Barley Chitinase Genes Expression Revamp Resistance Against Whitefly (Bemisia Tabaci) in Transgenic Cotton Plants

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Abstract

Chitinase is a hydrolytic enzyme that works against various plant pests, including whiteflies, as the exoskeleton of insects is made up of chitin. Based on their capability of hydrolyzing chitin, the present study was conducted to determine the expression of chemically synthesized barley *ChI* and *ChII* genes in cotton (*Gossypium hirsutum*) through Agrobacterium-mediated transformation. Out of fifty-five putative transgenic cotton plants, fifteen plants were able to survive and shifted to the field. The amplification of 447 bp and 401 bp by using gene-specific primers for *ChI* and *ChII,* respectively, confirmed the five positive transgenic cotton plants (T_0 generation), which were further evaluated for their mRNA expression. The T_0 transgenic cotton plants with the highest mRNA expression and better yield performance in the field were chosen for generation advancement of T_0 to T_1. The highest mRNA expression levels of 3.5-fold in P10(2) for the *ChI* gene and 3.7-fold in P2(1) for the *ChII* gene were observed in T_1 generation cotton plants. Flourescent in situ hybridization (FISH) confirmed a single copy number of *ChI* & *ChII* (hemizygous) on chromosome number 6. Furthermore, the efficacy of transgenes on whiteflies was evaluated through an insect bioassay, where after 96 hours of infestation, the mortality rate of whiteflies was calculated to be 78-80% in transgenic cotton plants. The number of eggs on transgenic cotton plants was calculated to range between 10-12/plant compared to the nontransgenic plants, where the egg number was calculated to be 90-100/plant. We conclude that the barley chitinase gene holds the potential to be used against all insects with chitin exoskeletons.

1. Introduction

Cotton (*Gossypium hirsutum L.*) is the major cash crop of Pakistan, which contributes an approximately 0.8% share of the gross domestic product (GDP) and a 4.1% value addition in the agriculture sector. A decline of 6-7% in cotton production was recorded in the recent past. Unfavorable weather conditions, decreased water availability and various pathogens are the major culprits of this reduction in cotton production (1). The whitefly (*Bemisia tabaci*) is one of the notorious cotton plant pests that causes severe crop losses in both fields and greenhouses. *Bemisia tabaci* not only decreases the rate of photosynthesis in plants but is also able to transmit a large number of plant pathogenic viruses, including torradoviruses, ipomoviruses, criniviruses, ipomoviruses, and begomoviruses (2). It feeds on the phloem sap and excretes honey dews that transmit viral infections. Approximately eleven hundred white fly species have been reported thus far, and three of them are predominantly known as vectors for plant viruses (3). However, *B. tabaci* is most significant among them and possesses the potential to directly transmit more than 250 species of begomoviruses (family Geminiviridae). *B. tabaci* has been reported to cause severe damage to tomato, tobacco, brinjal, chili, cotton, okra and potato worldwide (4). The annual loss due to white flies in many crops reaches up to billions of USDs. Several climatic factors trigger whitefly outbreaks, but the widespread use of insecticides is one of the major causes of insecticidal resistance. The excessive use of these chemicals also causes many health risks to consumers (5).

Modern biotechnology approaches therefore play a role in promoting plant defense mechanisms by introducing biological insect control strategies. Researchers have shown increasing interest in using biological control agents, including entomopathogenic fungi (EPFs), as an alternative to chemical control measures. *Metarhizium anisopliae* was first discovered to be effective against the greenhouse whitefly, and the pathogenicity of six isolates of *M. anisopliae* was tested on the *B. tabaci* Q biotype, where the results showed greater than 50% mortality in all six isolates (6). However, higher mortality of *B. tabaci* (97%) was observed under osmotic conditions upon infection with *M. anisopliae* isolated from *Coptotermes gestroi* (Rhinotermitidae: Isoptera). Chitinases are becoming more popular due to their effectiveness against white flies, termites, coconut beetles, grasshoppers and rice bug termites (7). Chitin is a poly-β-1,4-*N*-acetylglucosamine structural protein of pathogenic fungi, arthropods, molluskan shells and crustaceans. The fungal cell wall comprises almost 22-24%, while the insect exoskeleton is made up of approximately 40-45% chitin. Chitin degradation by chitinase, therefore, promptly results in insect death and increases insect mortality (8). Considering the significance of chitinases, the present study aimed to develop transgenic cotton conferring resistance to insects, especially *B. tabaci*. *Agrobacterium*-mediated transformation was used to transform the chemically synthesized barley *ChI* and *ChII* gene constructs. The developed transgenic cotton plants underwent molecular assessment to evaluate the effect of transgene expression. An insect bioassay against the whitefly *Bemisia tabaci* was conducted to evaluate the efficacy of transgenic cotton plants with *ChI* and *ChII*.

2. Materials And Methods

2.1. Plasmid designing, synthesis and cloning

The gene sequences of *ChI* and *ChII* were retrieved from (website name, e.g., NCBI) with accession numbers P11955.4 and ACJ68105.1, respectively. The codon-optimized sequences of *the ChI* and ChII genes under regulation of the constitutive promoter CaMV 35S and the respective restriction sites of *BamHI, KpnI, BamHI, and HindIII* were joined in silico in pUC57 using (https://(www.biobasic.com/gene-splash/) as shown in Figures 1 and 2, and the designed plasmids were chemically synthesized from (https://(www.biobasic.com/gene-splash/). The synthesized gene cassettes were cloned into the plant expression vector pCAMBIA 1301. Successful cloning was confirmed by restriction digestion analysis and polymerase chain reaction.

2.2. *Agrobacterium*-mediated Plant transformation

A local cotton *Gossypium hirsutum* variety, Klean cotton (CKC-1), was obtained from the seed biotechnology lab, CEMB, University of Punjab, Pakistan, and the genetic transformation of cotton was achieved using the shoot-apex-cut method as described by (9, 10). The processed embryos were transferred to MS broth containing *Agrobacterium tumefacien* harboring *ChI* and *ChII* gene constructs and incubated for one hour at 30°C with continuous shaking. The cotton embryos were allowed to dry on autoclaved filter paper followed by transfer to MS medium plates supplemented with kinetin (1 mg/mL) and 250 µg/mL cefotaxime and cocultivation on MS medium for the next 3 days at 25 ± 2°C in a growth

room with 16 hours of light and 8 hours of dark. Plantlets were shifted to glass test tubes (autoclaved) having MS media with selection cefotoxin (250 µg mL^{-1}), hygromycin (25 mg L^{-1}) along with B5 vitamins (thiamine-HCl; nicotinic acid: 50 mM: 10 mM; pyridoxine-HCl: 10 mM; myo-inositol: 100 mM; glycine: 2 mM) and kinetin (1 mg mL^{-1}) and kept under light until roots and shoots started emerging in the next 5-6 weeks by incubation at 25 ± 2°C in a growth room with 16 hours' light and 8 hours dark and 60 µE $m^{-2}s^{-1}$ light for in vitro growth. After 4-6 weeks, putative transgenic cotton plants were shifted to soil pots followed by shifting to the field under standard cultivation practices.

2.3. Confirmation of ChI and ChII genes in transgenic cotton plants through amplification

DNA isolation from transformed cotton plant leaves was achieved using protocols reported by (11) with little modification, and the presence of transgenes was detected by PCR amplification using gene-specific primers.

ChI (Act-F 5'_AACAGTGTGGTTCTCAGGCT_3' &

ACT-R 5'_ AAGTAGCCCCTCTCTCTTGC_3'), and

ChII (Act-F 5'_GCAGCTTTCTTCGGACAGAC_3' &

ACT-R 5'_ CCACATTCAAGACCGCCATT_3'),

The PCR conditions were optimized as follows: initial denaturation at 95°C for 5 min; 30 cycles of denaturation at 95°C for 30 seconds, annealing at 65°C (*chI* gene) and 63.5°C (*ChII* gene) for 45 secs; extension at 72°C for 60 secs; and a final extension at 72°C for 10 minutes. The amplified products were resolved on a 1.2% agarose gel and visualized under UV light.

2.4. Relative Expression of *ChI* and *ChII* genes in transgenic cotton plants

RNA isolation from transgenic cotton plant leaves was performed following the modified protocol by (12). A RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, K1622) was used for the synthesis of cDNA using one-step RT–PCR with random hexamers. The relative expression analysis of *the ChI* and ChII genes was performed through quantitative real-time PCR in transgenic and nontransgenic control cotton plants. Real-time PCR was performed in a 96-well plate iQ5 cycler (BIO-RAD) PCR machine using Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific, K0221). For data normalization, the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene was used as an internal control, and nontransgenic plants were used as a negative control. The GAPDH primer sequences are shown below in Table 1. The samples were analyzed in triplicate by using the following housekeeping gene-specific primers:

Table 1
GAPDH primer sequence and accession number

Gene name	5′-3′ sequence	Tm	Ps	Accession number
GAPDH	F-AGGAAGAGCTGCTTCGTTCA	60°C	106	XM_017782884.1
	R- CCGCCTTAATAGCAGCAGCTTTG			

2.5. Generation advancement of *ChI* and *ChII* Transgenic Cotton Plants

Transgenic cotton plants P1, P2, P3, P4 and P10 *harbored the ChI* gene, while P2 possessed both *ChI* and ChII in the T_0 generation. Transgenic plants that showed significant mRNA expression and possessed good morphological/physiological characteristics (i.e., showing better yield performance in the field) were chosen for further advancement of T_1 generation. Nontransgenic cotton plants were also raised as controls in a separate line to study their molecular and physiological characteristics in a comparative way.

2.6. Assessment of physiological traits of transgenic cotton plants

Various physiological parameters (photosynthetic rate, transpiration rate and gaseous exchange rate) were measured in triplicate using a CIRAS-3 portable photosynthesis system infrared gas analyzer (PP Systems, USA) on fully extended cotton leaves of both transgenic and nontransgenic (control) cotton lines. Measurements were made with specific adjustment of the molar flow rate of air at 403.3 µmol m-2s-1, atmospheric pressure at 99.9 kPa, water vapor pressure in the chamber at 6.0-8.9 mbar, PAR of leaf surface at 1000-1711 µmol m-2s-1, the temperature of a leaf at 28.4-32.4°C, ambient temperature 22.4-27.9°C and ambient CO2 concentration was set to be 352 µmol mol^{-1}.

2.7. Assessment of morphological Traits

Morphological characteristics (height, ginning out turn percentage (GOT%) and number of bolls per plant) of the transgenic cotton and nontransgenic cotton lines were also evaluated in T_1 progeny. Data from transgenic and control (nontransgenic) cotton lines were analyzed by using one-way analysis of variance (ANOVA) to determine any significant variation in the mentioned traits between transgenic and control cotton lines. GraphPad Prism software version 7 for Windows was used for all analyses.

2.8. Fluorescence In Situ Hybridization (FISH)

To determine the transgene location on the chromosome, *fluorescence in situ hybridization* (FISH) analysis was performed in advanced generations. The transgene was detected by labeling the probe with the Label IT Nucleic Acid Labeling kit (Mirus Bio LLC), Cy3, per the manufacturers' instructions. In situ hybridization was carried out on metaphase chromosomal spreads. Fluorescent signal detection was

performed using a fluorescence microscope (Olympus Model BX6 I). Blue (DAPI) and red63 filters were used to detect fluorescent signals.

2.9. Insect bioassay of whiteflies in transgenic cotton plants

The T2 generation of transgenic cotton plants was grown in a glasshouse at 37 ± 2°C, 14 L/10 D and ≈60% humidity. The plants were left uninfested for seven days by carefully isolating them in a net cage. Whitef culture was held on nontransgenic cotton plants in a separate greenhouse maintaining similar conditions. Approximately 0- to 24-hour-old whiteflies were caught using a manual aspirator and kept on ice to reduce environmental stress. The 15-20 whitefly pairs were released carefully on cotton plants (4-6 leaf stage) inside the cage (as shown in Figure 3). The bioassay was performed using three biological replicates from both transgenic and nontransgenic control cotton lines. After 96 hours of infestation, the mortality data were calculated using the following formula:

$$\%\text{Mortality} = \frac{\text{Numberofdeadlarvae}}{\text{Totalnumberoflarvae}} \times 100$$

Microscopic observation was performed to calculate the number of eggs on the lower and upper surfaces of the leaves.

2.10. Statistical analysis

GraphPad Prism (version 7.0) was used for all analyses. The values presented in the table and figures are the means plus standard deviation (mean ± STD). Analysis of variance (ANOVA) was performed for the morphological and physiological parameters. The results of insect bioassays and the qPCR data were also analyzed using the same analysis of variance. To determine any significant differences among the variables, Dunnett's multiple comparison (where applicable) was applied. Significant differences were considered when the P value was less than or equal to 0.05 ($p \leq 0.05$).

3. Results

3.1. Confirmation of pCAMBIA1301_ChI&ChII through Restriction Digestion and Polymerase Chain Reaction

The digested plasmid was resolved on a 0.8% gel, and the restricted fragments of 1485 bp and 1685 bp confirmed the successful cloning of *ChI* and *ChII* gene cassettes, respectively, in pCAMBIA1301 vectors (as shown in Figure 3). Likewise, the PCR amplicon at 447 bp and 401 bp further confirmed the successful ligation of *ChI* and *ChII* genes in pCAMBIA 1301, as shown in Figure 4.

3.2. Transformation and Confirmation of (pCAMBIA1301_*ChI*) &(pCAMBIA1301_*ChII)* Construct into *Agrobacterium tumefaciens*

The amplification products of 447 bp and 401 bp obtained by using gene-specific primers for *ChI* and *ChII* confirmed successful introduction of recombinant plasmids into *Agrobacterium* (as shown in Figure 5).

3.3. Molecular Analyses of Putative Transgenic Cotton Plants (T_0 Progeny)

Out of fifty-five plants (T_0) that were shifted in the field, only fifteen plants survived, and five plants (P1, P2, P3, P4, and P10) were able to be amplified by specific primers of *Ch I*, while amplification of *Ch II* was obtained only in cotton plants (P2) along with *Ch I*. *Ch II* was not amplified in any of the other tested cotton plants except P2 (as shown in Figure 6).

3.4. Quantitative real-time qRT–PCR analysis of *ChI* and *ChII*

Quantitative real-time qRT–PCR was used to measure the relative mRNA expression of *ChI* and *ChII* genes in the T_0 generation of transgenic cotton plants. The mRNA transcripts of T_0 transgenic cotton plants (P1, P2, P3, P4 and P10) expressing *ChI* and *ChII* genes were reverse transcribed into complementary DNA (cDNA) using oligo (dt) random oligomers. The synthesized cDNA of these transgenic cotton lines (P1, P2, P3, P4 and P10) was then amplified exponentially using a real-time thermocycler machine employing gene-specific real-time primers. The mRNA expression level of *ChI* was found to be 7.5-fold higher than that in nontransgenic control cotton plants, while the expression level of *ChII* was 8.7-fold higher than that in nontransgenic cotton plants in the T_0 generation, as shown in Figure 7.

3.5. Generation advancement of *ChI* and *ChII* Transgenic Cotton Plants

The progeny of these five cotton plants were raised to advanced T1 generation lines, as each was confirmed as a separate insertion. Conventional PCR amplification was used to confirm successful gene inheritance in the advanced generation of transgenic cotton lines, as shown in Figures 8 and 9. The amplification of 447 bp was evident in three cotton plants of the progeny 1 (Line 1) while amplification of 447 bp & 401 bp was found only in two plants of progeny 2 (Line 2), similarly five plants were found to harbor *ChI* in plant progeny 3 (Line 3) while Progeny of plant 4 and 5 were found to have gene amplification of *ChI* in one plant each.

Similarly, plants with the highest mRNA expression in the T_0 generation were further evaluated for their expression in the T1 generation. In the T_1 generation of transgenic cotton plants, the highest mRNA expression of 3.5-fold in P10(2) for the *ChI* gene and 3.7-fold in P2(1) for the *ChII* gene was measured in comparison to the nontransgenic control cotton (i.e., nontransgenic plants), as shown in Figure 10.

3.5. Morphological and Physiological Characteristics of Transgenic Cotton Plants in Advanced Generation

3.5.1. Plant yield: Approximately 129 g of cotton yield was calculated in transgenic cotton plants [p1(2)] expressing the *ChI* gene, while a maximum cotton yield of 130 g was determined in [p2(1)] transgenic

cotton plants expressing the *ChII* gene. For all the genes, almost all transgenic plants were found to have a significant ($p<0.0001$) increase in yield compared with their nontransgenic counterparts except in P4(3) and P6(4) transgenic plants, in which no statistically significant ($p <0.0001$) differences were observed in comparison to the control plants, as shown in figure 11 (A)&(B).

3.5.2. Plant height: Nearly all the transgenic plants expressing either the *ChI* gene or the *ChII* gene were found to be significantly ($p < 0.05$) taller than the negative control plants. Transgenic plants [p1(2) and p10(4)] expressing the *ChI* gene were found to have maximum heights of 98 and 98.5 inches, respectively. Correspondingly, maximum heights of approximately 98.5 and 98.7 inches were also calculated in similar transgenic plants [p2(1) and p2 (5)] expressing the *ChII* gene. On the other hand, nonsignificant ($p < 0.001$) results in height in comparison to the control were observed in P4(3) and P6(4) transgenic plants expressing *ChI* similarly transgenic plants[p2(2), p2(3) and p2 (4)] showed nonsignificant ($p < 0.001$) results in comparison to control for *Ch II* genes, respectively as presented in figure 12 (A)&(B).

3.5.3. Number of bolls per plant: Transgenic plant P1(2) expressing the *ChI* gene was found to have a maximum number of bolls per plant (102). A maximum of 103 bolls per plant was obtained in the cotton transgenic plants and P2(5)-expressing *ChII* genes. Nearly all the transgenic plants showed a significant ($p < 0.0002$) increase in the number of bolls per plant in comparison to the control plants except the P4 (6) transgenic plants for the *ChI* gene and P2(4) in which no statistically significant ($p > 0.0002$) differences were observed in the number of bolls per plant compared with the control plants. Figure 13 (A) & (B).

3.5.4. Ginning out turn percentage%: To determine the ginning Out Turn, the lint and seeds were separated using a ginning machine the ginning out turn percentage (GOT %) was determined by dividing the lint weight with that of the seeds then multiplied by 100. The GOT percentage was found to be statistically nonsignificant ($p>0.001$) in almost all the plants except in the transgenic plants P1(2) and P2(1), in which a slight increase in the GOT percentage was evident in comparison with the control plants, as shown in figure 14 (A&B).

3.5.5. Photosynthetic rate: A portable infrared gas analyzer (IRGA) was used to determine the net photosynthetic activity in fully expanded transgenic cotton leaves and nontransgenic control plants. There were no statistically significant differences observed between the control and transgenic plants except in the transgenic plants P1(2) and P10 (4) expressing the *Ch I* gene and the P2 (1) transgenic plant expressing *Ch II*, where significant ($p<0.0044$) differences in photosynthetic rate were noticed compared with the nontransgenic/control plants. Figure 15 (A&B).

3.5.6. Transpiration and gaseous exchange rates: The transpiration rate of both transgenic and control plants was determined, and the data are presented in Figure 16 (A&B). Nearly all the transgenic plants

showed a somewhat statistically nonsignificant gaseous exchange rate compared with that of the control plants, as shown in Figure 17 (A&B).

3.6. Determination of Transgene Copy Number and Location

One plant from each transgenic cotton line in the T_2 generation was subjected to determination of copy number and transgene location at different stages of cell division, prophase, metaphase and interphase using ChI- and *ChII*-specific probes. The transgenic cotton plant from line L_3P_2 showed one copy number at chromosome number 6 for the *ChI* gene, while L_2P_2 also showed a single copy number for ChII at chromosome 6 *but* at different chromatids, whereas no signal was observed in the nontransgenic control cotton plant, as shown in Figure 18 (A, B & C).

3.7. Insect Bioassay

3.7.1. Whitefly Survival Percentage and Eggling on Cotton Plants

Whitefly eggs calculated on advance generation T_2S_2 line. Cotton plant leaves were kept under a microscope in comparison to control nontransgenic plants. The ratio of eggs in transgenic cotton leaves was 10 to 22%, while 90 to 98% was calculated in nontransgenic control cotton leaves (as shown in Figure 19). Similarly, the average mortality ratio of whiteflies was 70–80% in transgenic plants compared to 30% in nontransgenic control cotton plants (as shown in Figures 20 and 21). It is evident from the results that chitinase has an impact on the whitefly exoskeleton, which helps to reduce the overall population in the form of eggs as well as adults.

4. Discussion

Chitinases are proteins generated by plants as defense machinery against various pests, including fungi and insects, such as whiteflies, owing to their potential to hydrolyze chitin, an outermost exoskeleton of many insects and fungi. The expression of barley chitinase gene sequences in cotton has previously been used for its ability to develop fungus-resistant crops (13, 14). An attempt was made in the current study to develop insect-resistant cotton through the introduction of barley *ChI and ChII* genes by using the *Agrobacterium*-mediated shoot apex cut method of transformation, as was done by (15–18). The transformation efficiency was 1.17% against the 1.1% reported by (9) by using the same method. A total of five plants were amplified out of a total of fifteen plants shifted to a greenhouse by using gene-specific primers. The low transformation efficiency was attributed to the recalcitrant nature of cotton. Quantitative real-time PCR showed the highest mRNA expression levels of *ChI* and ChII in transgenic cotton plants, up to 7.5- and 8.7-fold in transgenic cotton plants P1 (expressing *Ch1*) and P2 (expressing *ChII*), respectively, compared with nontransgenic cotton plants in the T0 generation. Similar results were obtained by (19) while assessing the ExpA1 gene in transgenic cotton plants under different promoters. (20) reported a similar pattern of transgene expression in transgenic cotton for the expression of the PME gene against insects.

Transgenic cotton plants in T_0 with the highest mRNA expression and better yield performance in the field were chosen for generation advancement of T_0 to T_1. The progeny of these five plants were raised to T1 generation lines and evaluated as separate insertions. The amplification product of 447 bp for *ChI* and 401 bp for *ChII* confirmed the transgene in the T1 generation. The mRNA expression in the T1 generation was quantified to be 3.5-fold in P10 (2) of the *ChI* gene and 3.7-fold in P2 (1) of the *ChII* gene in comparison to the control (i.e., nontransgenic cotton plants). The results are in accordance with (3) and (21) when evaluating transgenic cotton by adopting the same method of transformation. The transgenic cotton plants showed a significant increase in morphological characteristics compared with physiological characteristics and photosynthetic rates, which can be attributed to their insect resistance being healthier, with photosynthetic rates ranging from 5.5 to 5.8 mol $CO_2 m^{-2} s^{-1}$ in P10 (4)-expressing *ChI* genes and P2(1)-expressing *ChII*. Improved photosynthetic rates were also reported by (22), who used the barley *chitinase II* gene to create resistance against fungi. The results are also in accordance with (23), who demonstrated that exogenous DNA in a host cotton genome can affect plant growth and photosynthesis, while (24) reported that a significant difference between transgenic cotton and nontransgenic cotton occurred but not always throughout the growing season, in different experiments or for all transgenic cotton lines. (9) reported improved physiological performance of cotton transformed with Phytochrome B. An important aspect of chitinase activity was visualized, and transgenic cotton plants expressing *ChI* and *ChII* showed a high mortality rate of whiteflies feeding on transgenic cotton plants. The statistical results were in accordance with the report of (7, 25, 26–29).

5. Conclusion

From the results, it can be concluded that barley *ChI* and *ChII* have potential against insects with chitin as an exoskeleton. Further field trials have proven their field efficacy and their potential to be used as candidate genes for whitefly resistance in transgenic cotton plants. Moreover, the plants produced can prove to be excellent breeding material for utilization in institutional variety development programs.

Declarations

Compliance with institutional guidelines: The Experimental research and field studies on cotton plants was in compliance with national and Institutional (CEMB, University of the Punjab) guidelines.

Ethical Approval: This article does not contain any studies with animal/human participants performed by any of the authors.

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Conflict of interest: We, the authors Samina Bashir, Amina Yaqoob, Rohina Bashir, Shanila Bukhari, Muhammad Azam Ali, Tayyab Husnain, Abdul Qayyum Rao and Ahmad Ali Shahid, declare that we have no conflicts of interest in submitting or publishing this manuscript entitled “Barley chitinase gene

expression revamps resistance against whiteflies (*Bemisa tabaci*) in transgenic cotton plants" in the Molecular Biology Reports journal.

Author contribution:

SB performed the molecular analysis and wrote the main manuscript. AY performed the field work and prepared the figures. RB assisted in research work and data acquisition. SB assisted in field word and data evaluation. MA assisted in data interpretation. TH supervised the whole research work and provided the facilities. AQR and AAS reviewed and proofread the manuscript.

Availability of Data and Materials: The sequences of chitinase genes, *ChI* and *ChII* were retrieved from the Nucleotide domain of NCBI website (https://www.ncbi.nlm.nih.gov/nucleotide/) with accession numbers of P11955.4 and ACJ68105.1 respectively. The *ChI* and *ChII* genes are mentioned in the section 2.1 of methodology. Our study doesn't involve the isolation of any new novel gene. Instead, it involves the transformation of the already reported *ChI* and *ChII* genes (accession numbers given) in to the local cotton variety.

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Figures

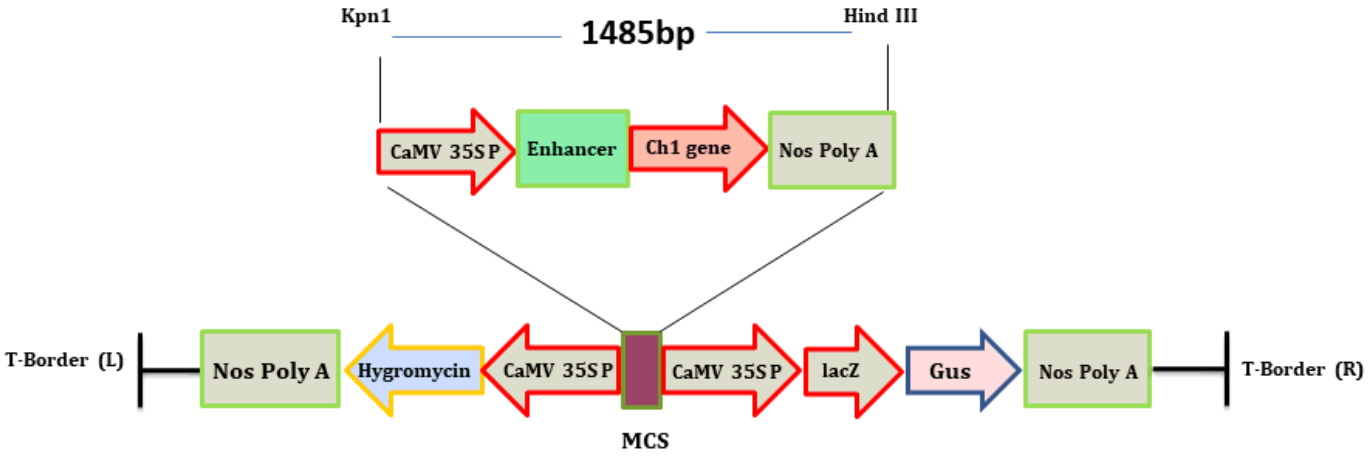

Figure 1

Schematic diagram of chitinase I cassette (*pCH I)*

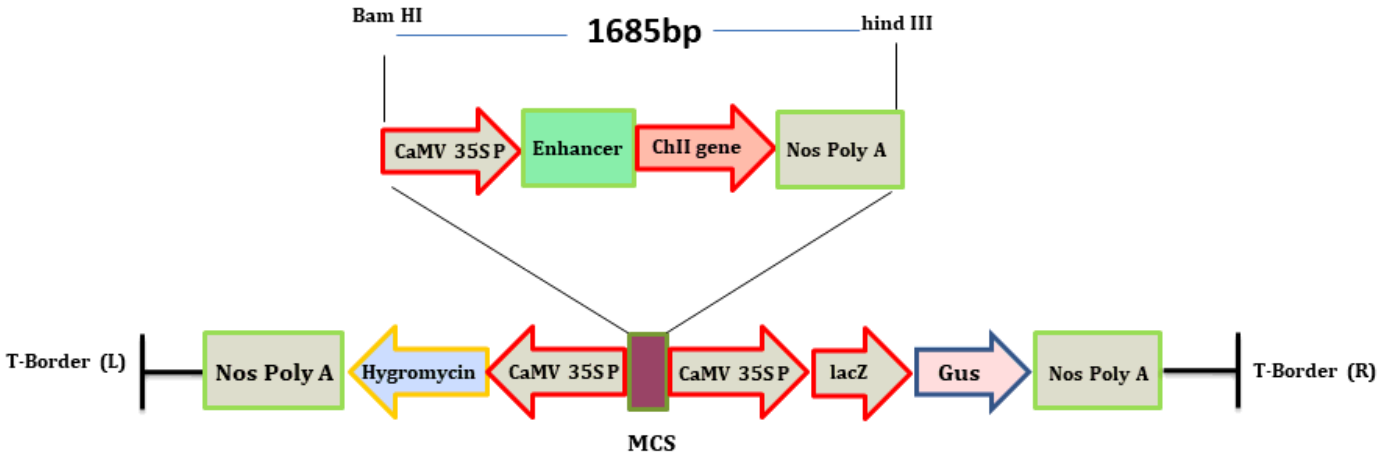

Figure 2

Schematic diagram of chitinase II cassette (*pCH II)*

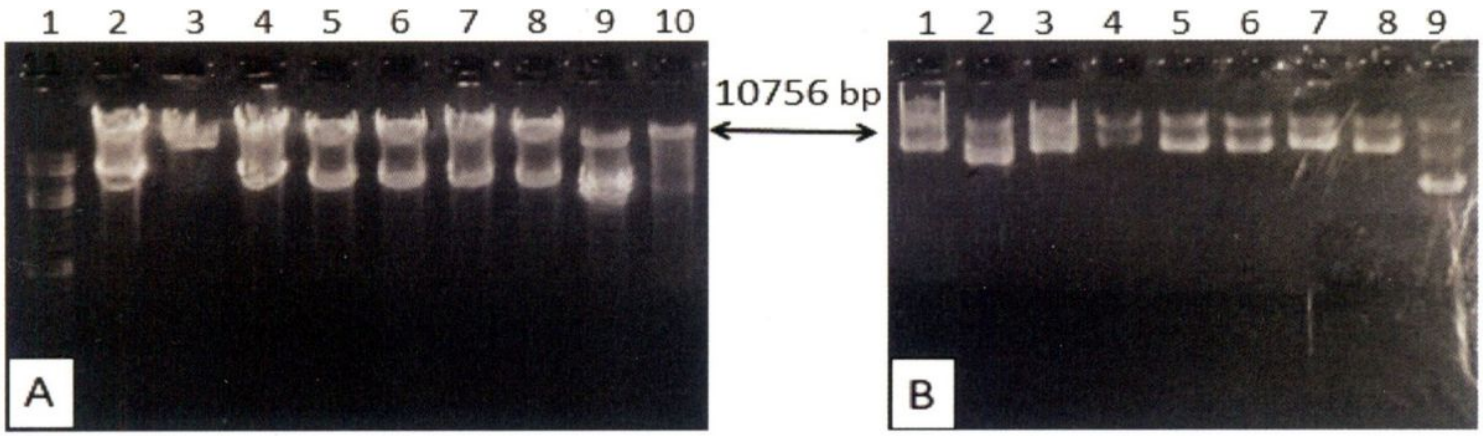

Figure 3

A) Screening of *E.coli* colonies for (pCAMBIA 1301_ *ChI)* by Restriction digestion;Lane1:1kb ladder;,Lane 2-10:Digested pCAMBIA with *Hind III & KpnI* and 1485bp released fragment of *ChI* gene cassette;: (B)Screening of *E.coli* colonies for (pCAMBIA 1301_ *ChII)* by Restriction digestion; Lane1-8:Digested pCAMBIA with *Hind III & BamHI* and 1685bp released fragment of *ChII* gene cassette.

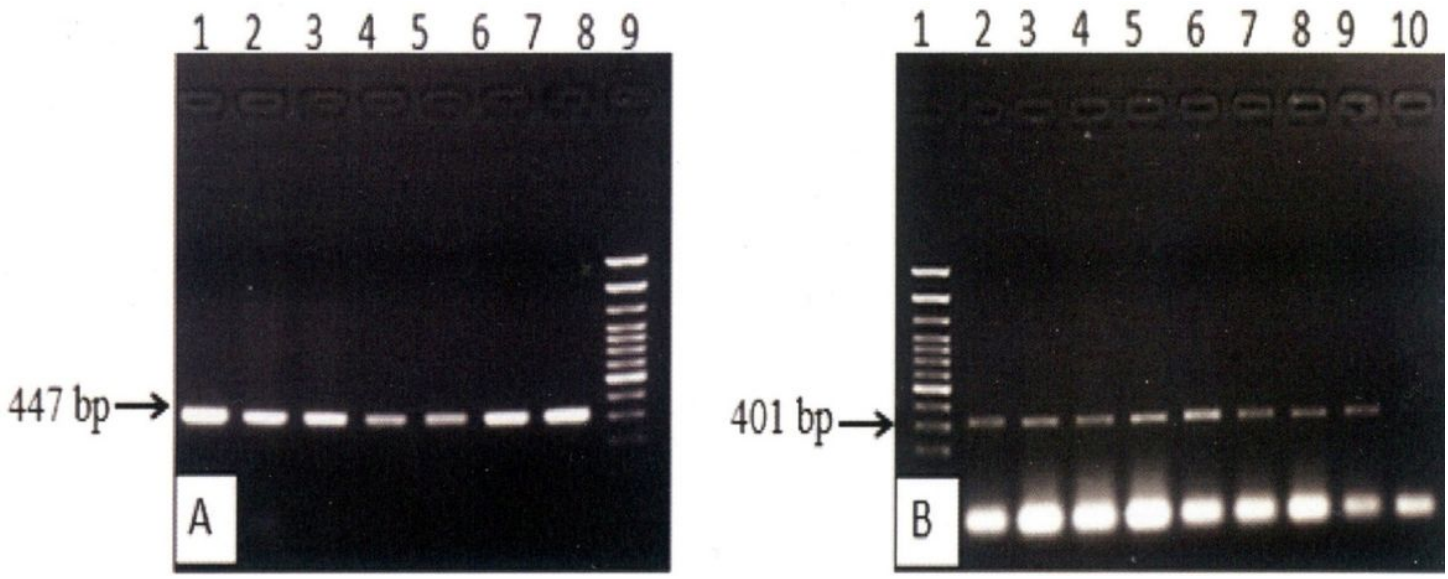

Figure 4

(A)Screening of *E.coli* colonies for *ChI* gene in pCAMBIA 1301 through PCR amplification; Lane 1-8: 447bp *ChI* amplicon, Lane 9 :100bp DNA ladder (B) Screening of *ChII* gene in pCAMBIA 1301 through PCR amplification; Lane 1: 100 bp DNA ladder Lane 2-9: 401 bp *ChII* amplicon, Lane 9 Unamplified negative colonies.

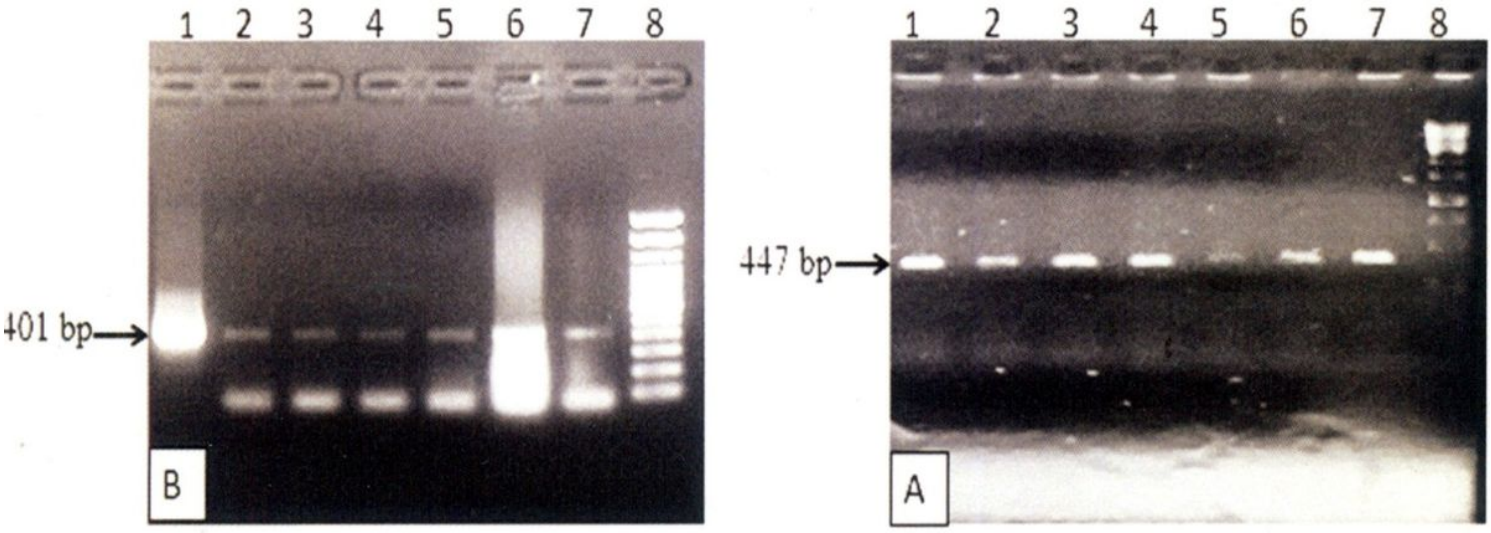

Figure 5

A) Confirmation of (pCAMBIA1301_*ChI*) in *Agrobacterium tumefaciens* LBA4404 through colony PCR; Lane 1: Positive control plasmid having (pCAMBIA1301_*ChI*); Lane 2-7: 447bp *ChI* amplicon containing colonies; Lane8: 100bp DNA ladder(B):Confirmation of (pCAMBIA1301_*ChII*) in *Agrobacterium*

tumefaciens LBA4404 through colony PCR; Lane1:positive control plasmid having (pCAMBIA1301_*ChII*) Lane 2-7: Screened colonies having 401bp *ChII* amplicon ; Lane 8: 100bp DNA ladder.

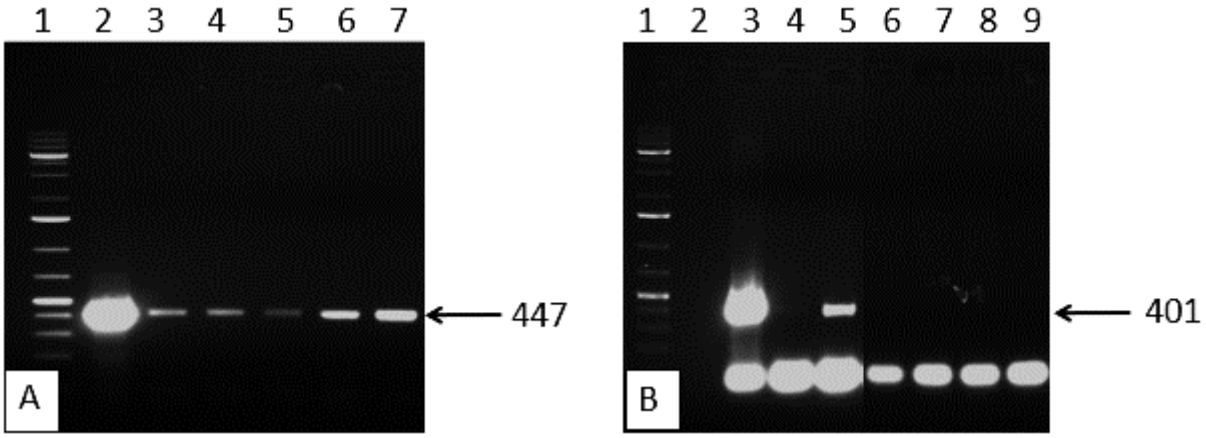

Figure 6

(A) PCR analysis of putative transgenic cotton plants in T_0 progeny for *Ch1* gene Lane 1: 100bp DNA ladder;Lane2: Positive control (plasmid pCAMBIA-1302 *Ch1*);Lane 3-7:Transgenic plants P1, P2, P3, P4, and P10 respectively) with amplification of 447bp fragment;(B) PCR analysis of putative transgenic cotton plants in T_0 progeny for *ChII* gene Lane 1:100bp DNA ladder;Lane 2: Negative control; Lane 3:Positive control (plasmid pCAMBIA_1301 *ChII*)) Lane 5: Pu tative transgenic plant 2 with amplification of 401bp fragment;Lane 4,6,7,8,9: are Negative plants (Non-transgenic plants).

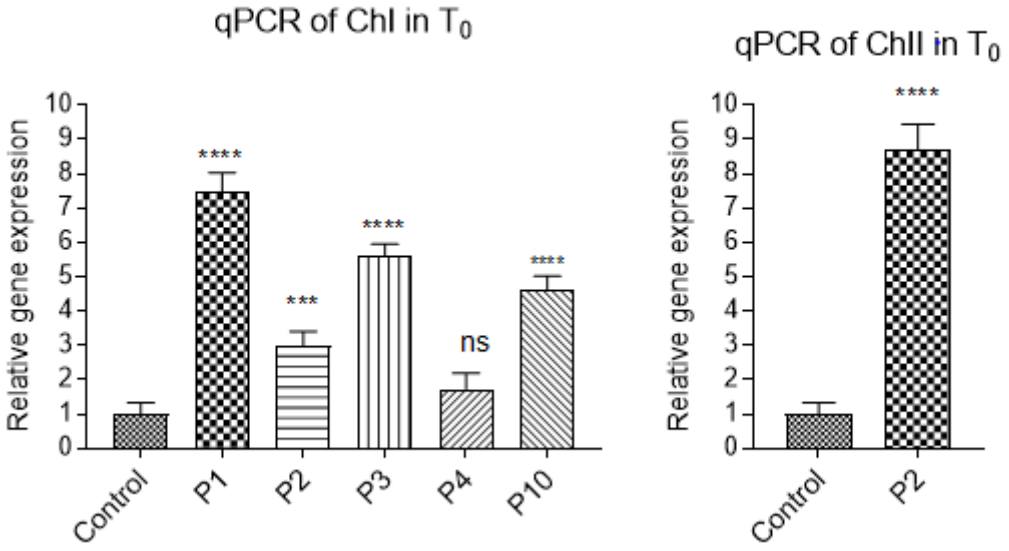

Figure 7

(A) Relative expression of *ChI* gene.(B) Relative expression of *ChII* gene. All values represent the average of technical and biological replicates. A steric indicate significance difference (**** P value <0.0001; *** P < 0.0005)

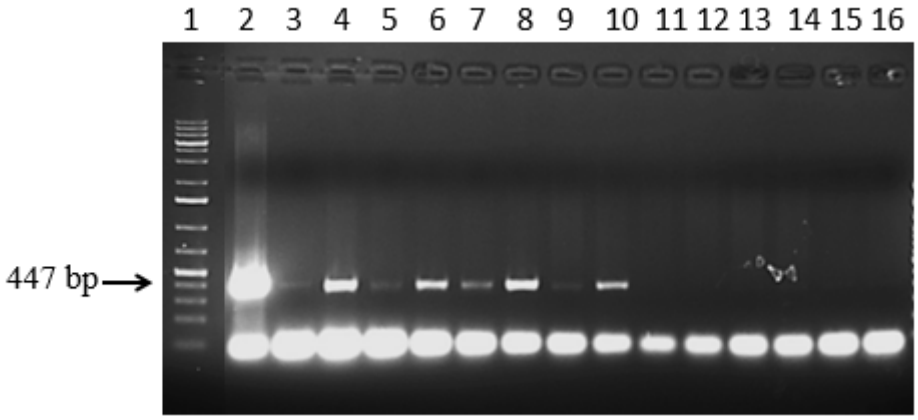

Figure 8

Analysis of transgenic cotton plants expressing *ChI* gene through PCR in T1 progeny;Lane 1:100 bp DNA ladder;Lane 2: positive control (Pcambia 1301_*ChI)*;Lane 3-10: Transgenic plants from with the amplification of 447 bp fragment ;Lane 11-16: Non-amplified segregated plants (Genomic DNA extracted from non-transgenic cotton plants)

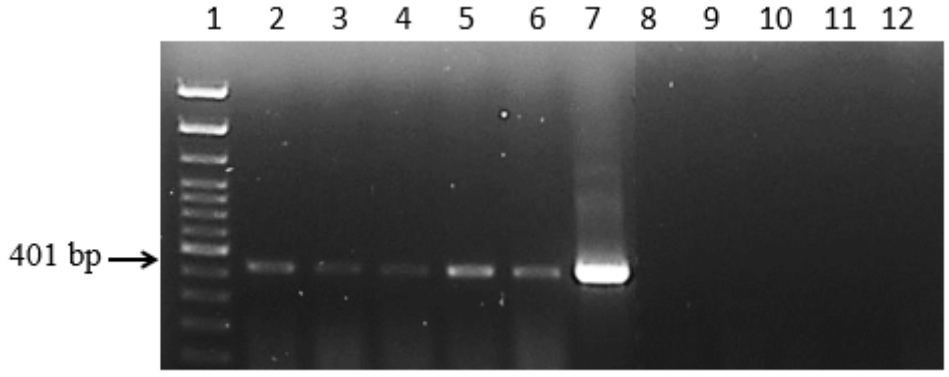

Figure 9

Amplified product transgenic cotton plants expressing *Ch II* gene by using gene specific primers in T1 progeny; Lane 1:100 bp DNA ladder;Lane 2-5: Transgenic plants with the amplification 401bp fragment;Lane7: positive control (Pcambia 1301_*Ch II)*;Lane 8-12: Non amplified segregated plants (Genomic DNA extracted from non-transgenic cotton plants)

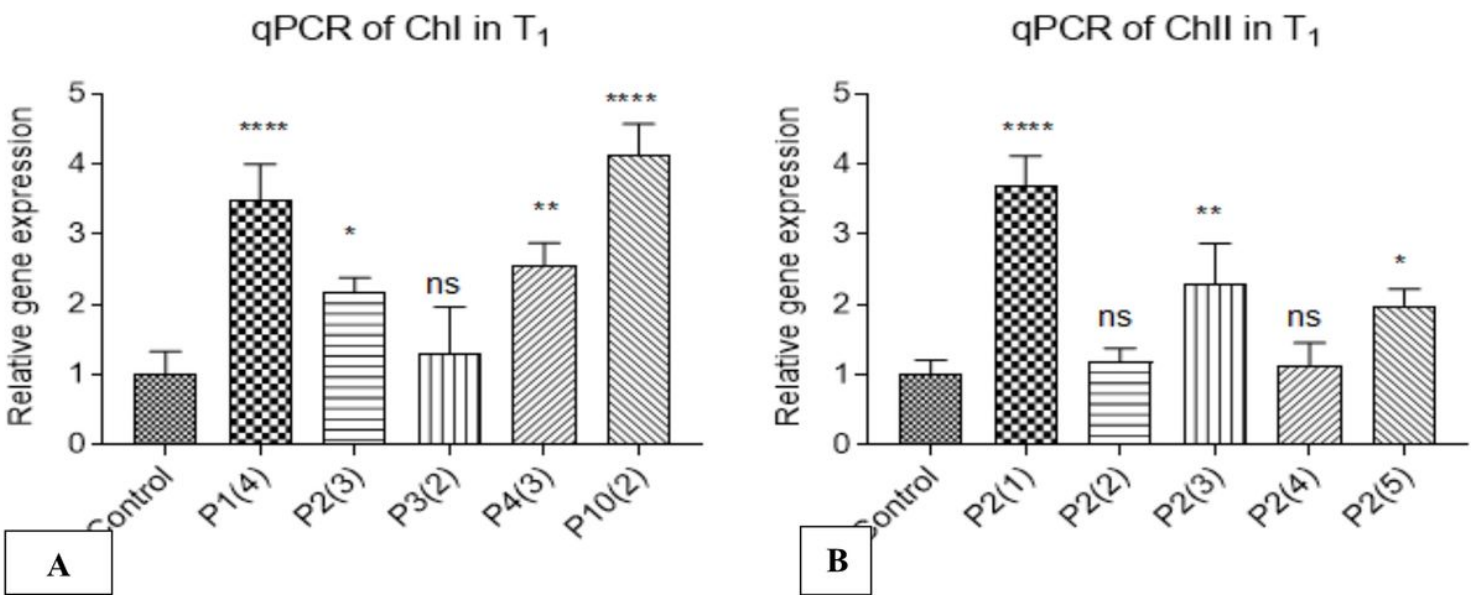

Figure 10

(A) Relative expression of *ChI* gene. A steric indicate significance difference (**** P value <0.0001; *** P < 0.0038; ;*P 0.0267) (B) Relative expression of *ChII* gene. All values represent the average of technical and biological replicates. A steric indicate significance difference (**** P value <0.0001; *** P < 0.0026; ;*P 0.0181)

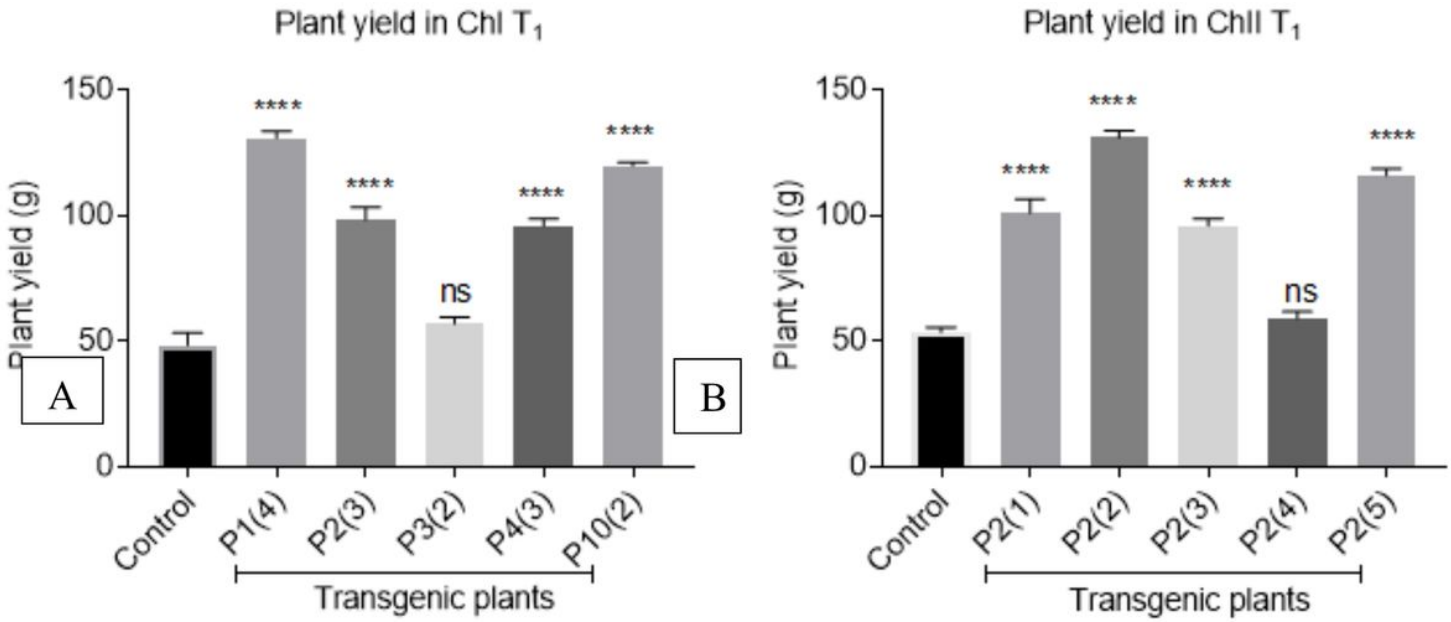

Figure 11

(A) Comparison of average yield per plant in transgenic line harboring *ChI* gene and Non-transgenic control line. (B) Average yield per plant of transgenic line harboring *ChII* gene and Non-transgenic control line. Each bar represent average of biological triplicate from control and transgenic lines. One way ANOVA anlysis was performed for statistical analysis. A steric indicate significance difference (**** P value <0.0001).

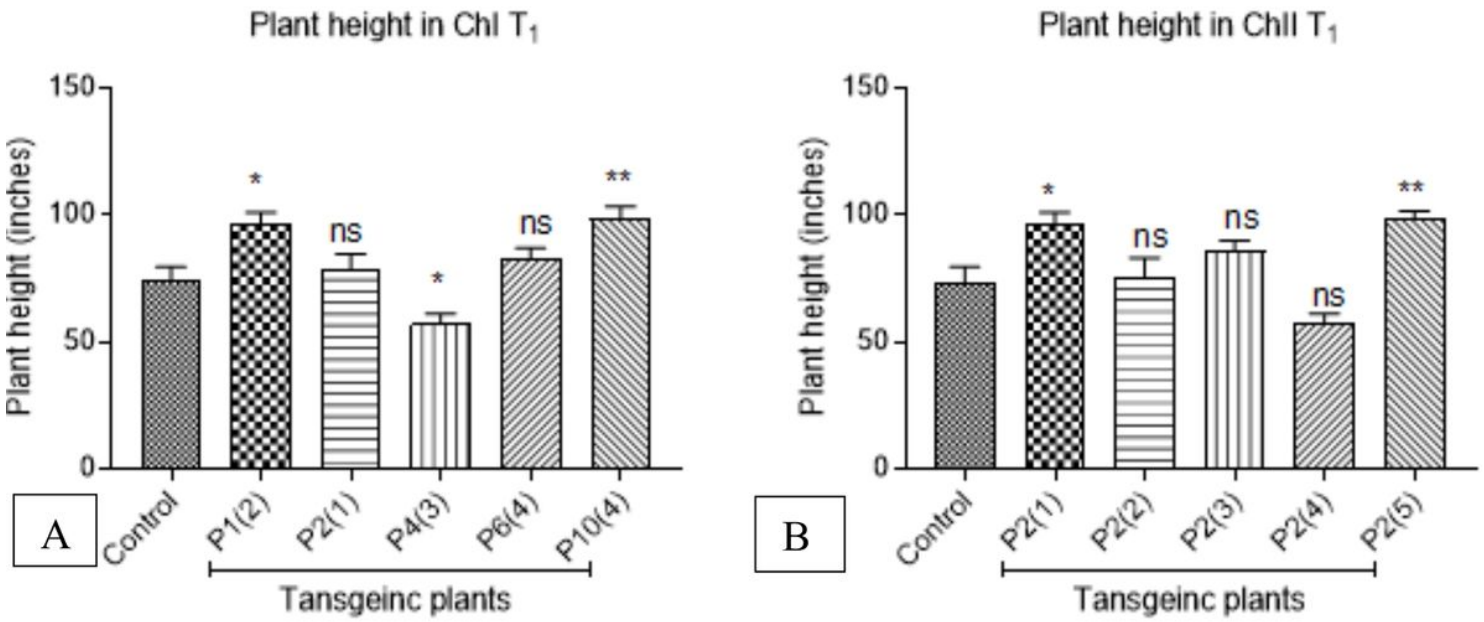

Figure 12

(A) Comparison of plant height in transgenic line harboring *ChI* gene and Non-transgenic control line. (B) Comparison of plant height in transgenic line harboring *ChII* gene and Non-transgenic control line Each bar represent average of biological triplicate from control and transgenic lines. One way ANOVA anlysis was performed for statistical analysis. A steric indicate significance difference (**P value <0.0016; *P value <0.0086)

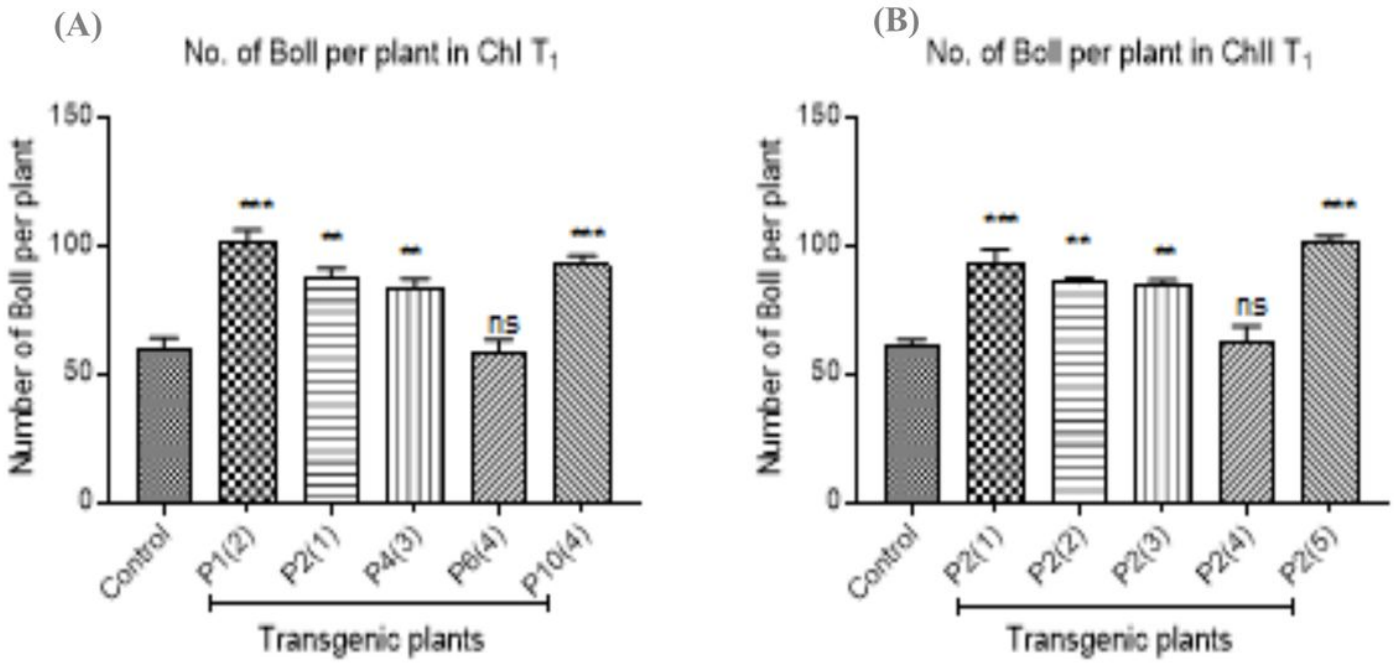

Figure 13

(A)Comparison of number of bolls per plant in transgenic line harboring *ChI* gene and Non-transgenic control line. (B) Comparison of number of bolls per plant of transgenic line harboring *ChII* gene and Non-transgenic control line. Each bar represent average of biological triplicate from control and transgenic lines. One way ANOVA anlysis was performed for statistical analysis. A steric indicate significance difference (***P value <0.0002; **P value <0.0021)

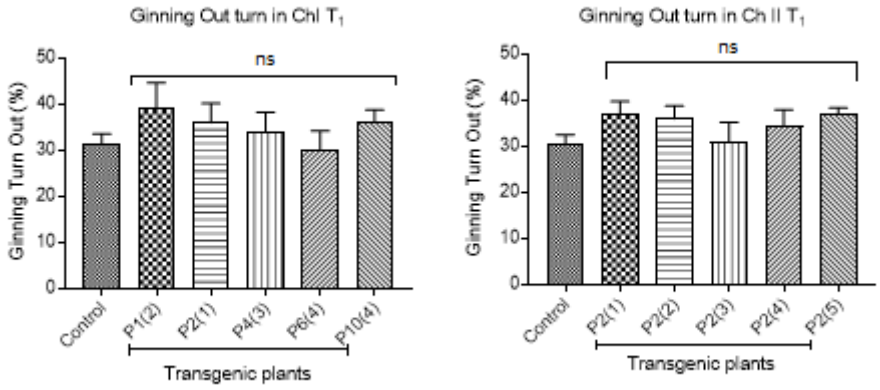

Figure 14

(A) Comparison of Lint (GOT) percentage of transgenic line harboring *ChI* gene and Non-transgenic control line. (B) Comparison of Transgenic line harboring *ChII* gene and Non-transgenic control line showing lint (GOT) percentage. Each bar is representative of mean value of three plants yield. One way ANOVA analysis was performed for statistical analysis.

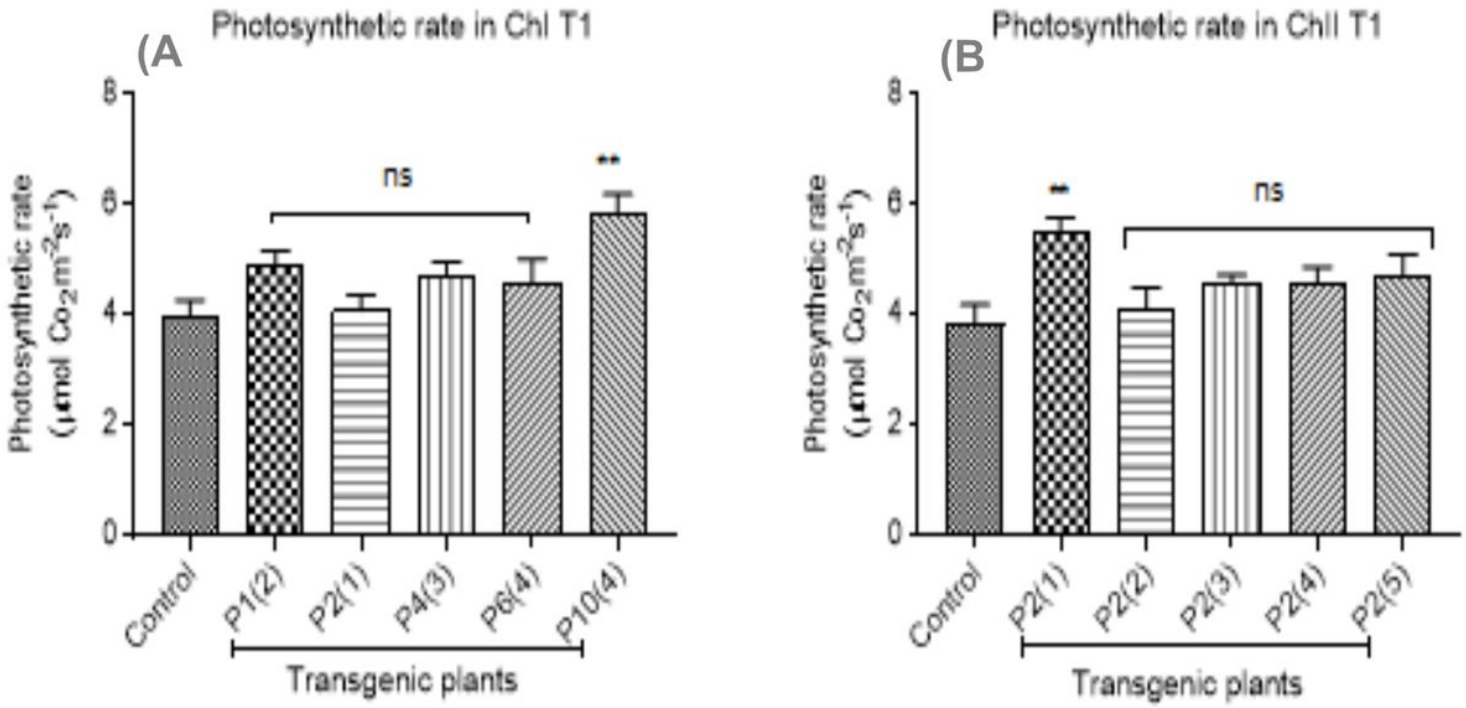

Figure 15

(A) Comparetive analysis of Photosynthetic rate of transgenic line harboring *ChI* gene and Non-transgenic control line. (B) Comparetive analysis of photosynthetic rate of Transgenic line harboring *ChII* gene and Non-transgenic control line. Each bar represent average of biological triplicate from control and transgenic lines. One way ANOVA anlysis was performed for statistical analysis. A steric indicate significance difference (**P value <0.0044).

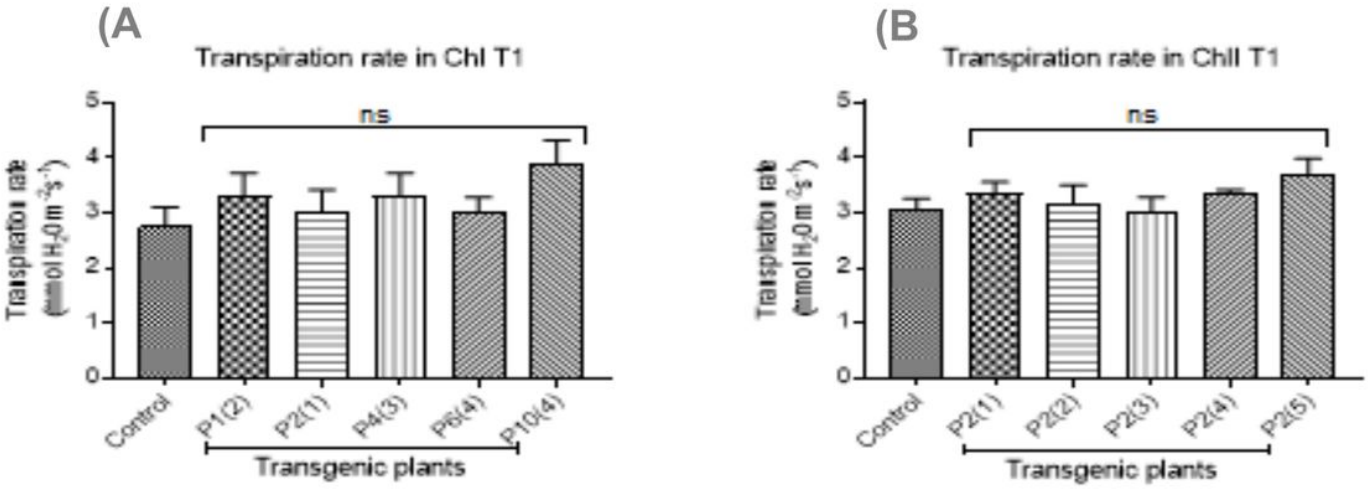

Figure 16

(A)Comparetive analysis of respiration rate of of transgenic line harboring *ChI* gene and Non-transgenic control line. (B)Comparative analysis of Transgenic line harboring *ChII* gene and Non-transgenic control line showing respiration rate. Each bar is representative of mean value of three plants. One way ANOVA analysis was performed for statistical analysis.

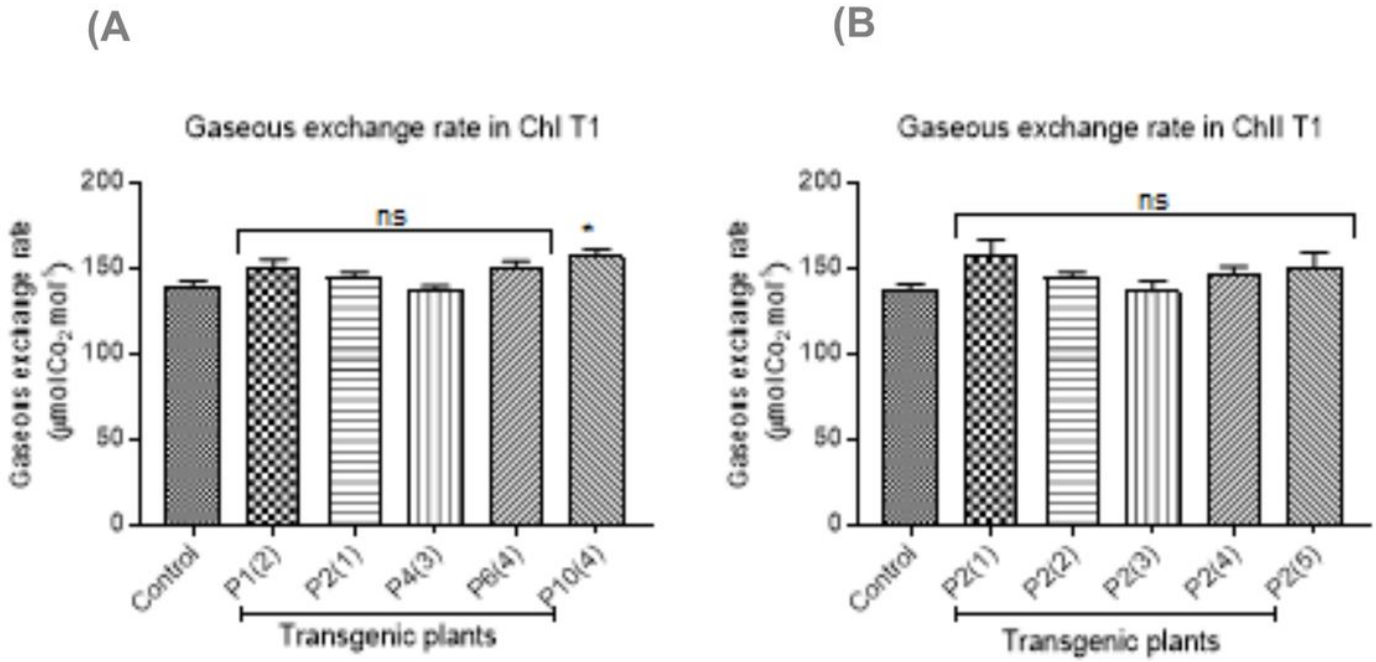

Figure 17

(A) Analysis of gaseous exchange rate in Transgenic line harboring *ChI* gene in contrast to Non-transgenic control line. (B) Analysis gaseous exchange rate in Transgenic line harboring *ChII* gene in contrast to Non-transgenic control line. Each bar represent average of biological triplicate from control and transgenic lines. Oneway ANOVA anlysis was performed for statistical analysis. A steric indicate significance difference (* P value <0.0117).

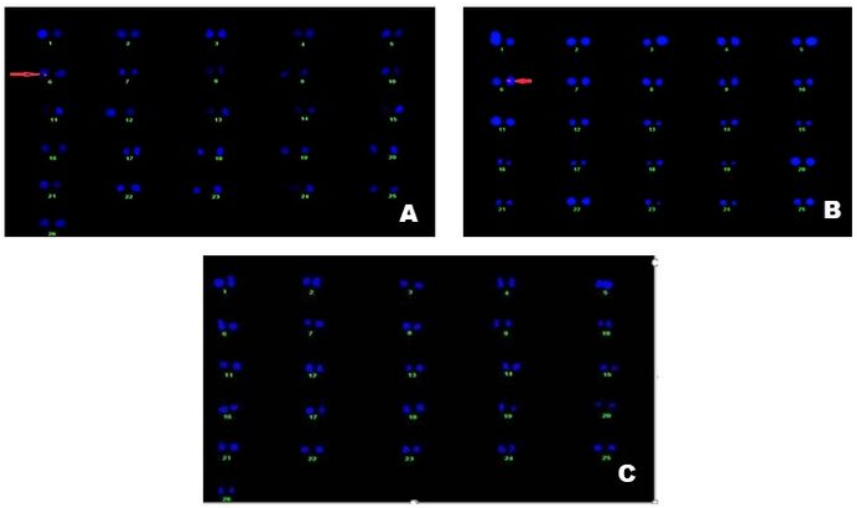

Figure 18

(A-B) FISH analysis; Determination of copy number and transgene location (C) No signal was observed in non-transgenic plant

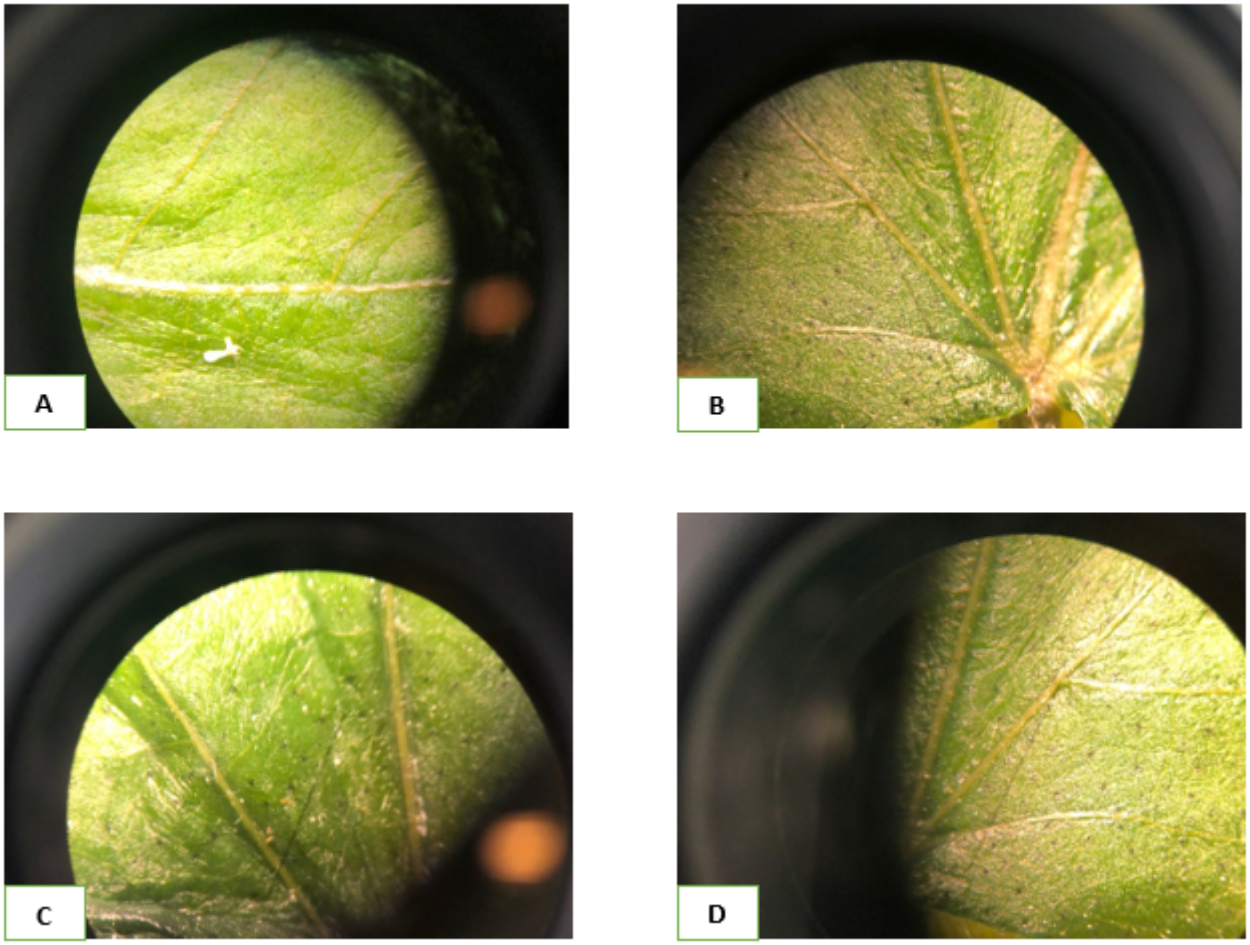

Figure 19

(A-B) Number of eggs produced by whitefly (*Bamisia tabaci*) were less on transgenic cotton leaf when observed under microscope. (C-D) Number of eggs produced by whitefly (*Bamisia tabaci*) on Non-transgenic cotton leaf were more when observed under microscope.

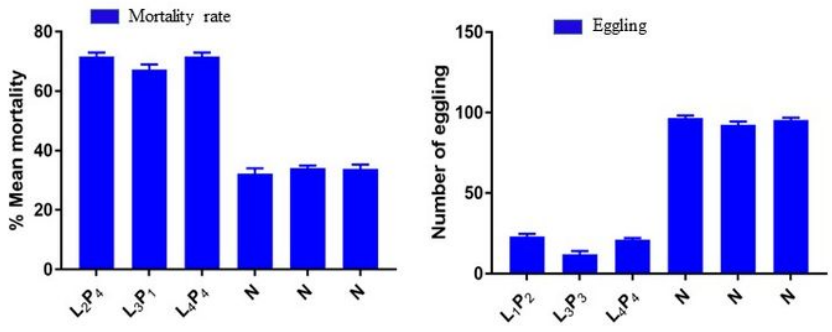

Figure 20

Percentage mortality and number of eggling in adult *Bamisia tabaci* feeding on transgenic (L_1P_2, L_3P_3, L_4P_4) and non-transgenic (N) control line. Number of plants n=3.

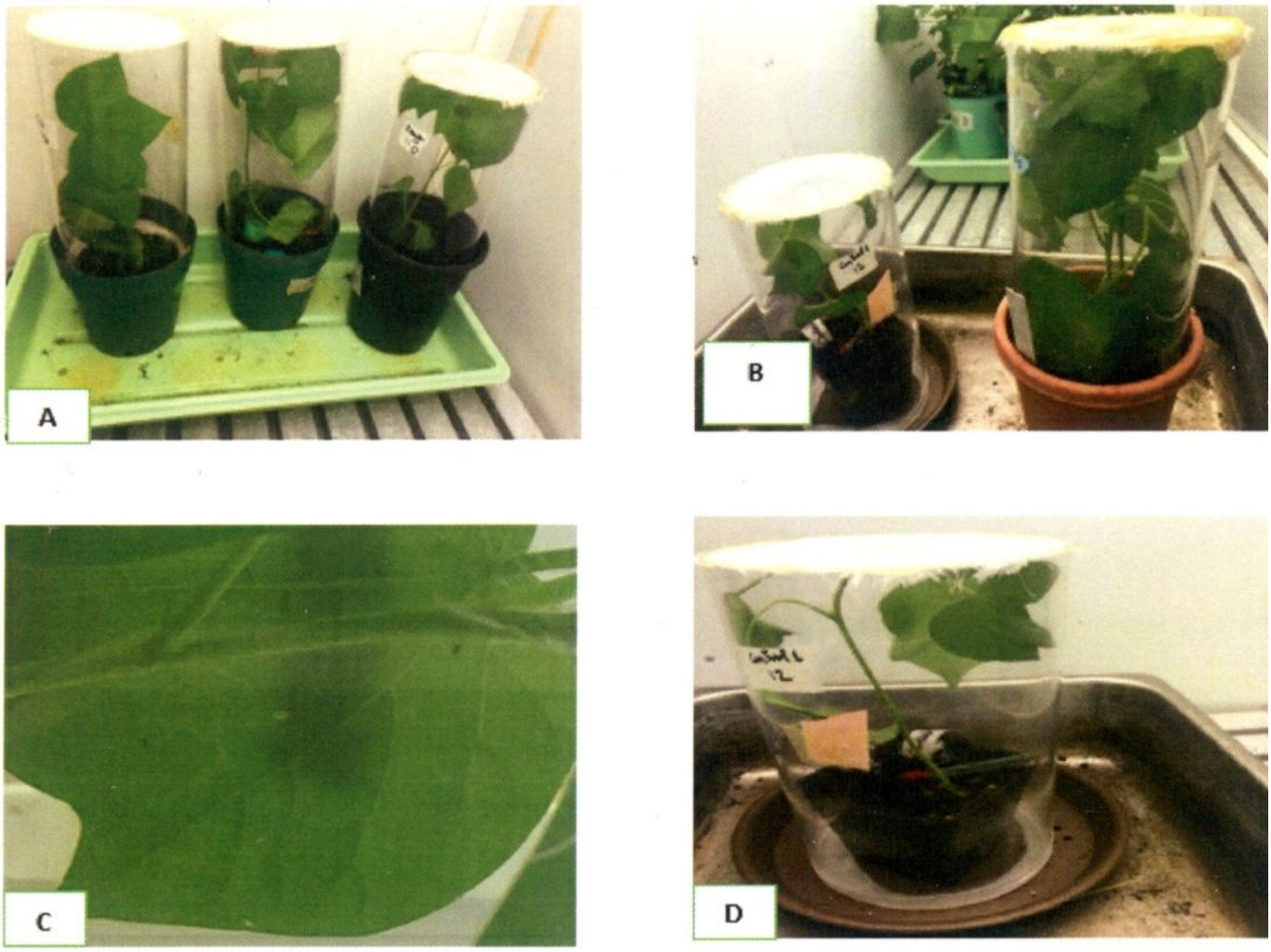

Figure 21

(A)Transgenic plant with *Bamisia tabaci* in glass cages (B) Non-transgenic plants with *Bamisia tabaci* in glass cages. (C) Counting of Whitefly feeding on non-transgenic control leaf (D) Counting of Whitefly feeding on transgenic control leaf.