones of Chitinase1b and negative control (vector backbone control) was inoculated in 25 ml of BMG medium (Buffered minimal glycerol media) and incubated at 30ºC with 250 rpm agitation. Cells were pelleted at 1500-3000 g, for 5 min at OD_{600}~2.0 and re-suspended in 100 ml of BMM medium (Buffered minimal methanol media) and MM (Minimal methanol media) media to a final OD_{600}~1.0 for gene expression induction and conditions (four conditions) as mentioned below.

Condition 1: BMM media at 25ºC; Condition 2: BMM media at 30ºC
Condition 3: MM media at 25ºC; Condition 4: MM media at 30ºC

BMM (Buffered Minimal Methanol) (1 liter)

100 mM potassium phosphate, pH 6.0 (Combine 132 mL of 1 M K_2HPO_4, 868 mL of 1 M KH_2PO_4 and confirm that the pH = 6.0 (if the pH needs to be adjusted, use phosphoric acid or KOH). 1.34% YNB, 4×10^{-5}% biotin, 0.5% methanol. Add 100 mL 10X GY for BMG. Autoclaved 700 mL water for 20 minutes. Cool to room temperature, then add the following and mixed well: 100 mL 1 M potassium phosphate buffer, pH 6.0, 100 mL 10X YNB (Yeast Nitrogen base), 2 mL 500X B (Biotin), 100 mL 10X M.

Methanol was added to media every 24 hrs to a final concentration of 0.5% to maintain induction. Sample time points- 6hrs, 12hr, 24hr, 36hr, 48hr, 60hr, 72hr, 84hr and 96hr. 1ml of culture was sampled each time and centrifuged (5000 rpm) to collect supernatant. Supernatant was concentrated and analyzed for expression on 12% SDS-PAGE gel.

Gel electrophoresis for protein separation

The 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was prepared according to protocol Laemmli (1970). Vertical slab gel, containing 12% (w/v) resolving gel and 5% stacking gel concentration of acrylamide, were run at a constant current of 15 mA for 5 h. Gel was stained in 0.25% Coomassie-Brilliant blue R-250, dissolved in 50% methanol and 10% acetic acid in distilled water. The gel were destained for 5 h in 5% methanol and 7% acetic acid in distilled water and documented the gel for analysis.

Chitinase enzyme activity

Chitinase enzyme activity was assayed spectrophotometrically using colloidal chitin as the substrate according to Sun *et al* (2006). Enzyme activity was measured by quantifying the reducing end product N-acetamino-glucose product from colloidal chitin. Enzyme solution in buffer was taken in different vial 1 ml each (0.1 M sodium phosphate buffer) for different pH gradients (pH 5, 5.5, 6, 7) and incubated at 25˚C, 30˚C, and 35˚C. Reaction was ended by adding 2 ml dinitrosalicylic acid reagent and heating in boiling water for 5 min. Centrifuged at 6000 rpm for 10 min to collect the supernatant and subjected to spectrophotometric reading at 530 nm. Enzyme activity was calculated as one unit equivalence to the liberation of 1µg N-acetamino-glucose per minute.

Results and Discussion

In the present investigation, tea [*Camellia sinensis*] chitinase 1b gene has been synthesized based on codon optimization and cloned in plasmid vector pPIC9K. Tea chitinase gene (909 bp) was successfully cloned into the pPIC9K cloning as well as expression vector for efficient expression in host cell, *Pichia pastoris* GS115. The chitinase 1b gene construct is then introduced into host cell, *Pichia pastoris* for its expressivity through electroporation and it was inserted into the *Pichia* GS115 genome as a multicopy manner for overexpression of the recombinant chitinase gene. In the first batch, no multicopy clones were found (Fig. 1) after 8 days of incubation, so the screening was repeated with fresh transformation (Fig 2.) and four colonies appeared on YPD plates with geneticin as selection marker as a Mut^+ positive colonies (Fig. 3). Colonies also appeared after 24 h of incubation in YPD plates with no antibiotic, geneticin (Fig.4). Methanol fast utilize colonies Mut^+ was appeared during 80 h of incubation on minimal methanol plates (Fig. 6) and other colonies were found on minimal dextrose (MD) medium after 48 h of incubation (Fig.5). Methanol at the final concentration of 0.5% was added to the medium to induce the chitinase1b gene expression. Colony PCR confirming the gene insert into the *Pichia* genome in the HIS4

region. It was confirmed by amplifying Chitinase1b gene using AOX forward and reverses primer in colony PCR. The chitinase1b gene amplified product size was ~1400 bp (Chitinase1b 909 bp + 480 vector backbone) (Fig.7). Agarose gel (1%) electrophoresis was done according to the standard protocol of Sambrook *et al* (2001) and gel was stained with ethidium bromide and photographed. Expected size of colony PCR product was found on the lanes 3 and 4 (Fig.7) which were selected for small scale expression trail in *Pichia pastoris* host cell. Small scale expression trial was carried out to find out the optimal conditions for expression of tea chitinase1b gene in *Pichia pastoris* GS115. The overexpression of the recombinant vector (pPIC9K-chi1b) was achieved at 48 h, 60 h, 72 h, 84 h in BMM medium at 25° C and in MM medium at 30° C. SDS-PAGE analysis revealed the accumulation of 32 kDa and 40 kDa chitinase 1b proteins during 48 h of incubation in the medium. There was no induction of protein expression upto 36 h of incubation (Plate 1, Fig. 1-2). In comparison to the negative control sample (vector without chitinase 1b gene insert) (Plate 1, Fig. 3, lanes 2, 7, 12 at 60 h, 72 h, and 84 h respectively) it was found two prominent bands visible at ~40 kDa and ~32 kDa size (Plate 1, Fig. 4, lanes 1-4 at 84 h; lanes 6-9 at 96 h; Fig. 5, lanes 2-3 for 72 h, lanes 5-6 for 60 h, lanes 8-9 for 72 h, lanes 11-12 for 84 h). The chitinase protein of 32 kDa size was observed during 48-72 hrs of incubation using condition 4 (MM media at 30ºC- Plate 1 (Fig 1-5). This corresponds to the theoretical molecular weight of tea chitinase ~32 kDa as expected with no post translational modifications (PTMs). On the other hand, 40 kDa protein was visualized at 60 hrs of incubation using condition 1 (BMM media at 25ºC, Plate 1, Fig. 5, lane- 5). The higher molecular weight may be due to post translational modification (could be glycosylation) of chitinase during controlled expression. Since both ~32 kDa and ~40 kDa protein band are not visualized in negative control, the expression of chitinase 1b could be concluded. We adopted the temperature conditions 25 ºC and 30 ºC for appropriate expression and for secretion of the chitinase protein into the medium. Supernatants were collected through centrifugation and analyzed by SDS-PAGE. Results show that the recombinant protein remains in the supernatant fraction which was secreted out due to the presence of α signal secretion factor bases 949-1218 in the vector pPIC9K. The different *Pichia* medium supernatants will vary in protein concentration primarily due to the amount of secreted protein. *Pichia* secretes very few native proteins. If the protein concentration of the medium is >50 µg/mL, 10 µL of medium will give a faint band on a Coomassie-stained SDS-PAGE gel. The molecular

weight of chitinase is 35 kDa that was isolated and purified by many workers. Isolation and characterization of chitinase genes from pitchers of the carnivorous plant *Nepenthes khasiana* has been conducted by Eilenberg *et al* (2006). Samac *et al* (1990) have critically demonstrated the isolation and cloning of chitinase gene in model plant *Arabidopsis thaliana*. Datta *et al.,* (1999) have shown that transgenic rice plant over expressing PR-5 gene can be more resistance against sheath blight disease pathogen *Rhizoctonia solani.* Even though chitinases have been shown to inhibit the hyphal tip growth of many fungi *in vitro.* Genetic transformation of pigeon pea with rice chitinase gene had been investigated by Kumar *et al.,* (2004) for enhanced resistance activity against pathogenic attack. In a recent study by Mincoff *et al*. (2006) an antifungal protein of about 30 kDa was isolated from *Sorgham bicolor* L. using chromatographic technique showed antifungal activity (18-36 μg/ml). Chitinase gene specific transcript accumulation was recorded during induced systemic resistance (ISR) induction with methyl jasmonate to analyze the defense mechanism in tea (Roy and Chakraborty, 2012). The chitinase activity measured in the present study was a acidic in nature because it was worked optimum level at pH 5.5, and may be present in plant vacuole/apoplast. Result was coincided with the previous finding of Wang *et al* (2009) and Mayer *et al* (1996). The optimum temperature was 30˚C, which was nearly consisted with the other study (Fukamizo *et al* 2009).

Fig 1. Transformed clones of Chiti1b in MD plates (Batch I)

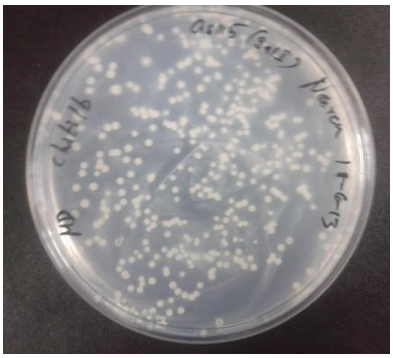

Fig 2. Transformed clones of Chiti1b in MD plates (Batch II)

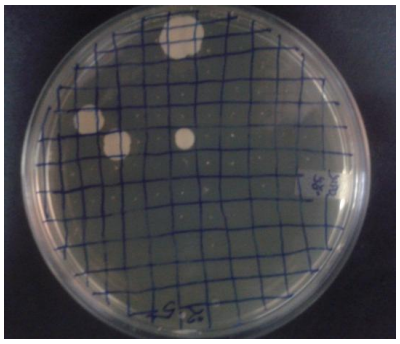

Fig 3. Multicopy screening- colonies appeared after 8 days of incubation in YPD plates with 0.25mg/ml geneticin

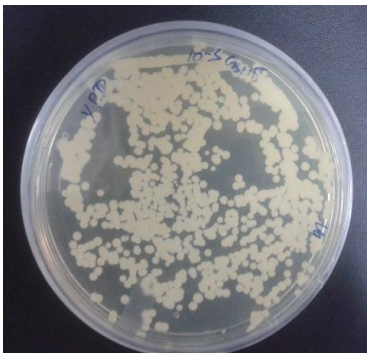

Fig 4. Colonies appeared after 24 hours of incubation in YPD plates with no geneticin.

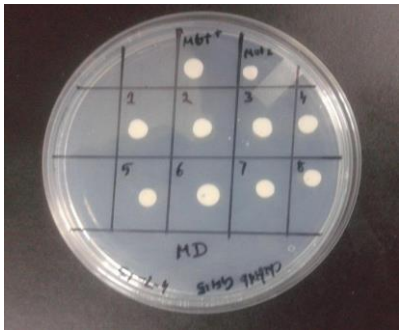

Fig 5. After 48 hours of incubation on minimal dextrose plates

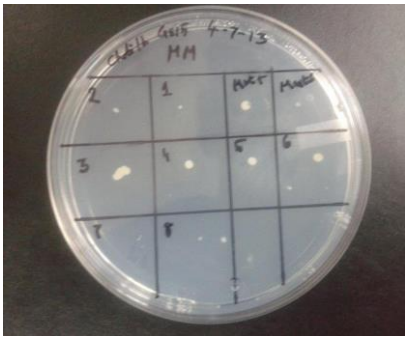

Fig 6. After 80 hours of incubation on minimal methanol plates

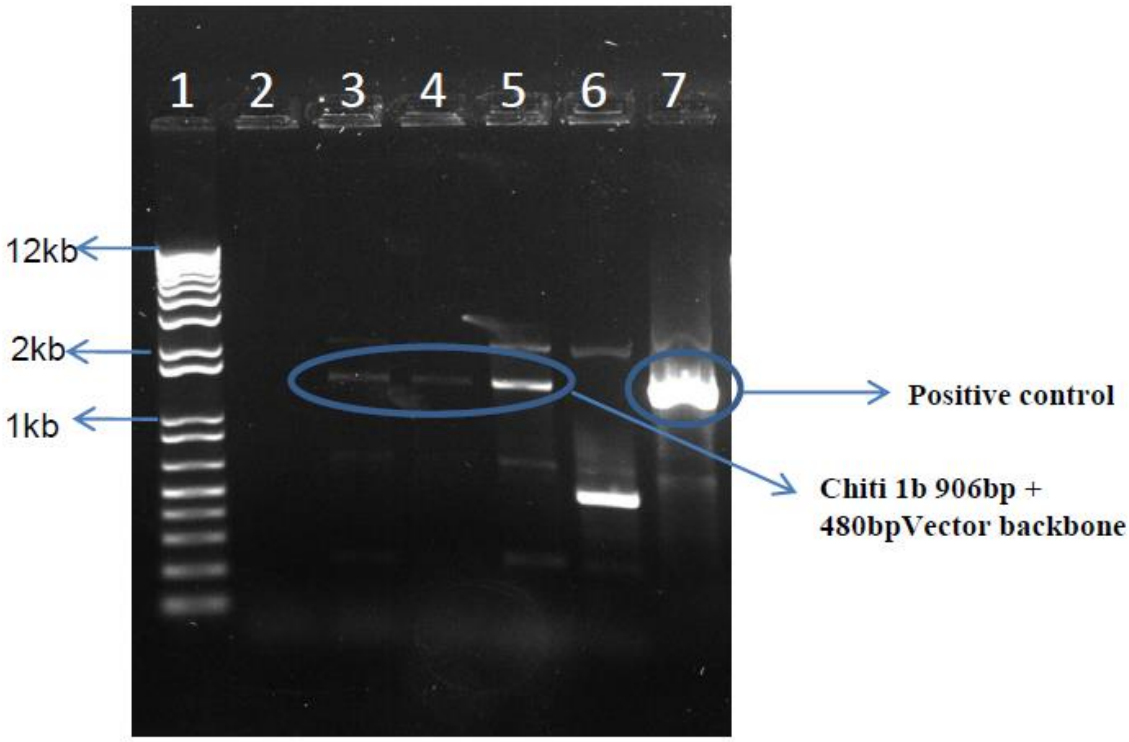

Fig 7. Lane1: 1kb plus ladder, Lane 2-6 Chiti1b clones, Lane7: positive control (chiti1b in pPIC9k vector amplified with the forward and reverse primers)

Plants over-expressing chitinases under the control of a strong constitutive promoter have been engineered and have shown improved resistance against fungal pathogens under laboratory conditions (Broglie *et al*., 1991). These enzymes can inhibit the growth of fungal hyphae *in vitro* (Schlumbaum *et al.*, 1986; Huynh *et al*., 1992). Some chitinases are induced following pathogen infection (Wu *et al*., 1994), and the overexpression of at least some chitinases in transgenic plants causes significant reductions in pathogen damage (Broglie *et al*., 1991). Chitinase gene specific genomic DNA has been amplified and cloned into pGEM-T vector by Roy and Chakraborty (2009). Taken together, these observations support the notion that a primary function of plant chitinases is in defending plants against attack by fungal pathogens, although there is also evidence that chitinases may function as lysozymes degrading bacterial cell walls and may play a role in developmental processes.

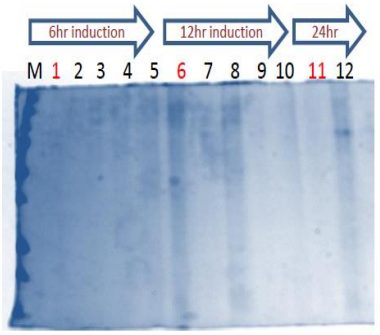

Fig 1. 12%SDS PAGE showing samples from Chitinase expression trial: M: Protein marker. Lanes 1,6 and 11: Negative control at 6, 12 and 24 hours, respectively. Lanes 2 - 5: expression condition 1 - 4 at 6hr, respectively. Lanes 7-10 expression condition 1 - 4 at 12hr, respectively. Lane 12: expression condition 1 at 24hr.

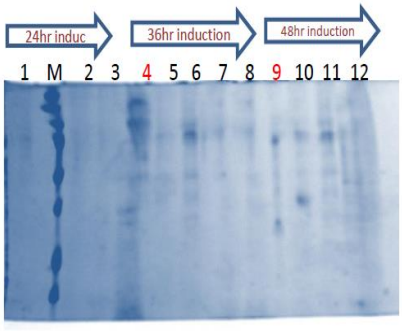

Fig 2. 12%SDS PAGE showing samples from Chitinase expression trial: M: Protein marker. Lanes 4 and 9: Vector backbone at 36 hr and 48 hr, respectively. Lanes 1-3: expression condition 1 – 4 at 24 hours. Lanes 5-8 : expression condition 1 - 4 at 36 hours. Lanes 10-12: expression condition 1 - 3 at 48 hours

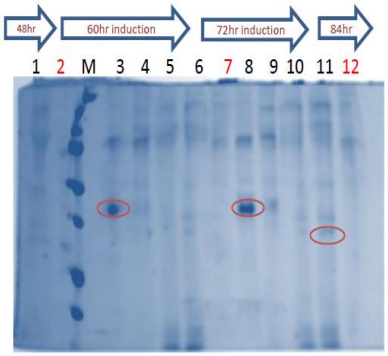

Fig 3. 12%SDS PAGE showing samples from Chitinase expression trial: M: Protein marker. Lanes 2, 7 and 12: Vector backbone at 60 hr, 72 hr and 84 hr respectively. Lane 1: expression condition 4 at 48 hr. Lanes 3-6: expression condition 1-4 at 60 hr. Lanes 8-11: expression condition 1-4 at 72 hr.

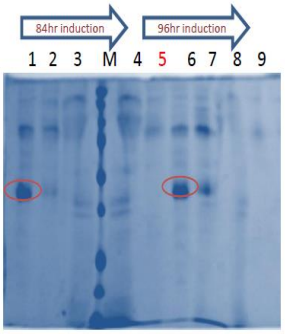

Fig 4. 12%SDS PAGE showing samples from Chitinase expression trial : M: Protein marker. Lane 5: Vector backbone at 96 hrs. Lanes 1-4: Positive clone in different condition 1-4 at 84 hrs. Lanes 6-9: expression condition 1-4 at 96 hr.

Sample at 48 hours, 60 hours, 72 hours and 84 hours of BMM at 25^{0}C and MM at 30^{0}C were analyzed in SDS-PAGE again to confirm the expression

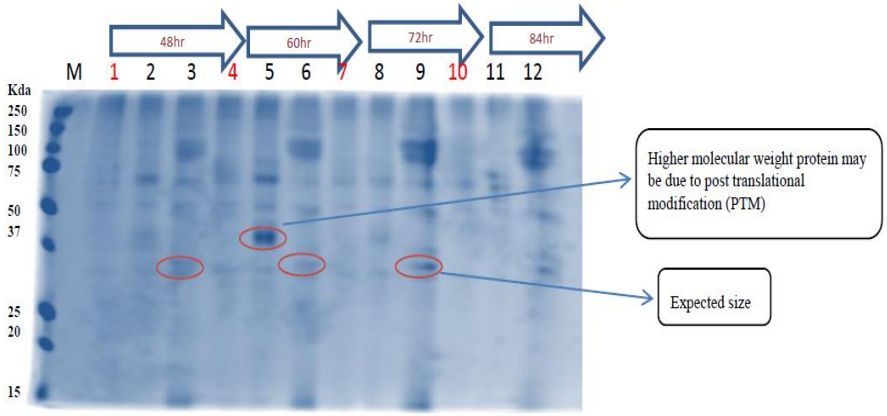

Fig 5. 12% SDS-PAGE showing samples from chitinase expression trial: M: Protein marker. Lanes 1, 4, 7 and 10: Vector backbone at 48 h, 60 h, 72 h and 84 h respectively. Lanes 2 and 3: expression condition 1 and 4 at 48 h. Lanes 5 and 6: expression condition 1 and 4 at 60 h. Lanes 8 and 9: expression condition 1 and 4 at 72 h. Lanes 11 and 12: expression condition 1 and 4 at 84 h.

Plate 1: Small scale trials for optimization of Chitinase gene expression conditions for visible observation on 12% SDS-PAGE (Fig. 1-5).

The production of transgenic plants with enhanced resistance to attack by the fungal pathogen *Rhizoctonia solani* has been developed (Broglie *et al*., 1991). Kirubakaran and Sakthivel, (2007) had cloned and over expressed the barley chitinase gene in *E. coli*, which was believed to be involved in plant defense responses to pathogen infection. Chitinase gene expressed in the host plant was improved the defense mechanisms against pathogenic fungi (Schickler and Chet 1997). The expressible chitinase1b gene investigated in the present study can be introduced into tea (*Camellia sinensis* L.) genome for enhancement of defense strategy against pathogenic attack.

Conclusion: Tea chitinase1b gene has been synthesized after codon optimization and cloned into vector pPIC9K successfully. Recombinant vector containing chitinase1b gene construct was introduced into host cell, *Pichia pastoris* GS115 through electroporation. Due to the presence of α-signal factor in the vector pPIC9K, foreign protein will be secreted out into the medium. Small scale trial of chitinase1b gene expression was carried on different media and in different conditions (MM, BMM media and 25ºC, 30ºC temperatures) and secreted out chitinase1b protein was isolated from the culture medium. Chitinase1b protein was analysed on 12% SDS-PAGE gel electrophoresis. Chitinase1b protein of 30 kDa and 40 kDa was found on gel after 48 h of incubation in MM and BMM media with 0.5% methanol as inducer. The protein size of 40 kDa may be due to post translational modification. It is confirming that the gene introduced into *Pichia* genome was chitinase1b and it was expressed in *Pichia* because negative control without gene could not give any protein band on SDS-PAGE gel.

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