

## Molecular cloning and expression of tea chitinase gene in *Pichia pastoris*

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### ABSTRACT

Tea is one of the important beverages of the world. 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  $\beta$ -glucanase, chitinases and others. Tea chitinase gene (909 bp) was synthesized based on chitinase 1b mRNA information and cloned into pPIC9K vector for efficient *in vitro* expression in eukaryotic system. Recombinant plasmid vector pPIC9K carrying chitinase1b insert was introduced into host cell *Pichia pastoris* strain GS115 through electroporation at the histidine locus to generate Mut<sup>+</sup> phenotype. Transformed host cells were placed on minimal dextrose (MD) and minimal methanol (MM) agar plates for selection of Mut<sup>+</sup> 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 were inoculated 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. 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. The knowledge gained in the present investigation will help to design the tea chitinase functionality against fungal diseases.

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

### 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<sup>1-4</sup>. Chitinases (PR-3) catalyze the hydrolysis of the  $\beta$ -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<sup>5-17</sup>. 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<sup>3, 9</sup>. The pattern of transcripts accumulation has been studied in six-typical defense response genes, POX (peroxidase), PR-1, PR-2 ( $\beta$ -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<sup>18</sup>. Chitinase gene specific transcript accumulation was studied in tea after induction with methyl jasmonate<sup>17</sup>. The acidic and basic chitinase genes have isolated and characterized fully in *Arabidopsis thaliana*<sup>9</sup>. Genes for chitinases have been analyzed at the molecular evolutionary level in maize and family poaceae<sup>15, 19</sup>. Plant-pathogen co-evolution is analyzed in *Arabidopsis* sp, in relation to class-I chitinases<sup>20</sup>. Some researcher has isolated and cloned chitinase-I gene from winged bean seed and characterized its structure in relation to defense system<sup>5</sup>. Chitinase gene has been isolated and cloned in model plant *Arabidopsis thaliana*<sup>9</sup>. Transgenic rice plant over expressing PR-5 gene can be more resistance against sheath blight disease pathogen *Rhizoctonia solani*<sup>10</sup>. So, there is no report on tea chitinase gene synthesis, cloning and expression in eukaryotic system. The present investigation is carried out to clone the synthesized chitinase1b gene in appropriate vector and expression in eukaryotic host *Pichia pastoris*.

#### **MATERIAL AND METHODS**

Tea Chitinase 1b gene was synthesized (909 bp) based on mRNA information (by C-CAMP, Bangaluru) of the NCBI Genebank (Acc.no- ) and inserted into plasmid vector pPIC9K for cloning and expression studies.

#### **Gene cloning in vector pPIC9K**

The chitinase1b gene was cloned in pPIC9K vector (Invitrogen, USA) which is a *Pichia pastoris* GS115 based vector for multicopy integration and secreted protein expression (due to  $\alpha$ -factor secretion signal attachment). Chitinase 1b gene has been inserted at *HIS4* region to make it Mut<sup>+</sup> 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<sup>+</sup> in GS115). 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).

#### **Transformation into *Pichia pastoris* (GS115) by electroporation**

Grow host cell, *Pichia pastoris* in YPD medium in a 50 mL conical flask at 30°C overnight and collected host cell for transformation at OD<sub>600</sub> value ~1.3-1.5. Electroporation was carried out following manufacturer's protocol (Invitrogen, USA). In brief, above pellet cells about 80  $\mu$ L was mixed with 10  $\mu$ L TE Buffer and transferred them to an ice-cold 0.2 cm electroporation cuvette. 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. Content is transferred to a sterile microcentrifuge tube and spread 200–600  $\mu$ L 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<sup>+</sup>/Mut<sup>S</sup> transformants**

Individual colonies were plated on minimal dextrose (MD) plates and minimal methanol (MM) plates for screening Mut<sup>+</sup> (methanol fast utilizer) and Mut<sup>S</sup> (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 (according to Invitrogen protocol). Briefly, cells were lysed by treating with zymolase to release genomic DNA. This was used as template for PCR. AOX 5 $\square$  forward primer and AOX 3 $\square$  reverse primers were used to amplify the Chit1b 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 Chit1b gene, respectively. Thermal conditions were as follows- initial one cycle 95°C for 5 minutes, then 30 cycles at 95°C for 1 minute, 54°C 1 minute, and 72°C 1 minute. One final cycle was included at 72°C for 5 minutes. PCR product was separated on 1% agarose gel electrophoresis and visualized with UV light after staining with ethidium bromide. The expected size of PCR amplification product is 1400bp.

### 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 30°C with 250 rpm agitation. Cells were pelleted at 1500-3000 g, for 5 min at OD600  $\square$  2.0 and re-suspended in 100 ml of BMM medium (Buffered minimal methanol media) and MM (Minimal methanol media) media to a final OD600~1.0 for gene expression induction. Four different conditions were set up for gene expression analysis. These are as follows- Condition 1: BMM media at 25°C; Condition 2: BMM media at 30°C, Condition 3: MM media at 25°C; Condition 4: MM media at 30°C.

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 collected after centrifusing the culture 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<sup>21</sup>. 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 was 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 standard protocol<sup>22</sup>. 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 1 $\mu$ g N-acetamino-glucose per minute.

## RESULTS AND DISCUSSION

In the present investigation, tea [*Camellia sinensis*] chitinase 1b gene was expressed in eukaryotic host system, *Pichia pastoris* GS115. Tea chitinase gene (909 bp) was successfully inserted into the pPIC9K cloning as well as expression vector for efficient expression in host cell, *Pichia pastoris* GS115. Electroporatically transformed host cells were selected on YPD plates with geneticin as selection marker. Methanol fast utilizer colonies Mut<sup>+</sup> was appeared during the incubation (36-48 h) on minimal methanol plates (Fig.1). Methanol at the final concentration of 0.5% was added to the

medium to induce the chitinase1b gene expression. Chitinase 1b gene insertion was confirmed by amplifying Chitinase1b gene using AOX forward and reverse primer in colony PCR. The chitinase1b gene amplified product size was ~1400 bp (Chitinase1b 909 bp + 480 vector backbone). Amplified product was separated on 1% agarose gel and stained with ethidium bromide for visualization on UV-transilluminator. Expected size of colony PCR product was found on the lanes 3 and 4 (Fig. 2) which were selected for small scale expression trial 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 chitinase 1b proteins during 48 h of incubation in the medium. There was no induction of protein expression upto 36 h of incubation (Fig. 3). The chitinase protein of 32 kDa size was observed during 48-72 hrs of incubation using condition 4 (MM media at 30°C) (Fig. 3). 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 25°C). 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 °C and 30 °C 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  $\alpha$  signal secretion factor bases 949-1218 in the

vector pPIC9K. *Pichia* secretes very few native proteins. 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<sup>6</sup>. Chitinase gene is cloned in model plant *Arabidopsis thaliana*<sup>9</sup>. Datta *et al.* have shown that transgenic rice plant over expressing PR-5 gene can be more resistance against sheath blight disease pathogen *Rhizoctonia solani*<sup>10</sup>. 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 some group for enhanced resistance activity against pathogenic attack<sup>12</sup>. In a recent study it has been found that an antifungal protein of about 30 kDa was isolated from *Sorghum bicolor* L. using chromatographic technique showed antifungal activity (18-36  $\mu$ g/ml)<sup>23</sup>. Chitinase gene specific transcript accumulation was recorded during induced systemic resistance (ISR) induction with methyl jasmonate to analyze the defense mechanism in tea<sup>17</sup>. The chitinase activity measured in the present study was acidic in nature because it worked optimum level at pH 5.5, and may be present in plant vacuole/apoplast. Result was coincided with the previous findings<sup>24-25</sup>. The optimum temperature was 30°C, which was nearly consisted with the other study<sup>26</sup>. 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<sup>11</sup>. These enzymes can inhibit the growth of fungal hyphae *in vitro*<sup>13-14</sup>. 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<sup>11</sup>. Chitinase gene specific genomic DNA has been amplified and cloned into pGEM-T vector for its functionality<sup>16</sup>. 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.

The production of transgenic plants with enhanced resistance to attack by the fungal pathogen *Rhizoctonia solani* has been developed<sup>11</sup>. Kirubakaran and Sakthivel (2007)<sup>27</sup> 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<sup>28</sup>. 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 and inserted into vector pPIC9K successfully for foreign gene expression. Recombinant vector containing chitinase1b gene construct was introduced into host cell, *Pichia pastoris* GS115 through electroporation. Small scale trial of chitinase1b gene expression was carried on different media and in different conditions (MM, BMM media and 25°C, 30°C 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 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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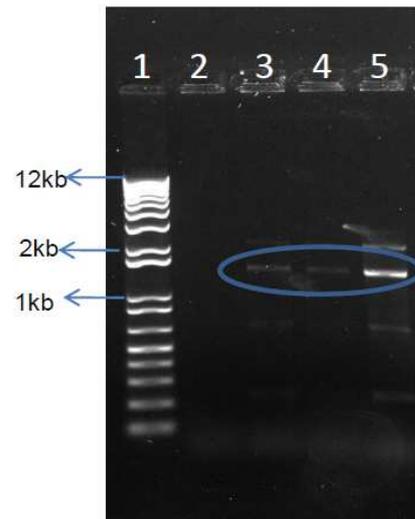
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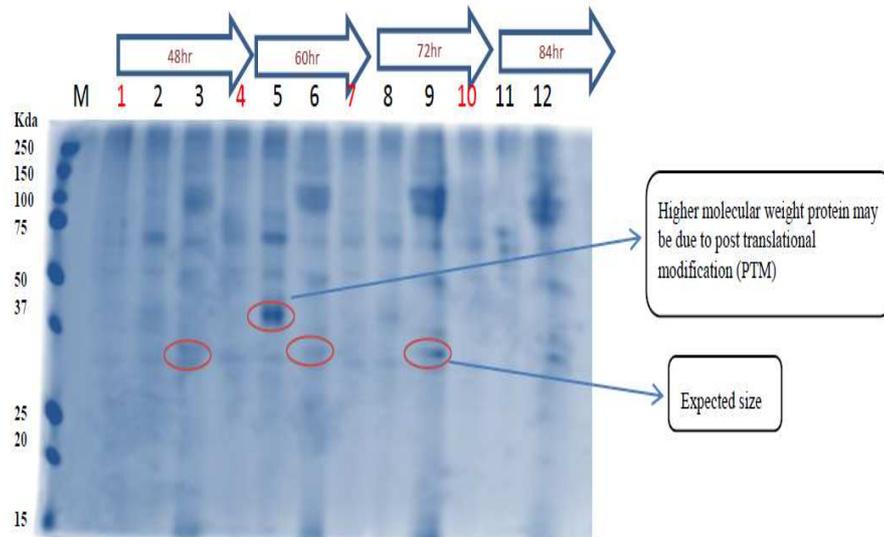


**Figure 1.** Transformed clones of Chitinase 1b on MD plate.



**Figure 2.** Colony PCR product 1400 bp separated on 1% agarose gel lanes 3-5 (circled).

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



**Figure 3.** 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.