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# Transgenic Peanut (*Arachis hypogaea* L.) Plants Conferring Enhanced Protection Against Fungal Pathogens by Expressing *Tc chitinase-I* Gene

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**Abstract**

In this study, an efficient *Agrobacterium*-mediated genetic transformation method followed by *Tc chitI* gene for enhanced expression in peanut (*Arachis hypogaea* L.) cv ICG 7827 is reported. *A. tumefaciens* strain LBA 4404 harboring the plant transformation plasmid pBinAR contains the *Tc chitI* gene and *nptII* gene as a selectable marker. The precultured leaflet (LL) explants were infected with *A. tumefaciens* containing *Tc chitI* gene and cocultivated on SIM (MMS+10 mg/L BAP+1 mg/L NAA+2 mg/L $AgNO_3$) for four days. After co-cultivation, these explants were transferred to selection medium (SIM+75 mg/L Kan+250 mg/L Cefotaxime). In $T_0$ generation, 70 % of transformation efficiency was recorded. $T_1$ generation derived from the primary transgenic ($T_0$) events revealed a Mendelian inheritance pattern (3:1) for the *Tc chit I* transgene. $T_1$ transgenic peanut plants were tested for resistance against *C. arachidicola*, *C. personatum,* and *P. arachidis* by infection with the microspores using detached leaf assay. The *Tc chitI* gene expressed in $T_1$ transgenic plants have shown longer incubation, longer latent period, and lower infection frequencies than non-transformed (WT) plants. A significant negative correlation was recorded between chitinase activity and the frequency of infection to the *C. arachidicola*, *C. personatum* and *P. arachidis* pathogens.

*Keywords*: *Agrobacterium*-mediated transformation; Fungal resistance; *Tc chitinase-I* gene; *Cercospora arachidicola; Cercospora personatum*; *Puccinia arachidis*.

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## 1. Introduction

Peanut (*Arachis hypogaea* L.) is an important oilseed crop that has received more attention for its improvement in recent years. It is more susceptible to several pathogens such as fungi, bacteria, viruses, insects, and nematodes. The fungi and viruses are the most common disease-causing agents in peanuts worldwide. Among fungal diseases, three major foliar diseases, namely early leaf spot (ELS-*Cercospora arachidicola*), late leaf spot (LLS-*Cercosporidium personatum*), and rust (*Puccinia arachidis*) are the most

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widely distributed and economically important diseases of groundnut. Foliar fungal diseases are the major production constraints of groundnut worldwide wherever the crop is grown. These diseases can cause more than 70 % loss in yield besides adversely affecting the quality of the produce (pods, seeds, and haulms) [1]. Late leaf spot is a major and widely distributed disease. It can cause defoliation to reduce pod and fodder yields by about 50 % and adversely affect the quality of its produce [2]. Rust is also an economically crucial disease-causing yield loss ranging from 10-52 %, in addition to a decline in seed quality. Chemical measures can control foliar diseases, but they increase production costs, thus beyond the reach of small and marginal farmers, and pollute the environment. Agricultural practices such as fungicide application and crop rotation are commonly used to fight these fungal diseases, but their effectiveness is limited. A realistic long-term solution to managing the disease is by breeding fungal-resistant cultivars.

Nevertheless, effective resistant genes are not always available in domestic cultivars, and resistance is rapidly overcome by new races of pathogens. In addition, conventional breeding strategies are extremely time-consuming. Therefore, the development and growing of resistant cultivars is the best viable option to minimize the economic losses of farmers and maintain the good quality of the product. The application of the biotechnological method for gene transfer provides a powerful tool to improve the fungal disease resistance in groundnut. Recent advances in gene transfer technology led to the transfer of desirable gene(s) into various crop plants. The studies on the genetic engineering of plants with chitinase genes become very important for the fungal disease control mechanism. Chitinases (E.C.3.2.1.14) are poly (1, 4-(N-acetyl-β-D-glucosaminide))-glycanohydrolases and catalyze the hydrolytic cleavage of the β-1,4-glycoside bond of N-acetylglucosamine (GlcNAc), the principal monosaccharide in the chain. They are present in various organisms and are important pathogenesis-related (PR) proteins involved in plant immune systems [3]. During pathogen invasion, the chitinases, directly and indirectly, inhibit fungal growth. They directly hydrolyze fungal cell walls, which contain chitin, the substrate for the enzyme, and by this action, fungal hyphal lysis and inhibition of fungal growth occur [4,5]. The chitinases also release elicitors from the fungal cell walls by their enzymatic action, and these elicitors induce various defense responses indirectly in plants [6]. The activity of elicitor-inducible chitinases was more beneficial for disease resistance as their activity will increase several-fold upon the invasion of a pathogen. Hence, proper selection of chitinase genes is essential for developing transgenic plants with enhanced disease resistance.

Several plant chitinase genes have been cloned and characterized [7]. Several transgenic plants (e.g., rice, tobacco, canola, tomato, and cucumber) overexpressing chitinases have been produced to increase resistance to fungal pathogens, with varying levels of protection [8-11].

Several reviews and research articles have also stressed the advantages of using chitinase for plant protection because these enzymes are fungicidal, part of the plant defense system and are not harmful to host or other plants [12]. Hence, efforts focused on the transgenic expression of the chitinase gene in plants and significant importance in the

resistance to fungal diseases had been recorded [11,13-21]. Based on the reports, it is observed that the chitinase gene plays a vital role in controlling plant fungal pathogens. In particular, the class I chitinases, which accumulate high levels in vacuoles in response to wounding and pathogen infection, have been reported to be important [11,22,23].

In view of this, in the present investigation, we have attempted to develop the transgenic groundnut plantlets with the integration of *Tc chitinase-I* gene through *Agrobacterium*-mediated genetic transformation by using leaflet (LL) explants from foliar disease susceptible peanut cv ICG 7827.

## 2. Materials and Methods

### 2.1. *Plant material*

Peanut cultivar ICG 7827 was chosen as the plant transformation material sensitive to fungal pathogens, leaf spots, and rust diseases. The mature seeds were obtained from the germplasm bank of ICRISAT, Patancheru, Hyderabad, Telangana, India.

Mature groundnut seeds were washed under running tap water for 10-15 min followed by liquid detergent Tween-20 (5 %-v/v) treatment for 5 min. It was repeated twice, followed by washing with sterile distilled water thoroughly. Later the seeds were surface sterilized with 0.1 % (w/v) $HgCl_2$ for 8 min followed by rinsing in sterilized distilled water for 3-4 times, dried on sterile tissue paper under aseptic conditions, and inoculated on ½ strength liquid Modified Murashige and Skoog's (MMS) medium for seedlings [24]. The leaflet (LL) explants from two weeks old *in vitro* grown seedlings were cut into 0.8-1.0 $cm^2$ size and precultured on shoot induction medium (SIM) containing MMS medium+10 mg/L BAP+1 mg/L NAA+2 mg/L $AgNO_3$ for 3 days.

### 2.2. *Gene constructs*

*Agrobacterium tumefaciens* strain LBA4404 harboring the binary plasmid pBinAR (13.7 Kb) was used for genetic transformation of *A. hypogaea* cv ICG 7827. The binary vector pBinAR carrying *Theobroma cacao chitinase-I (Tc chitI)* gene with a *nptII* selectable marker gene was used. The T-DNA portion of pBinAR having *nos-npt* II cassette in RB and 770bp *Eco*RI/Hind III fragment, containing the CaMV 35S promoter, a partial pUC18 polylinker and the OCS terminator in LB and selectable marker gene (*nptII*) driven by the NOS promoter and PNOS terminator sequences respectively (Fig. 1).

### 2.3. *Transformation method*

*Agrobacterium*-mediated genetic transformation of peanut cv ICG 7827 with the *Tc chit I* gene has been carried out by the following method of Rajinikanth *et al.* [25]. The precultured LL explants were infected with *A. tumefaciens* LBA 4404 harboring binary vector pBinAR containing *Tc chitI* gene and *nptII* as selectable marker gene and LL explants cocultivated on SIM containing MMS+10 mg/L BAP+1 mg/L NAA+ 2 mg/L

$AgNO_3$ for four days. After co-cultivation, these LL explants were shifted on to selection medium (SIM+75 mg/L Kan+250 mg/L Cefotaxime). After two weeks of incubation, the explants with $Kan^R$ shoots were cultured on SIM+50 mg/L Kan for further proliferation of shoots. Subsequently, the $Kan^R$ shoots were elongated, rooted, and established the plantlets. The putative transformants ($T_0$) were obtained within four months of culture initiation. A set of non-transformed explants were also regenerated and established in the greenhouse as control. $T_0$ transgenic plants were maintained in the containment greenhouse facility, and seeds were harvested to obtain the $T_1$, $T_2$ generations. The transgenic plants in $T_0$, $T_{1,}$ and $T_2$ generations were subjected to analysis using standard procedures.

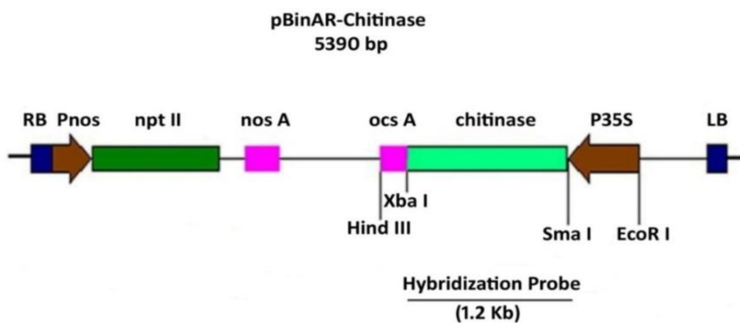


Fig. 1. Linear diagram of T-DNA portion of the pBinAR-Chitinase-I construct.

**2.4.** ***PCR and RT-PCR analysis of the transformants***

The genomic DNA was isolated from randomly selected putative transformants and one non-transformed (WT-wild type) plant (control) [25] and subjected to PCR amplification using the *Tc chit I* gene-specific primers (F) 5′-GGA AAA TGG TTG CCA GAG TCA GTGC-3′, (R) 5′-GCT ACA TTG AGT CCA CCG AGG GTC-3′ and *npt II* gene-specific primers(F) 5′-GCT TGG GTG GAG AGG GCT ATT-3′,(R) 5’-AGA ACT CGT CAA GAA GGC GA -3′. The PCR for *Tc chit I* gene was carried out by initial denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 58 °C for 1.30 min and 72 °C for 2 min and final extension at 72 °C for 10 min and *npt II* gene was carried out by initial denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 54 °C for 1 min and 72 °C for 1.30 min and final extension at 72 °C for 10 min. The amplified products were subjected to electrophoresis on 1.2 % agarose gel and visualized under gel documentation system, Biorad, USA (Fig. 3a-c). The randomly selected PCR-positive transgenic plants were used for RT-PCR analysis. Total RNA was isolated from leaf tissue of the transgenic plants using the TRIzol reagent according to the manufacturer's protocol, and RT-PCR analysis of the putative transformants was carried out using the Thermoscript RT-PCR system for 35-40 cycles using *Tc chit I* gene-specific primers for carrying out RT-PCR. One sample of RNA subjected directly to PCR without reverse transcription served as the negative control, and plasmid DNA from pBinAR-chitinase-I

served as the positive control. The amplified fragments were separated on 1.2 % agarose gel photographed under a gel documentation system (Fig. 3d).

### 2.5. *Southern blot analysis*

30 µg of genomic DNA from the transgenic and non-transformed (control) plants were digested with *Eco*RI, which cuts at restriction site within the plasmid DNA to determine the presence of the *Tc chit I* gene. According to the manufacturer's instructions, the digested DNA was separated by electrophoresis through a 1 % agarose gel and transferred onto a Nylon N+ membrane (Amersham Biosciences, UK). The 1.2 kb *Tc chitI* coding sequence fragment was used as a probe with a non-radioactively labeled (Alkphos Direct Labeling and Detection system of Amersham Biosciences, UK) (Fig. 4).

### 2.6. *Segregation analysis*

Inheritance of the transgene was studied using the Tc chitI gene's PCR screening in $T_1$ and $T_2$ generations. PCR +ve and –ve plants were identified, and a chi-square test was performed to validate the data for 3:1 segregation.

### 2.7. *Chitinase assay*

A colorimetric assay was performed with the leaves of 45 days old transformed and non-transformed (WT) control peanut plants using the previous method [26].

### 2.8. *Detached leaf assay for ELS, LLS, and rust diseases*

Disease evaluation of the $T_1$ transgenic peanut plants for early leaf spot (ELS), late leaf spot (LLS), and rust fungal pathogens was conducted by detached leaf assay technique [11].

### 2.9. *Data analysis*

The data collected on chitinase activity, ELS, LLS, and rust infection were subjected to analysis of variance (ANOVA), where the mean values in each treatment were compared using LSD at the 5 % level of significance (P=0.05). The values were means of ten replicates per event. The correlation analysis was done using Pearson correlation coefficient at a 5 % significance level among the transgenics and non-transformed control plants for infection frequency of three tested pathogens with chitinase activity.

## 3. Results and Discussion

Transgenic technology provides a powerful tool to enhance fungal disease resistance in the groundnut crop. This technology can enhance plant defense systems against fungal pathogens by producing high antifungal compounds. The studies on the genetic

engineering of plants with chitinase genes become essential for the fungal disease control mechanism. Several reviews and research articles have also stressed the advantages of using chitinase for plant protection because these enzymes are fungicidal, part of the plant defense system and are not harmful to host or other plants [7,12]. Hence, in the present study, we have developed the transgenic peanut cv ICG 7827 plants for resistance to leaf spot (ELS and LLS) and rust diseases by expressing an antifungal *T. cacao chitinase-I* gene.

Genetic engineering of plants involves the stable integration of foreign DNA sequences, usually into the nuclear genome of cells capable of giving rise to a whole transformed plant. There are numerous reports on the *in vitro* regeneration and genetic transformation of peanuts using various explants and transformation systems [27-31]**.** Each transformation system has its advantages and limitations. In the present investigation, we have developed the transgenic peanut plants expressing *Tc chitI* gene by using leaflet explants, and it showed 70 % of transformation efficiency.

Transgenic groundnut expressing a bacterial chitinase gene [32] and tobacco chitinase gene [33] were shown to possess enhanced resistance to LLS caused by *P. personatum* and rust caused by *P. arachidis.* The expression of rice chitinase and alfalfa glucanase genes in groundnut and observed enhanced resistance against sclerotinia blight in regenerated transgenic plants [34]. Expression of a barley oxalate oxidase in transgenic groundnut also enhanced resistance to sclerotinia minor [35]**.**

### 3.1. *Genetic transformation of peanut with Tc chit I gene*

A total of 60 precultured leaflet explants were infected with *A. tumefaciens* LBA 4404 harboring binary vector pBinAR containing *Tc chitI* gene and *nptII* as selectable marker gene cocultivated for four days. After co-cultivation, these explants were transferred onto the selection medium. The $Kan^R$ shoots were induced after two weeks of inoculation. The LL explants with $Kan^R$ shoots were further proliferated on SIM+50 mg/L Kan. 42 $Kan^R$ shoots were rooted and established the plantlets with 70% of transformation efficiency. The regenerated plants ($Kan^R$) showed average growth under greenhouse conditions and produced flowers and pods containing viable seeds (Fig. 2a-e).

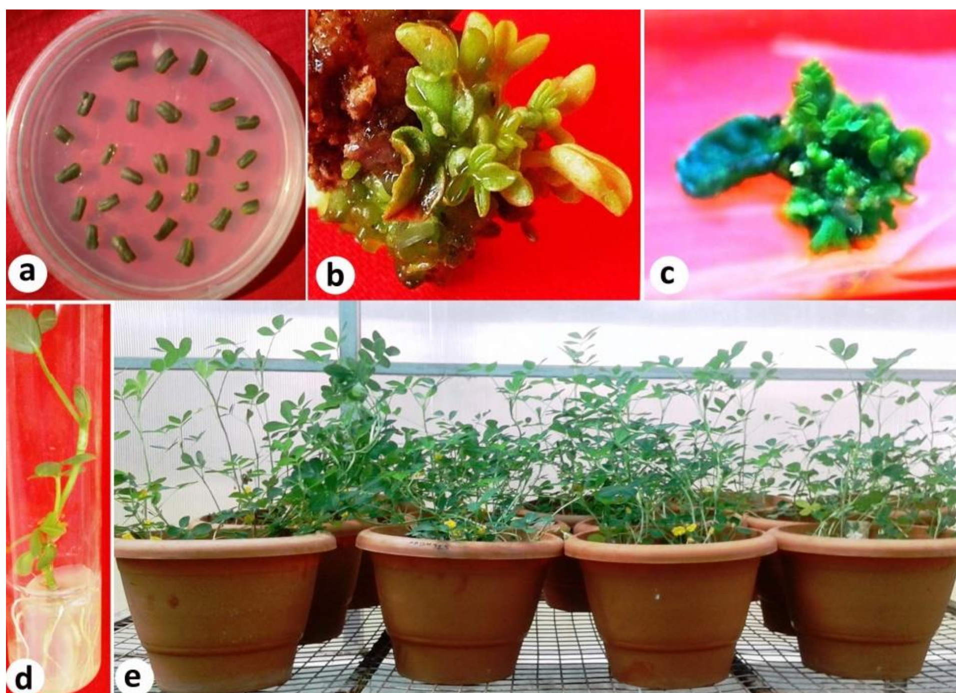


Fig. 2. *Agrobacterium* mediated genetic transformation in leaflet (LL) explants of peanut cv ICG 7827 by using binary vector pBinAR. **a)** Infected LL explants on SIM for co-cultivation; **b-c)** Induction of $Kan^R$ shoots on selection medium after 4 & 6 weeks of incubation respectively; **d)** *In vitro* rooting of the elongated micro shoot on RIM augmented with 1.0 mg/L NAA+50 mg/L Kan; **e)** $T_0$ plants are growing in plastic pots containing soil mix and maintain in the greenhouse.

## 3.2. *Molecular analysis of transgenics*

### 3.2.1. *PCR and RT-PCR analysis*

A total of 42 putative transgenic ($T_0$) peanut lines were transferred to a greenhouse for molecular analyses (Fig. 3). Total genomic DNA from putative peanut transformants was subjected to PCR analysis with *Tc chitI* and *nptII* gene-specific primers. The 1200 bp region of the *Tc chitI* gene was detected in $T_{0,}$ and $T_1$ transgenic plants with the transformation efficiency were 70 % (Fig. 3a-b). Randomly selected transformants of transgenic plants also showed amplification of 750 bp fragment of the *nptII* gene (Fig. 3c).

Expression of the introduced gene was analyzed by RT-PCR from the randomly selected $T_0$ and $T_1$ PCR positive plants. The expected 1200 bp amplified fragment corresponding to the *Tc chitI* gene was detected in all the selected plants for analysis (Fig. 3d).

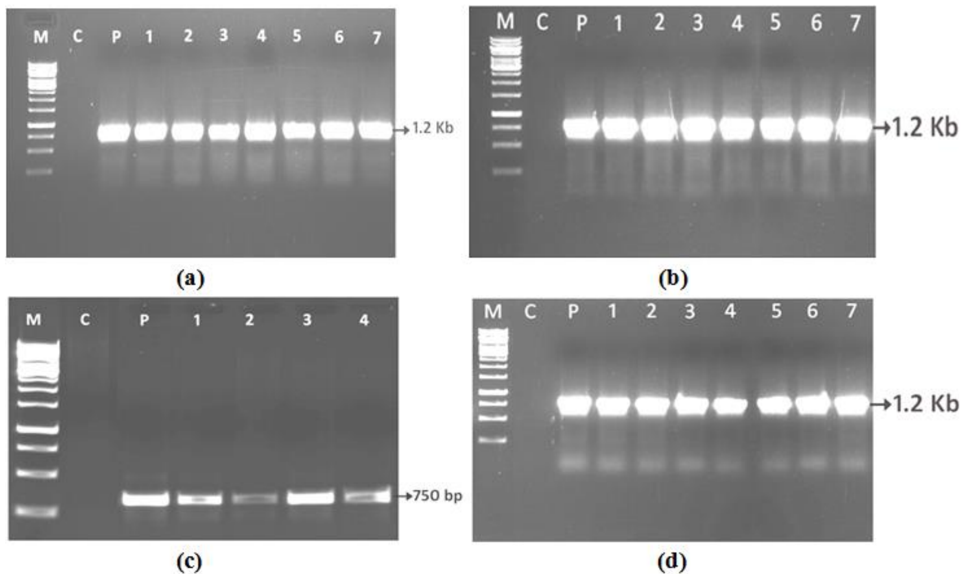


Fig. 3. Molecular analysis of $T_0$ and $T_1$ putative transformants of *A. hypogaea* cv ICG 7827 plants. a) PCR amplification of genomic DNA showing amplification of a 1200 bp fragment of the *Tc chitI* gene from $T_0$ plants; b) PCR amplification of genomic DNA showing amplification of a 1200 bp fragment of the *TcchitI* gene from $T_1$ plants; Lanes: 1-7: carry genomic DNA from $T_0$ and $T_1$ putative transformants respectively; c) PCR amplification of genomic DNA showing amplification of a 750 bp fragment of the *npt II* gene from $T_0$ and $T_1$ putative transformants, Lanes 1-4: Genomic DNA from $T_0$ and $T_1$ putative transformants; d) RT-PCR of the cDNA showing amplification of a 1200 bp fragment of the *Tc chitI* gene from $T_0$ and $T_1$ putative transformants, Lanes 1-4: cDNA from $T_0$ putative transformants, Lanes 5-7: cDNA from $T_1$ putative transformants. M: molecular size marker (1 Kb ladder), C: Non transformed control plant DNA (-ve control-WT), P: plasmid pBinAR DNA (+ve control).

### 3.2.2. *Southern blot analysis*

The Southern blot analysis was carried out to provide additional evidence of the Tc chit I gene integration. Randomly selected PCR and RT-PCR positive events were analyzed by Southern blot hybridization (*Eco*RI digested DNA) using a 1.2 Kb fragment as a probe (Fig. 4). The genomic DNA isolated from leaves of PCR and RT-PCR positive $T_0$ plants was subjected to Southern analysis. The results showed hybridization signals confirming that the transgene was successfully integrated into the plant genome. Southern hybridization analyses of PCR and RT-PCR positive events showed a single copy of the *Tc chit I* transgene in event number TC-1, TC-4, TC-7, TC-8, and TC-10 (Lanes 1-5). The non-transformed control plant did not show any hybridization signals (Lane C).

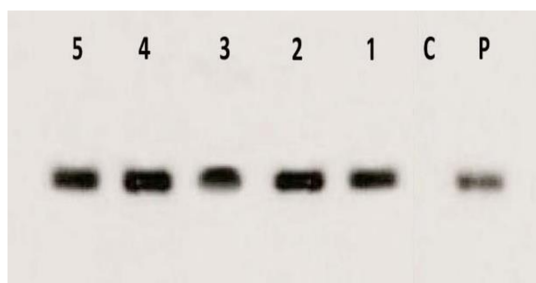


Fig. 4. Southern blot analysis of the genomic DNA from leaves of transgenics obtained through *Agrobacterium*-mediated genetic transformation. The genomic DNA of peanut transgenics was digested with *Eco*R1.

Lanes 1-5: *Eco*RI restricted genomic DNA from eventsTC-1, TC-4, TC-7, TC-8, and TC-10 showed a single copy number; Lane C: *Eco*RI restricted genomic DNA from control plants, Lane P: *Eco*RI restricted plasmid pBinAR:*TcchitI*.

3.2.3. *Segregation analysis of $T_1$ and $T_2$ transgenic plants*

The inheritance of transgene was studied from the PCR screening of the *Tc chitI* gene in $T_1$ and $T_2$ generation transgenic plants. The segregation pattern of PCR tested transgenic plants, and their progeny showed the Mendelian ratio (3:1 ratio) at p=0.05 in all the events in $T_1$ and $T_2$ progenies (Table 1). Gene segregation pattern was derived using a chi-square test. Ten events from the $T_1$ generation and ten from the $T_2$ generation were raised to study the segregation pattern. The positive plants were identified using PCR analysis in the progeny of $T_1$ and $T_2$ generations. In the $T_1$ generation, 9 out of 10 (90 %) found significance at p=0.05. Likewise, segregation analysis was also performed in the $T_2$ generation; 08 out of 10 events (80 %) in the $T_2$ generation were recorded significantly at p=0.05 %. Thus, segregation studies showed the Mendelian inheritance (3:1 ratio) of the *Tc chitI* gene in $T_1$ and $T_2$ progenies of peanut plants (Table 1).

In the present investigation, the integration of the transgene was confirmed by PCR, RT-PCR, and Southern blot analyses. Segregation studies showed a Mendelian ratio of 3:1 of the *Tc chitI* gene in $T_1$ and $T_2$ generation transgenic peanut plants. During the present investigations, in the $T_1$ generation, 9 out of 10 events (90 %) and $T_2$ generation 8 out of 10 events (80 %) showed a Mendelian segregation ratio of 3:1. The inheritance of the *Tc chitinase-I* gene in peanut ICG 13942 plants showed the Mendelian 3:1 ratio [11]. The 3:1 segregation ratio was obtained for promoterless *gus:nptII* bifunctional fusion gene [28] and *cry1 EC* gene [36] through Chi-square analysis in transgenic peanut as we have observed in the present study.

Table 1. Inheritance (segregation) pattern of *Tc chitI* gene in $T_1$ and $T_2$ generations of cv ICG 7827.

| $T_1$ generation | | | | | $T_2$ generation | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Even No. | No. of plants tested | No. of PCR +ve plants | No. of PCR -ve plants | Chi-square (X2) | Even No. | No. of plants tested | No. of PCR +ve plants | No. of PCR -ve plants | Chi-square (X2) |
| TC-1 | 22 | 15 | 07 | 0.545 | TC-1-4 | 30 | 20 | 10 | 1.110 |
| TC-2 | 27 | 18 | 09 | 1.000 | TC-2-3 | 26 | 15 | 11 | 4.155 |
| TC-3 | 20 | 14 | 06 | 0.266 | TC-3-1 | 18 | 10 | 08 | 3.629 |
| TC-4 | 23 | 14 | 09 | 2.448 | TC-4-5 | 22 | 14 | 08 | 1.514 |
| TC-5 | 20 | 11 | 09 | 4.266 | TC-5-3 | 19 | 13 | 06 | 0.437 |
| TC-6 | 24 | 17 | 07 | 0.221 | TC-6-1 | 21 | 12 | 09 | 3.570 |
| TC-7 | 25 | 15 | 10 | 3.000 | TC-7-5 | 17 | 10 | 07 | 2.372 |
| TC-8 | 18 | 12 | 06 | 1.554 | TC-8-2 | 23 | 13 | 10 | 4.187 |
| TC-9 | 21 | 15 | 06 | 0.142 | TC-9-5 | 20 | 12 | 08 | 2.400 |
| TC-10 | 19 | 11 | 08 | 2.964 | TC-10-2 | 21 | 15 | 06 | 0.142 |

*$X^2$ value at 0.05 % probability at 1 df is 3.84. Calculated values below 3.84 were non-significant, and the samples fit for 3:1 segregation ratio.

### 3.2.4. *Chitinase activity in the transgenic plants*

The chitinase activity varied amongst the transgenic events expressing *Tc chitI* gene where 7.0 fold increase in the chitinase activity (0.30 to 1.54 U/mg protein) was recorded as compared to the non-transformed control plants (0.22 U/mg protein) (Fig. 5). Of the 10 $T_1$ transgenic events tested, 5 transgenic events (TC-1, TC-4, TC-7, TC-8, and TC-10) had significantly higher chitinase activity than the rest, which sustained in the $T_2$ progeny of three of these five events (TC-1-4, TC-4-2and TC-10-3).

The chitinase activity varied amongst the transgenic events expressing *Tc chitI* gene, were 7.0 fold increase in the chitinase activity (0.30 to 1.54 U/mg protein) was recorded as compared to the non-transformed control plants (0.22 U/mg protein) (Fig. 5). Similarly, enhanced chitinase activity was recorded in the transgenic plants expressing the other type of chitinase genes [8,10]. A 6.5- folds increase in chitinase activity in transgenic peanut cv ICG 13942 with *Tc chitI* gene was observed [11]; an over 5-folds increase in chitinase activity was reported in transgenic peanut plants with rice chitinase [37]. Over 14-folds increase in chitinase activity was found in transgenic peanut [20] and rice [10].

Although some of the transgenic events showed enhanced chitinase activity in our study, compared to their non-transformed control plants, these differed in their level of resistance to ELS, LLS, and rust diseases. This variation may be explained by differences in the biochemical composition and structure of the fungal cell wall, tissue and cellular localization of the recombinant chitinase, concordance in chitinase expression kinetics and the period of infection, and the type of interaction between the plant and the pathogen [9,38,39].

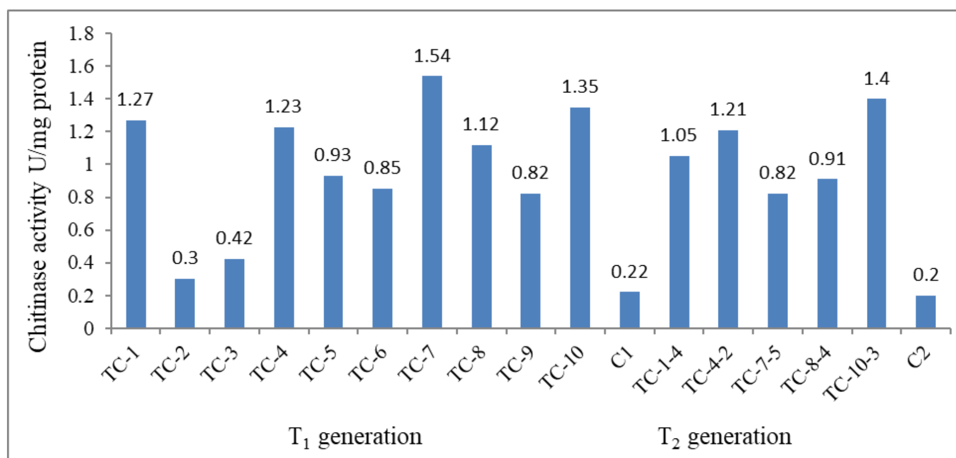


Fig. 5. Chitinase activity in the $T_1$ and $T_2$ transgenic peanut plants. Graph bars represent the mean±SD values of two replicates.

### 3.3. *Bioassay of transgenic plantlets challenged by the fungal pathogens*

The progenies of ten $T_1$ transgenic events were tested, and they showed significant differences for all the components of resistance to fungal pathogens, ELS, LLS, and rust diseases in detached leaf bioassay (Table 2).

For evaluation of ELS, the event TC-7 showed longer incubation (20 days), longer latent periods (25 days), and less no. of lesions (23 lesions) than their control plants (10 days IP, 14days LP, and 42 lesions). All transgenic events (TC-1, TC-2, TC-4, TC-05, TC-6, TC-7, TC-8, and TC-10) except events 3, 9 showed less LAD (4.12 to 10.43 %) than the control plants (11.40 %). Similarly, all the transgenic events showed lower Infection frequencies (1.92 to 4.86 $cm^2$) except transgenic events TC-2 (6.10 $cm^2$) and TC-9 (6.14 $cm^2$) than the control plants (5.12 $cm^2$). Thus, the event TC-7 showed the best results (longer IP 20 days, longer LP 25 days, lesser number of lesions per leaf 23, less LAD 4.12 %, and lower IF 1.92 $cm^2$) for all the resistance parameters tested in comparison to other events in $T_1$ plants (Table 2).

For evaluation of LLS, the event TC-10 and TC-7 showed longer incubation period (23 and 21 days), longer latent period (27 and 27 days), and fewer number of lesions per leaf (20 and 24) in $T_1$ transformed plants compared to non-transformed (control) plants (10 days IP, 16 days LP and 40 lesions). Except for the transgenic event TC-3 (LAD 13.12 % & IF 8.80 $cm^2$) all transgenic events (TC-1, TC-2, TC-4, TC-5, TC-6, TC-7, TC-8, TC-9, and TC-10) showed less LAD (4.04 to 10.75 %) and less IF (1.64 to 5.36 $cm^2$) compared to non-transformed counterparts (LAD 12.24 %, IF 7.12 $cm^2$). According to our observations, the event TC-10 showed better performance in all the recorded resistance parameters for LLS disease. They are longer IP (23 days), longer LP (27 days), lesser number of lesions per leaf (20), less LAD (4.04 %), and lower IF (1.64 $cm^2$) in comparison to other events in $T_1$ plants (Table 2).

$T_1$ transgenic events evaluated for rust disease showed a significant genotypic difference for all the components. Except for event TC-2 (IP-12 days, LP-19 days) and TC-3 (IP 10 days, LP-18 days), all the transgenic events TC-1, TC-4, TC-5, TC-6, TC-7, TC-8, TC-9, and TC-10 showed longer IP (15 to 26 days) and showed longer LP (20 to 29 days) than the non-transformed plants (13 days IP, 18 days LP). The event TC-1 showed a lesser number of lesions per leaf (22 lesions), less leaf area damage (4.82 %), and lower IF (2.23 $cm^2$) in comparison to the control plants (50 lesions, 14.34 % LAD, 8.04 $cm^2$ IF). Thus, the event TC-1 was performed better in all the parameters (longer IP-26 days, longer LP-29 days, lesser number of lesions per leaf-22, less LAD-4.82 %, and lower IF-2.23 $cm^2$) tested compared to other events screened for rust disease resistance (Table 2).

Thus, in the present investigation, the three transgenic events of TC-7, TC-10, and TC-1 displayed significantly higher resistance to *C. arachidicola* (ELS), *C. personatum* (LLS), and *P. arachidis* (rust) pathogens in $T_1$ plants, respectively (Fig. 6a-d).

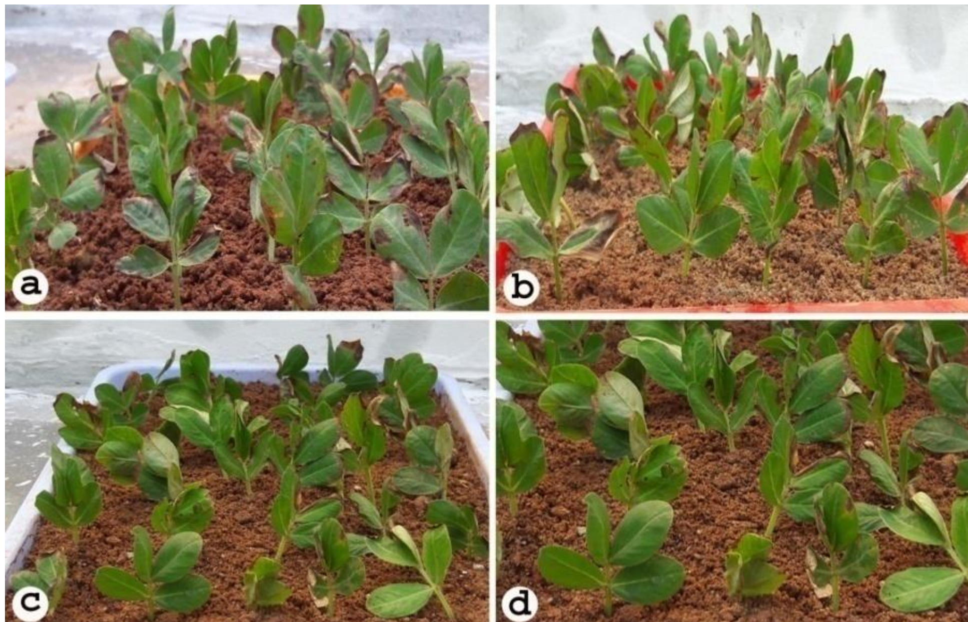


Fig. 6. a-c) Bioassay test of non-transgenic plants against ELS, LLS, and Rust diseases respectively; d) Transgenic groundnut plants expressing *TcchitI* gene in cv ICG 7827.

### 3.4. *Correlation between chitinase activity and infection frequency of fungal pathogens*

Disease severity correlated well with the chitinase activity and the infection frequency of ELS, LLS, and rust pathogens in the T1 transgenic plants with the Pearson correlation coefficients ranging from -0.8645 (P=0.05), -0.8036 (P=0.05) and -0.8475 (P=0.05), respectively. These results indicated that the transgenic events with high chitinase activity showed lower disease incidence and vice-versa (Fig. 7).

Table 2: Performance of peanut transgenic plants ($T_1$) carrying *Tc chitinI* gene against fungal pathogens *C. arachidicola, C. personatum* and *P. arachidis* in peanut cv ICG 7827.

| | Early Leaf Spot (ELS) | | | | | Late Leaf Spot (LLS) | | | | | Rust | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Event No. | IP | LP | NL | LAD | IF | IP | LP | NL | LAD | IF | IP | LP | NL | LAD | IF |
| TC-1 | 18b | 21b | 31cd | 7.68bd | 2.31cd | 20b | 24ab | 28cd | 6.43bd | 2.34cd | 26a | 29a | 22ade | 4.82ade | 2.23ade |
| TC-2 | 8ade | 12ade | 44ab | 8.26c | 6.10ab | 10ade | 14d | 43ab | 10.75b | 5.36b | 12d | 19cd | 45abc | 10.32b | 7.36b |
| TC-3 | 14bd | 16acd | 42b | 13.34a | 4.86abc | 10d | 12ade | 45a | 13.12a | 8.80a | 10ade | 18adc | 48b | 15.12a | 8.23a |
| TC-4 | 18abc | 17c | 31bd | 7.40cd | 3.80acd | 18abc | 22abc | 35c | 6.12cd | 2.80bd | 19c | 25abc | 35acd | 6.12d | 3.14cd |
| TC-5 | 15c | 20abc | 39bc | 8.13acd | 3.92c | 14acd | 20c | 35acd | 8.20c | 4.20bc | 19bc | 20acd | 39c | 9.02bc | 6.02c |
| TC-6 | 15acd | 15bd | 35c | 10.43bc | 4.24bc | 16bc | 20bc | 40bc | 10.14abc | 4.67c | 17acd | 22c | 44bc | 8.72c | 7.50abc |
| TC-7 | 20a | 25a | 23adc | 4.12adc | 1.92ade | 21ab | 27b | 24d | 4.68d | 1.92d | 24b | 28ab | 26cd | 6.23cd | 2.67d |
| TC-8 | 17bc | 20bc | 35acd | 11.12abc | 2.64bd | 16c | 19acd | 31bd | 6.84acd | 3.12acd | 20abc | 25bc | 34bd | 8.40acd | 4.87acd |
| TC-9 | 10cd | 14d | 44a | 12.68ab | 6.14a | 12bd | 17bd | 42abc | 8.56bc | 5.15abc | 15bd | 20b | 48ab | 9.46abc | 6.34bc |
| TC-10 | 19ab | 23ab | 26d | 4.83d | 2.10d | 23a | 27a | 20ade | 4.04ade | 1.64ade | 24ab | 27b | 26d | 6.84bd | 4.20bd |
| NT | 10d | 14cd | 42abc | 11.40b | 5.12b | 12cd | 10cd | 43b | 12.24ab | 7.12ab | 13cd | 18d | 50a | 14.34ab | 8.04ab |
| SE± | 1.231 | 0.657 | 2.312 | 0.923 | 0.745 | 2.342 | 1.453 | 4.213 | 0.564 | 3.121 | 1.452 | 0.345 | 2.312 | 0.879 | 0.231 |
| LSD% | 4.354 | 1.823 | 13.652 | 1.328 | 0.987 | 2.423 | 10.231 | 4.216 | 3.054 | 1.650 | 2.045 | 1.560 | 0.897 | 2.676 | 1.056 |
| Fp | >0.001 | >0.001 | >0.001 | >0.001 | >0.001 | >0.001 | >0.001 | >0.001 | >0.001 | >0.001 | >0.001 | >0.001 | >0.001 | >0.001 | >0.001 |

The values are the means of ten plants. Mean followed by the same letter are not significantly different at 5% level.
IP-Incubation period, LP-Latent period, NL-No. of lesions, LAD-Leaf area damage, IF-Infection frequency.
TC-1 to TC-10-Transformed, NT-Non Transformed.

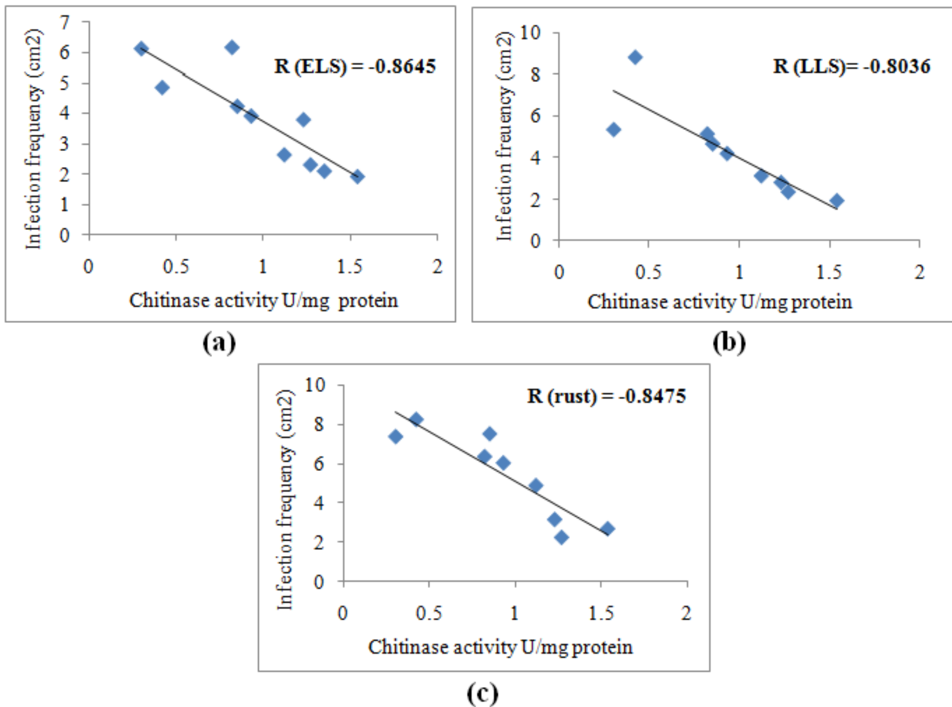


Fig. 7. Correlation of the chitinase activity in peanut cv ICG 7827 transgenic with the infection frequency.

a) Correlation between chitinase activity and *C. arachidicola* (ELS) infection frequency; b) Correlation between chitinase activity and *C. personatum* (LLS) infection frequency; c) Correlation between chitinase activity and *P. arachidis* (rust) infection frequency.

In the present study, correlation analysis showed a significant trend towards decreased disease severity in the transgenics with the increasing chitinase activity that confirmed that the inhibition observed was due to overexpressed *Tc chitI* protein. Similarly, a negative correlation between increased chitinase activity and resistance to ELS, LLS, and rust was reported in transformed peanut cv ICG 13942 [11]. A positive correlation between increased chitinase activity and resistance to ELS has also been shown earlier [33]. Likewise, various studies have also observed correlations on different crop species [10,40-42]**.**

For evaluating transgenic resistance against *C. arachidicola, C. personatum* and *P. arachidis* pathogens, a total of 10 $T_1$ events were tested. According to our observation, the events of TC-7, TC-10, and TC-1 performed better in all the tested parameters compared to other events screened for ELS, LLS, and rust disease resistance in T1 transgenic plants. The level of resistance to ELS, LLS, and rust in these transgenic peanut plants was higher than that identified in the non-transformed peanuts. In the present investigation *Tc chitinase* gene showed higher expression of enzyme activity with varying levels of resistance to ELS, LLS, and rust, respectively, when compared with transgenic plants using bacterial chitinase (*Bchit*) and rice chitinase (*RCG-3*) genes showed lesser expression of enzyme activity conjoined with varying levels of resistance to *C. arachidicola* [32,37]. The variability of pathogen resistance between transgenic events may be due to the localization of chitinase enzymes at the tissue and cellular levels [43]. Further use of rice chitinase (*Rchit*) in peanut transgenics displayed longer incubation and latent periods, lower infection rating, fewer lesions against late leaf spot (LLS), and rust diseases [20], as we have observed in the present investigations.

## 4. Conclusion

The results presented here demonstrate the successful insertion and expression of the *Tc chitiI* gene using the genotype-independent peanut cv ICG 7827. The stable transmission of the *Tc chitI* gene was further confirmed by PCR, RT-PCR, and Southern blot and expression analyses of progeny from transgenic lines. A strong negative correlation between the infection frequency of chitinase against fungal pathogens *C. arachidicola* (ELS), *C. personatum* (LLS), and *P. arachidis* (Rust) appeared to be enhanced in those lines, which were exhibiting a seven-fold increase in chitinase activity. Our present protocol is more suitable for the rapid development of transgenics in a recalcitrant system like a peanut. Further, the same technology can also be used for the genetic engineering of peanuts to introduce various agronomically important traits to develop tolerance against different types of biotic and abiotic stresses.

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