Journal of Cotton Research

RESEARCH

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Barley chitinase genes expression revamp resistance against whitefly (*Bemisia Tabaci*) in transgenic cotton (*Gossypium hirsutum* L.)

BASHIR Samina[1,2], YAQOOB Amina[1], BASHIR Rohina[3], BUKHARI Shanila[2,4], SHAHID Naila[1], AZAM Saira[1], BAKHSH Allah[1*], HUSNAIN Tayyab[1], SHAHID Ahmad Ali[1] and RAO Abdul Qayyum[1]

Abstract

Background Chitinase is an enzyme that hydrolyzes chitin, a major component of the exoskeleton of insects, including plant pests like whiteflies. The present study aimed to investigate the expression of chemically synthesized barley *ch1* and *chi*2 genes in cotton (*Gossypium hirsutum*) through *Agrobacterium*-mediated transformation. Fifty-five putative transgenic cotton plants were obtained, out of which fifteen plants successfully survived and were shifted to the field. Using gene-specific primers, amplification of 447 bp and 401 bp fragments confirmed the presence of the *ch1* and *chi*2 genes in five transgenic cotton plants of the T_0 generation. These five plants were further evaluated for their mRNA expression levels. The T_0 transgenic cotton plants with the highest mRNA expression level and better yield performance in field, were selected to raise their subsequent progenies.

Results The T_1 cotton plants showed the highest mRNA expression levels of 3.5-fold in P10 (2) for the *ch1* gene and 3.7-fold in P2 (1) for the *chi*2 gene. Fluorescent in situ hybridization (FISH) confirmed a single copy number of *ch1* and *chi*2 (hemizygous) on chromosome no. 6. Furthermore, the efficacy of transgenes on whitefly was evaluated through an insect bioassay, where after 96 h of infestation, mortality rates of whitefly were calculated to be 78%–80% in transgenic cotton plants. The number of eggs on transgenic cotton plants were calculated to be 0.1%–0.12 per plant compared with the non-transgenic plants where egg number was calculated to be 0.90–1.00 per plant.

Conclusion Based on these findings, it can be concluded that the chemically synthesized barley chitinase genes *(ch1* and *chi*2) have the potential to be effective against insects with chitin exoskeletons, including whiteflies. The transgenic cotton plants expressing these genes showed increased resistance to whiteflies, resulting in reduced egg numbers and higher mortality rates.

Keywords Chitinase, Cotton, White fly, Transgene, Bioassay

*Correspondence:
Bakhsh Allah
abthebest@gmail.com; qayyumabdul77@yahoo.com
Full list of author information is available at the end of the article

Introduction

Cotton (*Gossypium hirsutum* L.) is the most important cash crop and the backbone of textile industry in the world (Yavuz et al. 2020). Cotton contributes about 0.8% share in the gross domestic product (GDP) and 4.1% value addition in agriculture sector in Pakistan. The decline of 6%–7% in cotton production was recorded in the recent past. Unfavorable weather conditions decreased water availability and various pathogens were the major reason behind this reduction in cotton production (Jatoi 2021). The whitefly (*Bemisia tabaci*) is one of the notorious cotton pests that causes severe crop losses in both field and greenhouse. *B. tabaci* not only decreases the rate of photosynthesis in plants but also able to transmit a number of plant pathogenic viruses including torradoviruses, ipomoviruses, criniviruses, ipomoviruses, and begomoviruses (Sani et al. 2020). It feeds on the phloem sap and excretes out the honey dews which transmit viral infections. About eleven hundred whitefly species have been reported so far, and three of them are predominantly known as vectors for plant viruses (Sufyan Tahir et al. 2021). However, *B. tabaci* is the most significant among them which possess the potential to directly transmit more than 250 species of begomoviruses (Geminiviridae). *B. tabaci* has been reported to cause severe damage to tomato, tobacco, brinjal, chilli, cotton, okra, and potato worldwide (Vyskočilová et al. 2019). The annual losses due to the white fly in many crops have been reported upto several billion US dollars. Several climatic factors triggered the outbreak of whitefly, but the widespread use of insecticides is one of the major causes for insecticidal resistance. The excessive use of these chemicals also cause many health risk to consumers (Din et al. 2021). The modern biotechnology approaches are, therefore, playing their role in promoting the plant defense mechanisms by introducing the biological insect control strategies.

Biological control agents are considered more effective and an alternative to chemical control measures. The pathogenicity of six isolates of *Metarhizium anisopliae* showed greater than 50% mortality on the *B. tabaci* (whitefly) Q biotype (Iwanicki et al. 2019), but as a biocontrol agent. *M. anisopliae* might decay quickly because of UV (Ultraviolet) radiation, rain, or other environmental variables.

The chitinases are getting more popularity due to their effectiveness against the whitefly, termite, coconut beetle, grasshoppers, and rice bug termites (Anwar et al. 2019). Chitin is a poly-β-1, 4-*N*-acetylglucosamine structural protein of pathogenic fungi, arthropods, molluscan shells, and crustaceans. The fungal cell wall comprised of almost 22%–24% while insect exoskeleton is made up of about 40%–45% chitin. Chitin degradation by the chitinase, therefore, promptly resulted in the insect death and increased insects' mortality (Yang et al. 2019). The chitinase gene can provide targeted protection against specific pests that are susceptible to chitin degradation. It offers a more specific and effective defense mechanism against insects and fungi that rely on chitin for their structural integrity. By incorporating the chitinase gene, cotton plants can potentially reduce the reliance on chemical pesticides. This can lead to reduced environmental pollution, decreased risks to non-target organisms, and improved safety for farmers and consumers. Keeping in view the significance of chitinases, the present study was aimed at developing transgenic cotton conferring resistance to *B. tabaci*. The *Agrobacterium*-mediated transformation was used to transform the chemically synthesized barley *ch1* and *chi*2 genes in local cotton cultivars. Insect bioassay against whitefly was conducted to evaluate the efficacy of developed transgenic cotton lines.

Materials and methods

Construct designing, synthesis, and cloning

The gene sequences of *ch1* and *chi*2 were retrieved from National Center for Biotechnology Information (NCBI) with accession numbers as P11955.4 and ACJ68105.1, respectively. The constructs containing the codon-optimized sequences of *ch1* and *chi*2 genes were developed under the control of constitutive CaMV 35S promoter. *ch1* construct was developed by cloning gene with overhangs of *Bam*HI and *Kpn*I, whereas *chi*2 construct was developed by cloning *chi2* gene with overhangs of *Bam*HI and *Hin*dIII restriction sites. The construct was chemically synthesized from https://www.biobasic.com/genesplash/. The synthesized cassettes in plant expression vector pCAMBIA 1301 were confirmed through restriction digestion analysis and polymerase chain reaction (PCR) to determine the presence and correct insertion of DNA fragments within the vector.

Agrobacterium-mediated plant transformation

A local cotton variety, named CEMB Klean cotton (CKC-1), was obtained from the Seed Biotechnology Laboratory, Centre Of Excellence in Molecular Biology (CEMB), University of the Punjab, Pakistan, and the genetic transformation of cotton was achieved using the shoot-apex-cut method as described by Rao et al. (2011) and Bakhsh et al. (2012). Three to five days-old seedlings of cotton were used to excise embryo and the processed embryos were cut with sharp surgical blade and transferred to Murashige and Skoog (MS) broth containing *Agrobacterium tumefaciens* LBA4404 harboring *ch1* and *chi*2 gene constructs and were incubated for one hour at 30 °C with continuous shaking. The delinted cotton embryos

were allowed to be dried on an autoclaved filter paper followed by transferring to the MS medium plates supplemented with kinetin (1 mg·mL^{-1} and 250 µg·mL^{-1} cefotaxime and co-cultivated on MS medium for the next 3 days at 25 °C ± 2 °C in a growth room with 16 h light and 8 h dark. Plantlets were shifted to the glass test tubes (autoclaved) having MS media with selection cefotaxime (250 µg·mL^{-1}), hygromycin (25 mg·L^{-1}) along with B5-vitamins (50 mmol·L^{-1} thiamine-HCl, 10 mmol·L^{-1} nicotinic acid, 10 mmol·L^{-1} pyridoxine–HCl, 100 mmol·L^{-1} myo-inositol, 2 mmol·L^{-1} glycine) and kinetin (1 mg·mL^{-1}) were kept under light until roots and shoots starts emerging in next 5–6 weeks by incubation at 25 °C ± 2 °C in a growth room with 16 h light and 8 h 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.

Confirmation of *ch1* and *chi*2 genes in transgenic cotton plants

The DNA isolation from the transformed cotton leaves was achieved using protocols reported by Horne et al. (2004) with little modification and the presence of transgene was detected by PCR amplification using gene specific primers [*ch1* (Act-F 5'_AACAGTGTGGTTCTCAGGCT_3' and ACT-R 5'_ AAGTAGCCCCTCTCTCTTGC_3'), and *chi*2 (Act-F 5'_GCAGCTTTCTTCGGACAGAC_3' and ACT-R 5'_ CCACATTCAAGACCGCCATT_3')]. The PCR conditions were optimized as initial denaturation at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 30 s, annealing at 65 °C (*ch1* gene) and 63.5 °C (*chi*2 gene) for 45 s, extension at 72 °C for 60 s, and a final extension at 72 °C for 10 min. The amplified products were resolved on 1.2% (1.2 g·(100 mL^{-1}) TAE buffer) agarose gel and visualized under UV light.

Relative expression of *chi*1 and *chi*2 genes in transgenic cotton plants

The RNA isolation from transgenic cotton plant leaves was done following the modified protocol by Jaakola et al. (2001). Revert Aid 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 *ch1* and *chi*2 genes was done through quantitative real-time PCR in transgenic and non-transgenic control cotton plants. The real-time PCR reaction was performed in a 96-well plate iQ5 cycler (BIO-RAD) PCR machine using the Maxima SYBR Green/ROX qPCR Master Mix (2×) (Thermo Scientific, K0221). For the data normalization, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene was used as internal control and non-transgenic plants used as the negative control. Samples were analyzed in triplicate by using the following housekeeping gene specific primers (Table 1).

Generation advancement of *chi*1 and *chi*2 transgenic cotton plants

Transgenic cotton plants P1, P2, P3, P4, and P10 harboring *ch1* gene while P2 possess both *ch1* and *chi*2 in T_0 generation. Transgenic plants T_1 with the best molecular and good morphological/physiological characteristics were selected for further advancement. Non-transgenic cotton plants were also raised as the control in a separate line to study their molecular and physiological characteristics in the comparative way.

Assessment of physiological traits of transgenic cotton plants

The various physiological parameters (photosynthetic rate, transpiration rate, and gaseous exchange rate) were measured in triplicates using CIRAS-3 portable photosynthesis system infrared gas analyzer (PP Systems, USA) on a fully extended cotton leaf of both transgenic and non-transgenic (control) cotton line. Measurements were made with specific adjustment of the molar flow rate of air at 403.3 µmol·min^{-1} PAR of leaf surface at 1 000–1 711 µmol·m^{-2}·s^{-1}, the temperature of a leaf at 28.4–32.4 °C, ambient temperature 22.4–27.9 °C and ambient CO_2 concentration was set to be 352 µmol·mol^{-1}.

Assessment of morphological traits

Morphological characteristics (height**,** ginning out turn percentage (GOT, %), and the number of bolls per plant) of the transgenic cotton and non-transgenic cotton lines were also evaluated in T_1 progeny.

Fluorescence *in situ* hybridization (FISH)

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

Table 1 GAPDH primer sequence

5'-3' sequence	T_m/°C	Product size /bp
F-AGGAAGAGCTGCTTCGTTCA R- CCGCCTTAATAGCAGCAGCTTTG	60	106

microscope (Olympus Model BX6l). Blue (DAPI) and red63 filters were used to detect fluorescent signals.

Insect bioassay of whitefly in transgenic cotton plants

The T_2 of transgenic cotton plants were grown in a glasshouse at 37 °C ± 2 °C, 14 h light/10 h dark and about 60% relative humidity. The plants were left uninfected for seven days by carefully isolating them in a net cage. The insect bioassay was performed using three biological replicates from both transgenic and non-transgenic (control) cotton lines of T_2 were selected; approximately 0–24-h old whiteflies were caught using a manual aspirator and kept on ice to reduce the environmental stress. The 15–20 whiteflie pairs were released carefully on cotton plant (4–6 leaves stage) inside the net cage. After 96 h of infestation, microscopic observation was made to calculate the number of eggs on the lower and the upper surface of the leaves and count the number of adult/nymph white flies on transgenic/non-transgenic (control) cotton plants dead in 96 h. Mortality was calculated on both transgenic and non-transgenic (control) by using given formula:

$$\text{Mortality}(\%) = \frac{\textit{The number of dead adult/Nymph}}{\textit{Total number of adult/Nymph}}$$

Microscopic observation was made to calculate the number of eggs on the lower and the upper surface of the leaves.

Statistical and data analysis

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

Results

Confirmation of recombinant construct

The digested plasmid was resolved on 0.8% gel and the restricted fragments of 1485 bp (base pair) and 1685 bp confirmed the successful cloning of *ch1* and *chi*2 cassettes, respectively, in pCAMBIA1301vectors. Likewise, the PCR amplicon at 447 bp and 401 bp further confirmed the successful ligation of *ch1* and *chi*2 genes in pCAMBIA 1301 (Supplementary Fig. S1).

Transformation of (pCAMBIA1301 *ch1*) and (pCAMBIA1301_ *chi2*) constructs into *Agrobacterium tumefaciens* LB4404

The amplification product of 447 bp and 401 bp obtained by using gene specific primers of *ch1* and *chi*2 confirmed successful introduction of recombinant plasmids into *Agrobacterium* (as shown in supplementary Fig. S2A).

Molecular analyses of putative transgenic plants (T_0)

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 *ch1* while amplification of *chi*2 was obtained only in cotton plant (P2) along with *Ch1* (as shown in supplementary Fig. S2B).

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

Quantitative real-time qRT-PCR was used to measure the relative mRNA expression levels of *ch1* and *chi*2 genes in T_0. The mRNA transcripts of T_0 transgenic cotton plants (P1, P2, P3, P4, and P10) expressing *ch1* and *chi*2 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) were then amplified exponentially using real-time thermocycler machine employing gene-specific real-time primers. The mRNA expression level of *ch1* in T_0 was found to be 7.5-fold in P1, 2.9-fold in P2, 5.8-fold in P3, non-significant in P4 and 4.9-fold in P10 as compared with non-transgenic control cotton plants. The maximum obtained expression level was 7.5-fold for *chi*1 in P2 plant, while the expression level of *chi*2 was measured 8.7-fold in P2 plant than non-transgenic cotton plants in T_0 (Fig. 1A and B). The five transgenic plants in T_0 were raised to get T_1. The mRNA expression level of *ch1* in T_1 was found to be 3.5-fold in P1 line plant number 4, 2.4-fold in P2 line plant 3, non-significant in P3 line plant 2, 2.7-fold in P4 line plant 3, and 4.3-fold in P10 line plant 2 as compared with non-transgenic control cotton plants. Only plant 2 was positive for *chi*2 in T_0, further mRNA expression was studied in P2 lines, revealing the maximum obtained expression level was almost 3.7-fold (Fig. 1C and 1D).

Generation advancement of *ch1* and *chi2* transgenic cotton plants

The progeny of these five cotton plants was raised to advanced T_1 lines as each confirmed as separate insertion. Conventional PCR amplification was used to confirm the successful gene inheritance in the advanced generation of transgenic cotton lines as shown in Supplementary Fig. S3. The amplification of 447 bp was evident in three cotton plants of the progeny 1 (line 1) while amplification

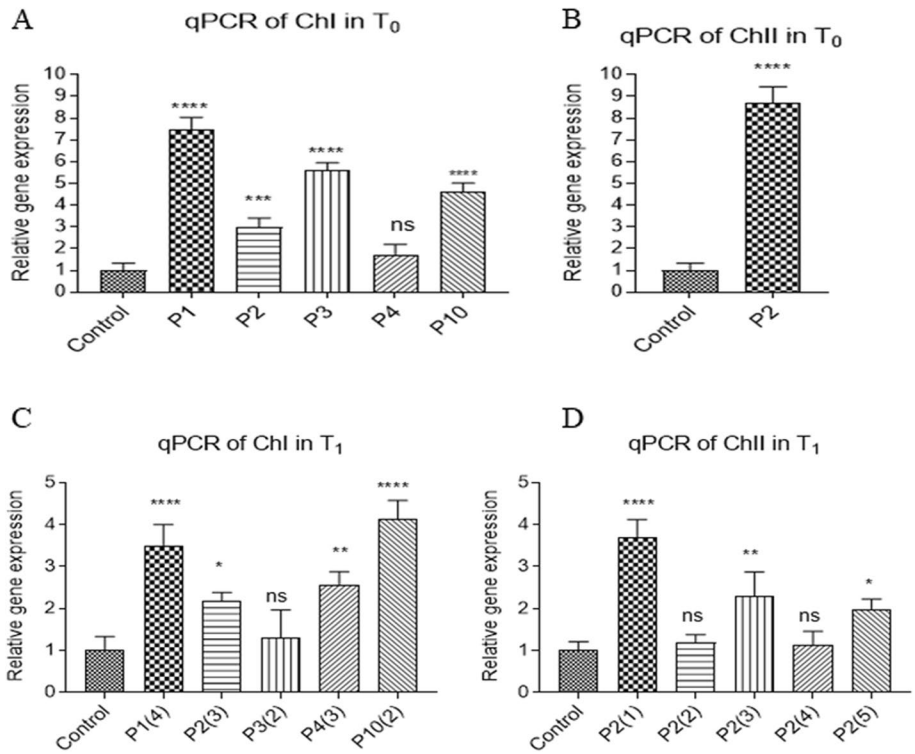

Fig. 1 **A** Relative expression of ChI gene. **B** Relative expression of ChII gene. All values represent the average of technical and biological replicates. A steric indicate significance difference (**** $P < 0.0001$; *** $P < 0.0005$). **C** Relative expression of ChI gene. A steric indicate significance difference (**** $P < 0.0001$; *** $P < 0.0038$;;*P 0.0267) **D** Relative expression of ChII gene. All values represent the average of technical and biological replicates. A steric indicate significance difference (**** $P < 0.0001$; *** $P < 0.0026$;;*P 0.0181)

of 447 bp and 401 bp was found only in two plants of progeny 2 (line 2), similarly five plants were found to harbor *ch1* in plant progeny 3 (line 3) while progeny of plant 4 and 5 were found to have gene amplification of *ch1* in one plant each. Similarly, plants having the highest mRNA expression in T_0 were further subjected to be evaluated for their expression in T_1. In T_1, the highest mRNA expression level of 3.5-fold in P10(2) for *ch1* gene and 3.7-fold in P2(1) for *chi*2 gene were measured in comparison to the non-transgenic control cotton (Fig. 1C and D).

Morphological and physiological characteristics of transgenic cotton plants

About 129 g of cotton yield was calculated in the transgenic cotton plants [p1 (2)] expressing *ch1* gene while a maximum of 130 g of cotton yield was determined in [p2 (1)] transgenic cotton plant expressing the *chi*2 gene. For all the genes, almost all transgenic plants had significant ($P < 0.000\ 1$) increase in yield compared with their non-transgenic counterpart (49 g) except in P4(3) and P6(4) transgenic plants where no statistically significant ($P < 0.000\ 1$) differences were observed in comparison to the control plants (Fig. 2 A1 and A2).

Nearly all the transgenic plants expressing either *ch1* gene or the *chi*2 gene were found to be significantly ($P < 0.05$) taller than the control plants. Transgenic plants [p1(2) and p10(4)] expressing *ch1* gene were found to have a maximum height of 98 and 98.5 inches, respectively. Correspondingly, a maximum height of about 98.5 and 98.7 inches were also calculated in similar transgenic plants [p2(1) and p2 (5)] expressing *chi*2 gene. On the other hands, non-significant ($P < 0.001$) results in height in comparison to the control (75 inches) was observed in P4(3) and P6(4) transgenic plants expressing *ch1* similarly transgenic plants [p2(2), p2(3) and p2 (4)] showed non-significant ($P < 0.001$) results in comparison to control for *chi*2 genes, respectively (Fig. 2 B1 and B2).

Transgenic plant P1(2) expressing the *ch1*gene was found to have a maximum number of bolls per plant (102). While a maximum of 103 bolls per plant were obtained in the cotton transgenic plant and P2(5) expressing *chi*2 gene. Nearly all the transgenic plants showed significant ($P < 0.0002$) increase in the number of bolls per plants in comparison to the control plants (60 bolls) except the P4 (6) transgenic plants for *ch1* gene and P2(4) where statistically significant ($P > 0.0002$)

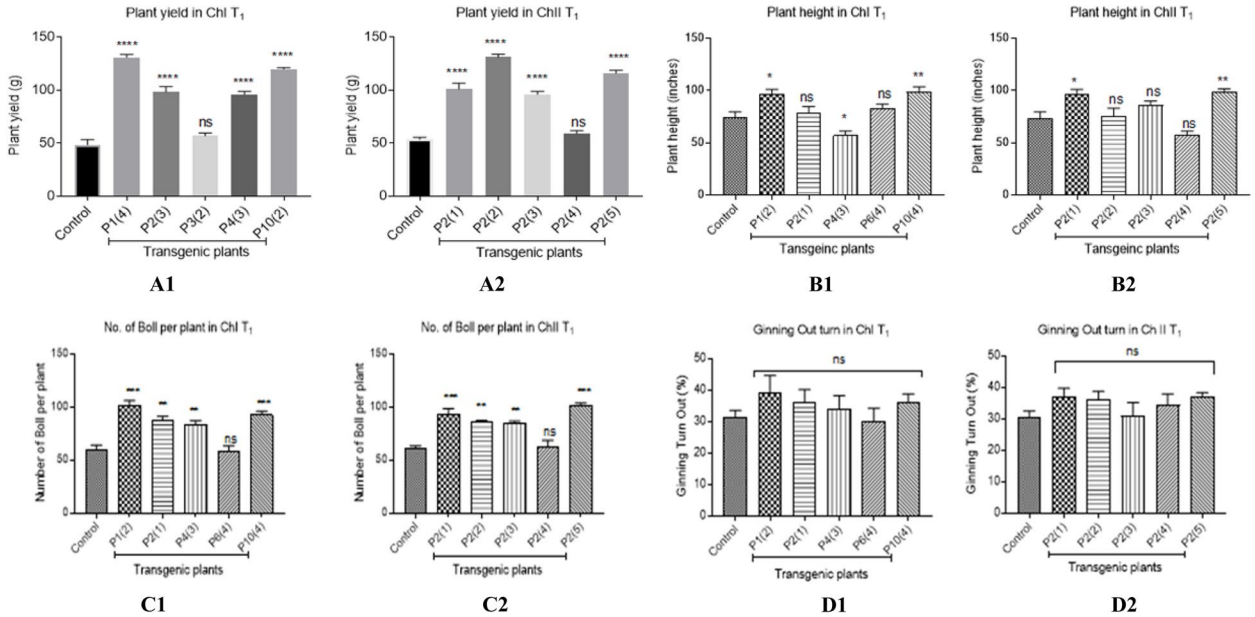

Fig. 2 (A1) Comparison of average yield per plant in transgenic line harboring *Chl* gene and non-transgenic control line; (A2) Average yield per plant of transgenic line harboring *ChII* gene and non-transgenic control line. Each bar represents an average of biological triplicate from control and transgenic lines. One way ANOVA anlysis was performed for statistical analysis. A steric indicate significance difference (*****P*< 0.0001). (B1) Comparison of plant height in transgenic line harboring *Chl* gene and non-transgenic control line; (B2) Comparison of plant height in transgenic line harboring *ChII* gene and non-transgenic control line each bar represents average of biological triplicate from control and transgenic lines. One way ANOVA anlysis was performed for statistical analysis. A steric indicate significance difference (***P*< 0.0016; * *P*< 0.0086). (C1) Comparison of the number of bolls per plant in transgenic line harboring *Chl* gene and non-transgenic control line; (C2) Comparison of the number of bolls per plant of transgenic line harboring *ChII* gene and non-transgenic control line. Each bar represents an average of biological triplicate from the control and transgenic lines. One way ANOVA anlysis was performed for statistical analysis. A steric indicate significance difference (****P*< 0.0002; ** *P* < 0.0021). (D1) Comparison of lint (GOT) percentage of transgenic line harboring *Chl* gene and non-transgenic control line. (D2) Comparison of transgenic line harboring *ChII* gene and non-transgenic control line showing lint (GOT) percentage. Each bar is representative of the mean value of three plants' yield. One way ANOVA analysis was performed for statistical analysis

differences were observed in the number of bolls per plant compared to the control plants (Fig. 2 C1 and C2).

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 non-significant ($P > 0.001$) 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 (Fig. 2 D1 and D2).

The portable infrared gas analyzer (IRGA) was used to determine the net photosynthetic activity in fully expanded transgenic cotton leaves and that of non-transgenic 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 *ch1* gene as well the P2 (1) transgenic plant expressing *chi*2 where significant ($P < 0.0044$) differences in photosynthetic rate were recorded when compared with the non-transgenic control plants (Fig. 3 A1 and A2).

The transpiration rates of both transgenic and the control plants were determined, and the data was presented in Fig. 3B1 and B2. Nearly all transgenic plants showed statistically non-significant gaseous exchange rate compared with the control plants (Fig. 3 C1 and C2).

Determination of the transgene copy number and location

One plant from each transgenic cotton line in T_2 was subjected for determination of the copy number and transgene location at different stages of cell division, prophase, metaphase, and interphase using *ch1* and *chi*2 specific probe. The transgenic cotton plant from line L_3P_2 showed one copy number at chromosome no. 6 for gene *ch1* while L_2P_2 also showed single copy no. for *chi*2 at chromosome no 6 but at different chromatids whereas, no signal was observed in the non-transgenic control cotton plant (Fig. 4).

Insect bioassays

Whitefly eggs were calculated on advance generation T_2S_2 line. Cotton plant leaves were kept under microscope in comparison to the control non-transgenic plants. The ratio

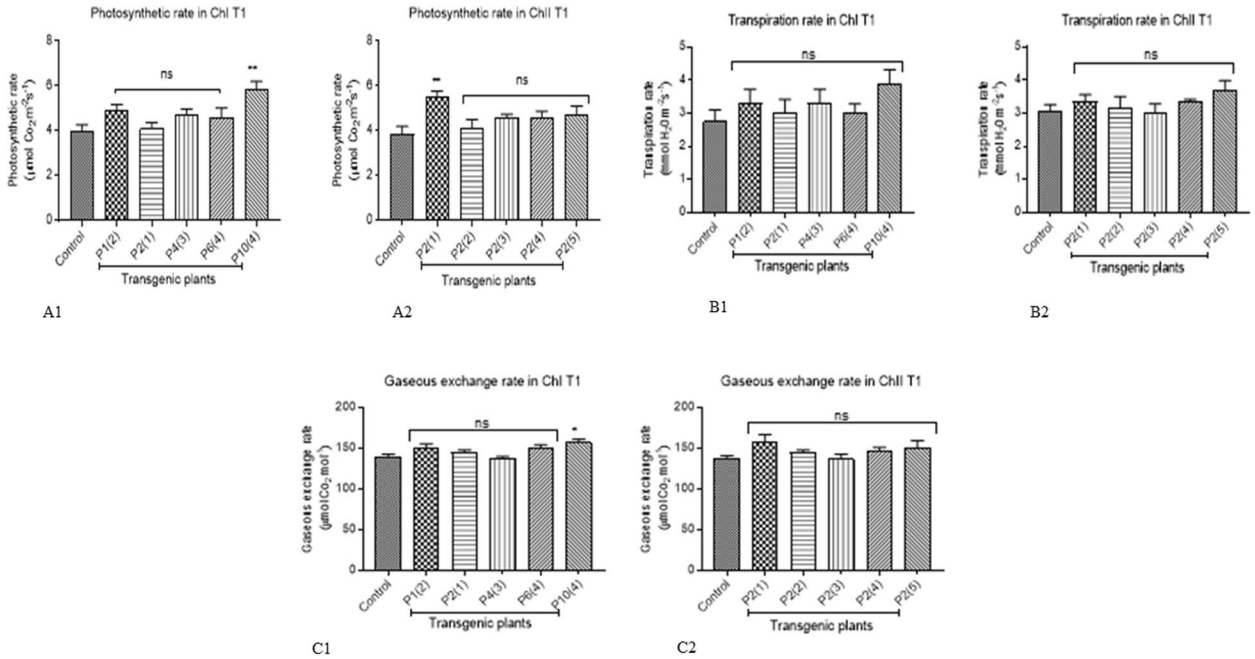

Fig. 3 (A1) Comparative analysis of Photosynthetic rate of transgenic line harboring *ChI* gene and non-transgenic control line; (A2) Comparetive analysis of photosynthetic rate of transgenic line harboring *ChII* gene and non-transgenic control line. Each bar represents an average of biological triplicate from control and transgenic lines. One way ANOVA anlysis was performed for statistical analysis. A steric indicate significance difference (**$P < 0.0044$). (B1) Comparative analysis of respiration rate of transgenic line harboring *ChI* gene and non-transgenic control line; (B2) Comparative analysis of Transgenic line harboring ChII gene and non-transgenic control line showing respiration rate. Each bar is representative of the mean value of three plants. One way ANOVA analysis was performed for statistical analysis. (C1) Analysis of gaseous exchange rate in transgenic line harboring *ChI* gene in contrast to non-transgenic control line; (C2) Analysis gaseous exchange rate in transgenic line harboring *ChII* gene in contrast to non-transgenic control line. Each bar represents an average of biological triplicate from control and transgenic lines. Oneway ANOVA anlysis was performed for statistical analysis. A steric indicate significance difference (*$P < 0.0117$)

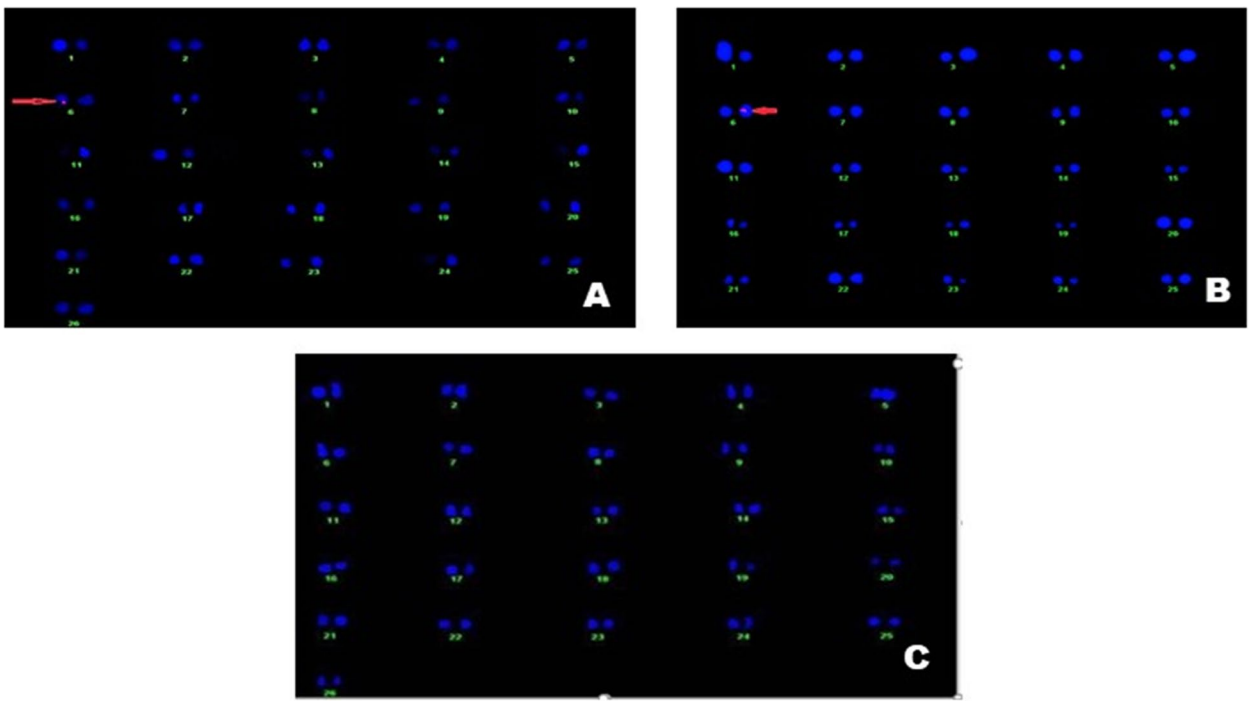

Fig. 4 **A-B** FISH analysis; Determination of the copy number and transgene location. (**C**) No signal was observed in non-transgenic plant

of eggs in transgenic cotton leaves was 10% to 22% while 90% to 98% was calculated in non-transgenic control cotton leaves (Fig. 5). Similarly, the average mortality ratio of whitefly was 70% to 80% found in transgenic plants as compared with 30% of whitefly in the non-transgenic control cotton plant (Fig. 6). It is evident from results that chitinase has its impact on whitefly exoskeleton which help in reduction of their overall population in the form of eggs as well as adults.

Discussion

Chitinases are proteins generated by plants as defence machinery against various pests including fungi and insects like whitefly owing to their potential to hydrolyse chitin an outermost exoskeleton of many insects and fungi. In plant transformation traits from different species can be introduced in desired plant species as acquired through conventional breeding technology (Ziemienowicz et al. 2014). The background knowledge of fungi and its relation with chitinase were utilized to make possible the expression of gene sequence in cotton for chitinase as protective barrier against broad range of fungi as was done by Bolar et al. (2000). Plants produce endochitinases that have been reported to confer resistance in plants against fungal disease (Jabeen et al. 2015; Iqbal et al. 2012). The expression of barley chitinase gene sequence in cotton had previously been used for its ability to develop

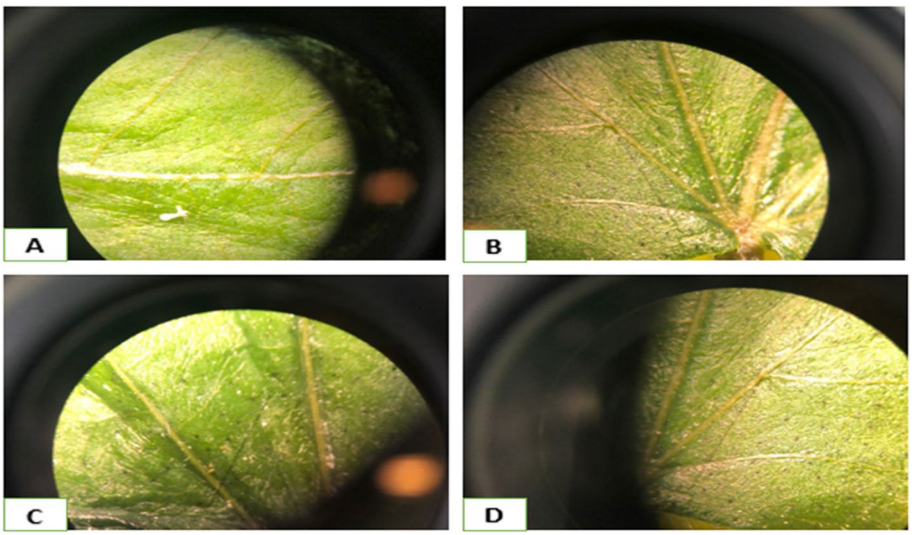

Fig. 5 **A-B** The number of eggs produced by whitefly (*Bamisia tabaci*) were less on transgenic cotton leaf when observed under microscope. **C-D** The number of eggs produced by whitefly (*Bamisia tabaci*) on non-transgenic cotton leaf were more when observed under microscope

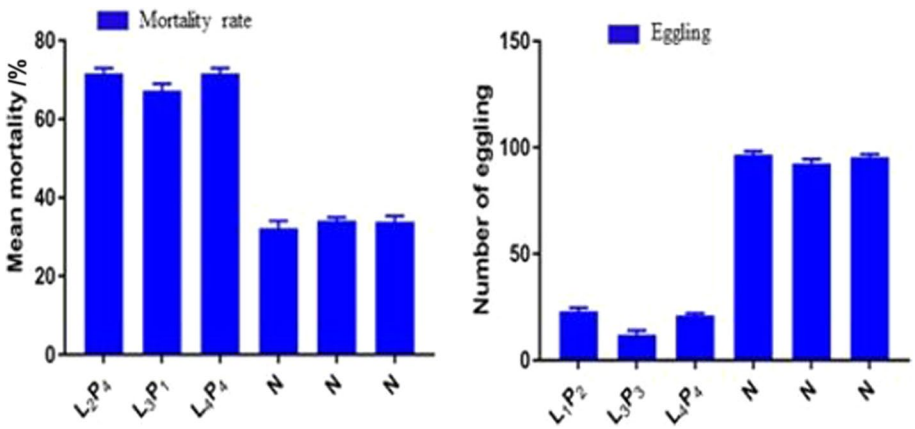

Fig. 6 Percentage mortality and number of eggling in adult *Bamisia tabaci* feeding on transgenic (L_1P_2, L_3P_3, L_4P_4) and non-transgenic (N) control line. The number of plants n = 3

fungus resistant crops (Kirubakaran et al. 2007; Toufiq et al. 2018).

An attempt was made in current study to develop insect resistant cotton through introduction of barley *ch1 and chi*2 genes by using *Agrobacterium*-mediated shoot apex cut method of transformation as was done by Puspito et al. (2015), Bajwa et al. (2015), and Liu et al. (2012). Transformation efficiency was 1.17% as against 1.1% reported by Rao et al. (2011) by using the same method. Total five plants were amplified out of total fifteen shifted to green house by using gene specific primers. The low transformation efficiency was attributed to recalcitrant nature of cotton.

Quantitative real-time PCR showed the highest mRNA expression level of *ch1* and *chi*2 in transgenic cotton plants up to 7.5- and 8.7-fold in transgenic cotton plant P1 (expressing *ch1*) and P2 (expressing *chi*2), respectively, than that of non-transgenic cotton plants in T_0. Similar results obtained by Yaqoob et al. (2020) while assessing ExpA1 gene in transgenic cotton plants under different promoters. Zubair et al. (2019) reported similar pattern of transgene expression in transgenic cotton for expression of PME gene against insects. Transgenic cotton plants in T_0 with the highest mRNA expression level and better yield performance were chosen for generation advancement of T_0 to T_1. The progeny of these five plants were raised to T_1 and evaluated further. The amplification product of 447 bp for *ch1* and 401 bp for *chi*2 confirm transgene in T_1. The mRNA expression levels in T_1 was quantified to be 3.5-fold in P10 (2) of *ch1* gene and 3.7-fold in P2 (1) of *chi*2 gene in comparison to the control (i.e., non-transgenic cotton plants). The results are in accordance with Sufyan Tahir et al. (2021) while evaluating transgenic cotton by adopting same methods of transformation. The evidence of enhanced protection against fungus pathogens through higher chitinase expression level has been reported by Shibuya et al. (2001).

The study suggests that endochitinases in combination with the recombinant technology can be a promising tool for improving plant resistance to fungal diseases. Hybridization, ffluorescent in situ hybridization (FISH) and southern hybridization techniques are used to find the transgene copy number and location on chromosomes but FISH was preferred on account of visual confirmation and accuracy (Tsuchiya et al. 2001). Many factors like the copy number and location of gene on chromosomes and promoters are directly related to transgene expression (Rao et al. 2011). The *ch1* transgenic cotton plant showed one gene copy number on chromosome number 9 while *chi*2 was reported to be on chromosome no. 10 whereas non transgenic plants did not show any signal.

Transgenic cotton plants were also evaluated for any insertional impact on essential characteristics of plants. Endochitinase expression in transgenic cotton plants showed no morphological abnormalities as reported on broccoli by Mora et al. (2001). The photosynthetic rates which can be attributed to their insects' resistance as being healthier with photosynthetic rate ranged from 5.5 to 5.8 $mol{\cdot}m^{-2}{\cdot}s^{-1}$ (CO_2) in P10 (4) expressing *ch1* gene and P2(1) expressing *chi*2, respectively. The improved photosynthetic rates were also reported by Khan et al. (2017) when barley *chitinaseII* gene was used to develop resistance against fungi. The gaseous exchange rate was statistically similar ($P>0.05$) in almost all the transgenic cotton plants except P10 (4) expressing *ch1* gene in which slightly higher ($P<0.05$) gaseous exchange rate was calculated in comparison to the control plants. The results are also in accordance with Wang et al. (2015) who demonstrated that exogenous DNA in a host cotton genome can affect the plant growth and photosynthesis while Sun et al. (2009) reported significant difference between transgenic cotton and non-transgenic cotton occurred but not always throughout the growing season or in different experiments or for all transgenic cotton lines. While all the transgenic cotton plants showed almost similar transpiration rate as compared with the control cotton plants. However, a few transgenic cotton plants P10 (4) and P2 (1) expressing *ch1* and *chi*2, respectively, showed relatively higher ($P<0.05$) transpiration rate as compared with the control cotton plants. These light fluctuation in physiological performance can be attributed to genomic combination of cotton plants rather than transgene. Rao et al. (2011) reported improved physiological performance of cotton transformed with Phytochrome B gene. The results of transgenic cotton plants with improved high mortality rate of whitefly were in accordance with report of Faria et al. (2001), Vicentini et al. (2001), Quesada-Moraga et al. (2006), and Mascarin et al. (2013). The development of transgenic cotton plants with enhanced whitefly resistance using barley genes *ch1* and *chi*2 represents a promising step towards more sustainable and resistant cotton production. Further research can refine this technology and unlock its full potential for the benefit of farmers and the environment.

Conclusion

Transgenic cotton plants modified with barley *chi1* and *chi*2 genes have shown significant potential in combatting whiteflies. This further strengthens the use of *chi1* and *chi*2 as candidate genes for improving whitefly resistance in cotton on a larger scale. The enhanced resistance to whiteflies can be further incorporated into new cotton varieties through traditional cross-breeding techniques, leading to more resilient crops. By reducing reliance on

insecticides, transgenic cotton with improved whitefly resistance can contribute to more sustainable agricultural practices. This can minimize environmental damage and potentially lower production costs for farmers.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42397-024-00169-y.

Additional file 1: Supplementary Fig. S1. Schematic diagram of ***chi1*** cassette (A) Schematic diagram of ***chi2*** cassette (B). Screening of *E. coli* colonies for (pCAMBIA 1301_ ***chi1***) by restriction digestion; Lane1: 1 kb ladder; Lane 2–10: Digested pCAMBIA with *Hind*III and *Kpn*I and 1 485 bp released fragment of ***chi1*** gene cassette: Screening of *E. coli* colonies for (pCAMBIA 1301_ ***chi2***) by restriction digestion; Lane 1–8: Digested pCAMBIA with *Hind*III and *Bam*III and 1 685 bp released fragment of ***chi2*** gene cassette. Screening of *E. coli* colonies for ***chi1*** gene in pCAMBIA 1301 through PCR amplification; Lane 1–8: 447 bp ***chi1*** amplicon, Lane 9: 100 bp DNA ladder Screening of *ChiI* gene in pCAMBIA 1301 through PCR amplification; Lane 1: 100 bp DNA ladder Lane 2–9: 401 bp ***chi2*** amplicon, Lane 9 Unamplified negative colonies (C &D).

Additional file 2: Supplementary Fig. S2. {A (a)} Confirmation of (pCAMBIA1301_ ***chi1***) in *Agrobacterium tumefaciens* LBA4404 through colony PCR; Lane 1: Positive control plasmid having (pCAMBIA1301_ ***chi1***); Lane 2–7: 447 bp ***chi1*** amplicon containing colonies; Lane8: 100 bp DNA ladder{ A (b)}: Confirmation of (pCAMBIA1301_ ***chi2***) in *Agrobacterium tumefaciens* LBA4404 through colony PCR; Lane 1: positive control plasmid having (pCAMBIA1301_ ***chi2***) Lane 2–7: Screened colonies having 401 bp ***chi2*** amplicon; Lane 8: 100 bp DNA ladder. {B (a)} PCR analysis of putative transgenic cotton plants in T_0 progeny for ***chi1*** gene; Lane 1: 100 bp DNA ladder; Lane2: Positive control (plasmid pCAMBIA-1302 ***chi1***); Lane 3–7: Transgenic plants P1, P2, P3, P4, and P10, respectively, with amplification of 447 bp fragment. {B (b)}}PCR analysis of putative transgenic cotton plants in T_0 progeny for ***chi2*** gene; Lane 1:100 bp DNA ladder; Lane 2: Negative control; Lane 3: Positive control (plasmid pCAMBIA_1301 ***chi2***); Lane 5: Putative transgenic plant 2 with amplification of 401 bp fragment; Lane 4,6,7,8,9: Negative plants (Non-transgenic plants).

Additional file 3: Supplementary Fig. S3. (A) Analysis of transgenic cotton plants expressing ***chi1*** gene through PCR in T1 progeny; Lane 1:100 bp DNA ladder; Lane 2: Positive control (Pcambia 1301_ ***chi1***); 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). (B) amplified product transgenic cotton plants expressing ***chi2*** gene by using gene specific primers in T1 progeny; Lane 1: 100 bp DNA ladder; Lane 2–5: Transgenic plants with the amplification 401 bp fragment; Lane7: positive control (Pcambia 1301_ ***chi2***); Lane 8–12: Non amplified segregated plants (Genomic DNA extracted from non-transgenic cotton plants).

Acknowledgements
The authors are grateful for the research facilities provided by the Center of Excellence in Molecular Biology (CEMB), University of the Punjab.

Authors' contributions
Bashir S executed the research work and wrote the initial draft of the manuscript. Latif A performed field work and analyzed data. Bashir R and Bukhari S assisted in research work and data acquisition. Shahid N, Azam S, and Bakhsh A assisted in bioassays and data interpretation. Husnain T supervised the whole research work. Shahid AA and Rao AQ finally reviewed and presented in the current form.

Funding
No funding was obtained for this research work.

Availability of data and materials
The data generated and materials used in this study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
Authors declare that they don't have competing interests.

Author details
[1]Centre of Excellence in Molecular Biology, University of the Punjab, Lahore 53700, Pakistan. [2]Department of Botany, Kinnaird College for Women University, Lahore 54000, Pakistan. [3]Department of Botany, Government College University, Faisalabad 38000, Pakistan. [4]Institute of Botany, University of the Punjab, Lahore 54590, Pakistan.

Received: 3 September 2023 Accepted: 16 February 2024
Published online: 18 March 2024

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