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ORIGINAL PAPER

Expression of *Tcchitinase-I* gene in transgenic peanut (*Arachis hypogaea* L.) confers enhanced resistance against leaf spot and rust diseases

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Abstract
The disease-resistant transgenic peanut cv ICG 13942 plants were developed by using *Tcchitinase-I* gene. *Agrobacterium tumefaciens* strain LBA4404 harboring the binary vector (pBinAR) contains the chitinase (*Tcchitinase-I*) gene and neomycin phosphotransferase resistance (*nptII*) gene. The transformed shoots were developed on selection medium (MMS + 0.5 mg/L IAA + 15 mg/L TDZ + 100 mg/L Kan + 250 mg/L Cefotaxime) from deembryonated cotyledon (DC) explants. Established plantlets were screened for the presence of *Tcchitinase-I* and *nptII* genes. Stable integration and expression of the transgenes (T_0) were confirmed by using PCR, RT-PCR and Southern blot analyses. The transformation frequency 63.34% was recorded. All the transformed (T_0) plants were found normal, flowered and set seeds. After selfing the T_0 plants, a Mendelian inheritance pattern (3:1) for the transgene in T_1 progeny is revealed. T_1 transgenic peanut plants were evaluated for resistance against *Cercospora arachidicola, C. personatum* and *Puccinia arachidis* by infection with the microspores using detached leaf assay. These T_1 plants have shown longer incubation, latent period and lower infection frequencies in comparison to non-transformed (WT) plants. The *Tcchitinase-I* gene expression in resistant transgenic plants was compared to that of a susceptible control. A significant negative correlation was recorded between chitinase activity and the frequency of infection to the three tested disease causing agents.

Keywords *Tcchitinase-I* · *nptII* · DC · *Arachis hypogaea* · Early leaf spot · Late leaf spot · Rust

Introduction

Peanut or groundnut (*Arachis hypogaea* L.) is an important oilseed crop grown in the tropical and sub-tropical regions of the world. Due to various biotic stresses such as insect pests, bacterial and fungal diseases, the yield in peanut is decreased. Among the major fungal diseases, early leaf spot (ELS) (*Cercospora arachidicola*), late leaf spot (LLS) (*Cercospora personatum*) and rust caused by *Puccinia arachidis* are more destructive (50–70%) in peanut (Subrahmanyam et al. 1984). Chitin is an important cell wall component of fungi and this is degraded by chitinases. Chitinases (E.C. 3.2.1.14) are poly (1,4-(N-acetyl-β-D) glucosaminide))-glycanohydrolases. They directly hydrolyze fungal cell wall, chitin the substrate for the enzyme, and by this action fungal hyphallysis and inhibition of fungal growth occur (Patil et al. 2000). Thus, plants use one of the many natural defense mechanisms to resist against pathogens and accumulate proteins (e.g., chitinases) active against disease causing organisms. Where this mechanism is too weak or appears too late to induce full protection against pathogen, engineering the expression of a defense protein can enhance the resistance to fungal diseases (Broglie et al. 1991; Grison et al. 1996).

The use of fungicide to control the disease is often ineffective because the pathogen spreads rapidly under favorable conditions. The crop production heavily relies on chemicals for protection which is not viable as these chemicals provide ephemeral benefits often with adverse side effects (Kumar et al. 2008). The major destructive fungi, on the other hand, develop tolerance to most classes of fungicides and these can cause environmental pollution (Moham et al. 2003). In view

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of this, genetic engineering with chitinase is a powerful tool to improve the fungal disease resistance in plants.

Genetic engineering technology plays a great role in transfer of gene(s) of interest for developing disease resistance and improving quality and crop yield. By using this technology, the chitinase gene from different origins has been introduced into various crop plants for developing enhanced fungal resistance: tobacco (Zhu et al. 1994), rice (Lin et al. 1995), cucumber (Kishimoto et al. 2002), Italian ryegrass (Takahashi et al. 2005), cotton (Ganesan et al. 2009), banana (Sreeramanan et al. 2009) and peanut (Chu et al. 2008, 2013).

Though enhanced fungal resistance has been developed in peanut by using tobacco chitinase (Rohini and Rao 2001), barley oxalate oxidase (Livingstone et al. 2005), mustard defensin (Anuradha et al. 2008) and tobacco β-1,3-glucanase (Sundaresha et al. 2009), there is no report on *Tcchitinase-I* gene in peanut cvs. Hence, in the present study, we have developed the transgenic peanut cv ICG 13942 plants by using deembryonated cotyledon (DC) explants through *Agrobacterium*-mediated genetic transformation by using *Tcchitinase-I* gene for resistance to leaf spot (ELS, LLS) and rust diseases.

Materials and methods

Plant material

The mature seeds of peanut cv ICG 13942, obtained from the germplasm bank of ICRISAT, Patancheru, Hyderabad, Telangana, India were used. This variety is susceptible to leaf spot and rust fungal diseases.

Mature groundnut seeds of peanut cv ICG 13942 were washed under running tap water for 10–15 min followed by treating with liquid detergent Tween-20 (5%-v/v) for 5 min and it was repeated twice followed by rinsing in 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 under aseptic conditions and soaked for 24 h in sterile distilled water. These soaked seeds were dried on sterile tissue paper, dissected aseptically and removed the zygotic embryo. Now, these deembryonated cotyledons (DC) were cut longitudinally and precultured on shoot induction medium (SIM) containing Modified Murashige and Skoog's (1962) (MMS) medium + 0.5 mg/L IAA + 15 mg/L TDZ for 3 days.

Gene constructs

Agrobacterium tumefaciens strain LBA4404 harboring the binary plasmid pBinAR (13.7 Kb) was used for genetic transformation of groundnut cv ICG 13942. The binary vector pBinAR carrying *Theobroma cacao chitinase-I (Tcchitinase-I)* gene with a *nptII* selectable marker gene was used. The T-DNA portion of pBinAR having *nos-npt*II cassette in RB and 770 bp *Eco*RI/*Hind* III fragment contains 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. A 1.2Kb *Sma*I-*Xba*I fragement of *T. cacao* class I chitnase was taken out from pGH00.0126 vector and cloned intocorresponding sites of binary vector pBinAR (Fig. 1).

Transformation procedure

The precultured DC (deembryonated cotyledon) explants were infected with *A. tumefaciens* LBA 4404 harboring binary vector pBinAR containing *Tcchitinase-I* gene and *nptII* as selectable marker gene and cocultivated on SIM (shoot induction medium) containing MMS (modified MS medium) + 0.5 mg/L IAA + 15 mg/L TDZ for 4 days. After cocultivation, these explants were shifted onto selection medium containing SIM + 100 mg/L Kan + 250 mg/L Cefotaxime. After 2 weeks of incubation, the explants with Kan^R shoots were cultured on SIM + 50 mg/L Kan for further proliferation of microshoots. Subsequently, the shoots were elongated, rooted and established the plantlets by following our earlier study (Rajinikanth and Rama Swamy 2018). The putative transformants (T_0) were obtained within 4 months of culture initiation (Fig. 2a–e). The plants were regenerated from non-transformed explants and established in the greenhouse as control. T_0 transgenic plants were maintained in the greenhouse 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 analyzed using standard procedures.

PCR and RT-PCR analysis of the transformants

The genomic DNA was isolated from randomly selected putative transgenic plants and one non-transformed plant (control) according earlier method (Sharma et al. 2000) and

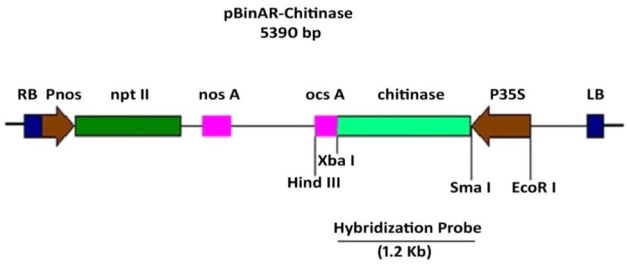


Fig. 1 Linear diagram of T-DNA portion of pBinAR-chitinase-I construct

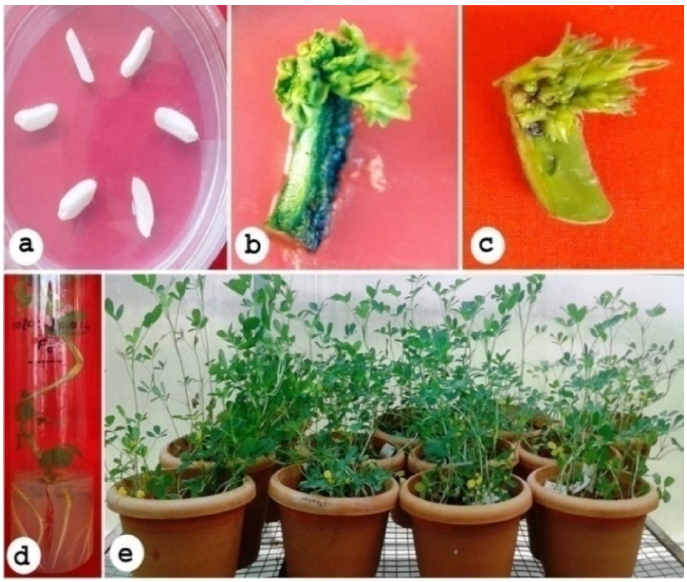


Fig. 2 *Agrobacterium*-mediated genetic transformation in deembryonated cotyledon (DC) explants of peanut cv ICG 13942 by using binary vector pBinAR. **a** Infected DC explants on SIM for cocultivation. **b, c** Induction of Kan^R shoots on selection medium after 4 & 6 weeks of incubation, respectively. **d** In vitro rooting of elongated microshoots on RIM augmented with 1.0 mg/L NAA + 50 mg/L Kan. **e** T_0 plants are shifted to plastic pots containing soil mix and maintained in the green house

subjected to PCR amplification using the *Tcchitinase-I* gene-specific primers:(F)5′-GGAAAATGGTTGCCAGAGTCA GTGC-3′, (R)5′-GCTACATTGAGTCCACCGAGGGT-3′ and *nptII* gene-specific primers: (F) 5′-GCTTGGGTG GAGAGGGCTATT-3′, (R) 5′-AGAACTCGTCAAGAA GGCGA-3′. The PCR analysis for *Tcchitinase-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 *nptII* 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, b). The randomly selected PCR-positive transgenic plants were used for RT-PCR analysis. Total RNA was isolated from leaf tissue of the putative transformants using the TRIzol reagent according to the manufacture's protocol and RT-PCR analysis of the putative transformants was carried out using the Thermoscript RT-PCR system for 35–40 cycles using *Tcchitinase-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 ultraviolet light (Fig. 3c).

Southern blot analysis

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

Segregation analysis

Inheritance of the transgene was studied by using the PCR screening of *Tcchitinase-I* gene in T_1 and T_2 generations. PCR + ve and –ve plants were identified and chi-square test was performed to validate the data for 3:1 segregation.

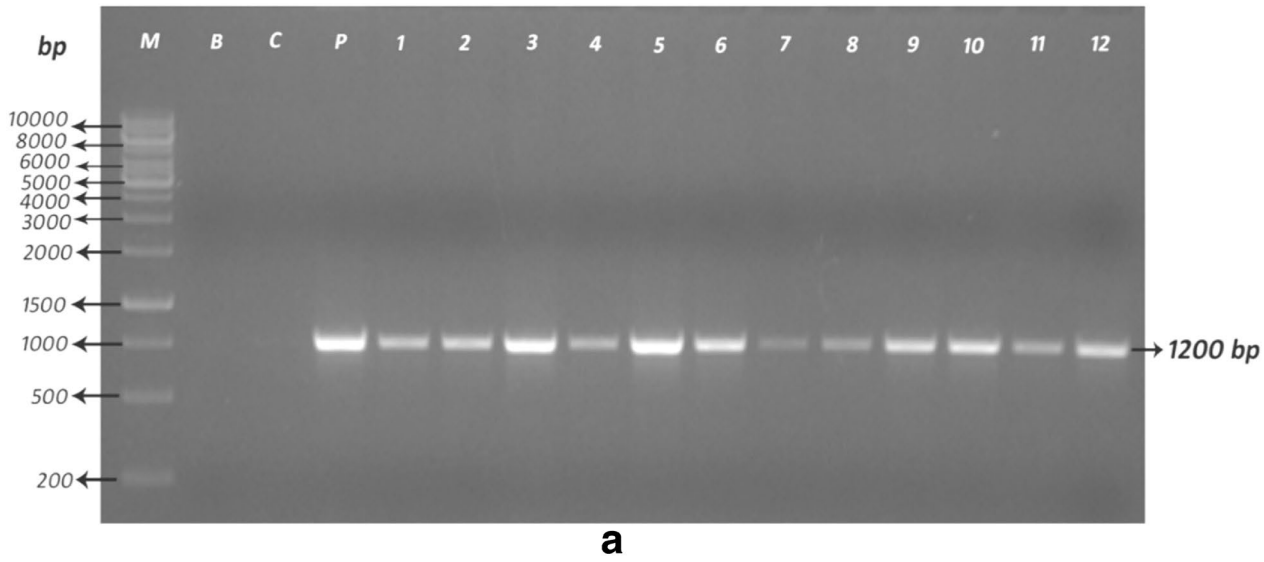
Chitinase assay

A colorimetric assay was performed with the leaves of 45-day-old transformed and non-transformed (WT) control peanut plants following the method of Mauch et al. (1984).

Detached leaf assay for ELS, LLS and rust diseases

Disease evaluation of the transgenic peanut plants for ELS, LLS and rust pathogens was conducted in T_1 generation plants by detached leaf assay technique as reported earlier.

When the plants (T_1) were 40 days old, the quadrifoliate leaves from either second or third fully expanded leaf of 10 selected PCR + ve transgenic plants per event were excised from pulvinus region and arranged in randomized block design in plastic trays containing sterile river sand. The leaves were immediately dipped in distilled water and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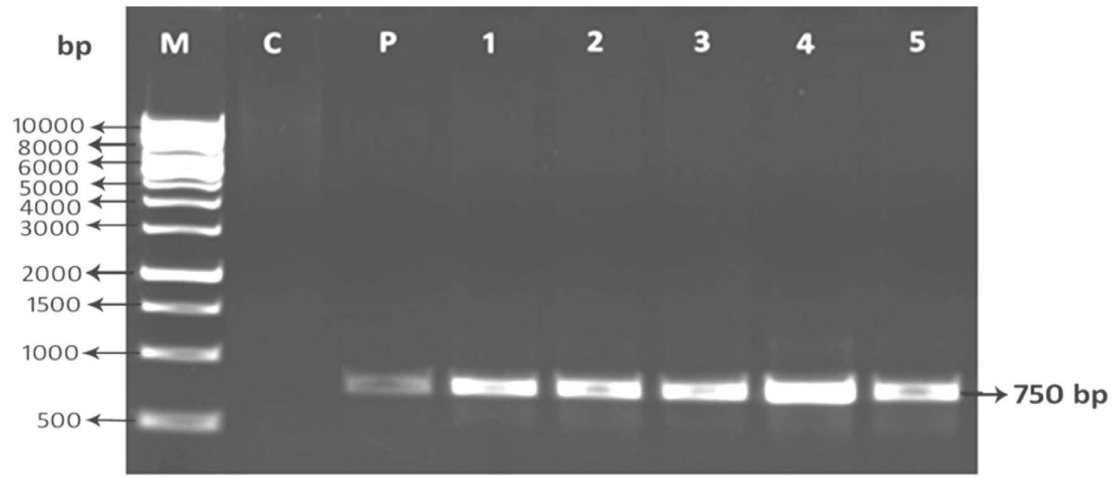
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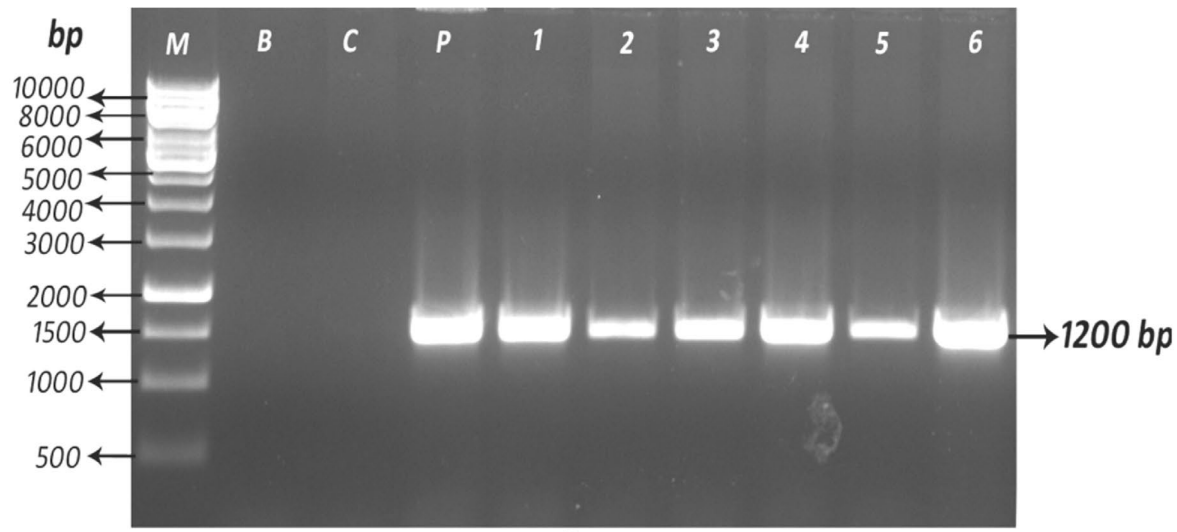
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◂**Fig. 3** Molecular analysis of the transformed peanut cv ICG 13942 plants. **a** PCR amplification of genomic DNA showing amplification of a 1200 bp fragment of the *Tcchitinase-I* gene, Lanes: 1–12: carry genomic DNA from T_0 putative transformants. **b** PCR amplification of genomic DNA showing amplification of a 750 bp fragment of the *nptII* gene, Lanes: 1–5: carry genomic DNA from T_0 putative transformants. **c** RT-PCR of the cDNA showing amplification of a 1200 bp fragment of the *Tcchitinase-I* gene, Lanes: 1–6: carry cDNA from T_0 putative transformants. M: molecular size marker (1 Kb ladder), B: blank, C: non-transformed control plant DNA (-ve control-WT), P: plasmid pBinAR (+ ve control)

were taken for planting in plastic trays having dimensions 39.5 cm × 29 cm × 7 cm. The trays were filled with a layer (approximately 1.5 cm thick) of sterile sand. The sand was kept moist with distilled water. Holes were made with the help of plastic droppers to place the leaves. In each hole, a leaf was planted and the lower portion of the rachis was covered with sand. Simultaneously, ten leaves of susceptible control plants were also planted in a separate tray. The Hoagland's nutrition solution (Hoagland and Arnon 1950) was supplied to leaves, which provided the essential nutrients. The trays were covered with clear polythene cover and incubated before inoculation for 24 h in a growth chamber for acclimatization. The day and night temperature in the growth chamber was maintained at 23 °C with relative humidity of 60% and illuminated with white light. The leaves were allowed to get acclimatized for 24 h after transferring to plastic trays filled with sterile sand and then challenged with ELS, LLS and rust disease pathogens separately by using the method of Prasad et al. (2013).

The experimental trays were examined daily beginning 6 days after inoculation (DAI) for incubation period (IP) and latent period (LP). After 28 days of inoculation, observations on lesions or pustules per leaf (LN, PN), percentage leaf area damaged (LAD%) and infection frequency (IF, defined as number of lesions or pustules/cm^2 leaf area) were recorded.

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 was 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 5% level of significance among the transgenics and non-transformed control plants for infection frequency of three tested pathogens with chitinase activity.

Results

Genetic transformation of peanut with *Tcchitinase-I* gene

Agrobacterium tumefaciens-mediated genetic transformation was carried out by using the binary vector pBinAR-*Tcchitinase-I* with DC explants of groundnut cv ICG 13942. A total of 38 primary transformants (T_0) were regenerated from 60 precultured DC explants following cocultivation with *A. tumefaciens* harboring the binary vector pBinAR-*Tcchitinase-I* with 63.34% of transformation efficiency. The regenerated plants (Kan^R) exhibited normal growth under greenhouse conditions and produced morphologically normal flowers and pods that contained viable seeds (Fig. 2a–e).

Molecular analysis of transgenics

Integration and expression of transgenes

The insertion of the *Tcchitinase-I* gene into the peanut through *Agrobacterium* gene transformation was initially screened by PCR analysis. The presence of 1200 bp region of the *Tcchitinase-I* gene was detected in 12 of the 38 transgenic plants produced with the binary plasmid pBinAR:*Tcchitinase-I* with the transformation efficiency is 63.34% (Fig. 3a). Randomly selected transformants of 5 of the 38 transgenic plants also showed amplification of 750 bp fragment of the *nptII* gene (Fig. 3b). Expression of the introduced gene was analyzed by RT-PCR from the randomly selected 6 T_1 and T_2 PCR-positive plants. The expected 1200 bp amplified fragment corresponding to the *Tcchitinase-I* gene was detected in all the plants that were selected for analysis (Fig. 3c). Randomly selected PCR- and RT-PCR-positive events were analyzed by Southern blot hybridization for copy number (*Eco*RI digested DNA) using 1.2 Kb fragment as probe (Fig. 4). The Southern analysis indicated the presence of three copies of the transgene in event number TC-1, TC-7, TC-12, TC-14, TC-18 (Lanes 3–7), while the event TC-20 (Lane 1) showed two copies of the transgene and event TC-9 (Lane 2) showed single copy of the transgene, whereas no transgene insertion was detected in non-transformed control plant DNA (Lane C). 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.

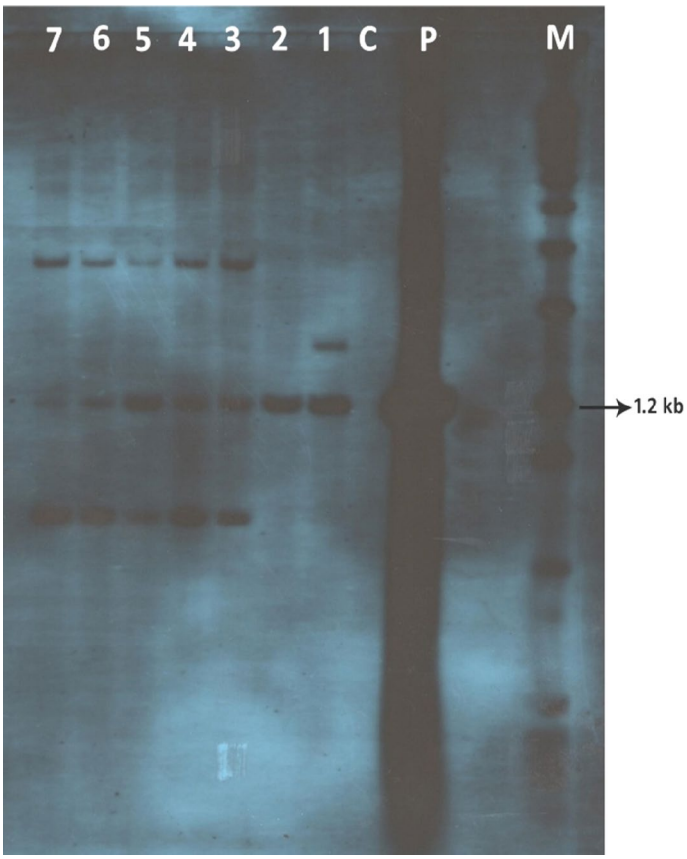

Fig. 4 Southern blot analysis of the genomic DNA from leaves of transgenics obtained through *Agrobacterium*-mediated genetic transformation. The genomic DNA of peanut transgenics was digested with *Eco*R1 to check the copy number of the integrated gene. Lanes: 1: carry *Eco*RI-restricted genomic DNA from event TC-20 showed two copy numbers, 2: carry *Eco*RI-restricted genomic DNA from event TC-9 showed one copy number, 3–7: carry *Eco*RI-restricted genomic DNA from events TC-1, TC-7, TC-12, TC-14, TC-18 showed three copy numbers, respectively, C: *Eco*RI-restricted genomic DNA from control plants, P: *Eco*RI-restricted plasmid pBinAR: *Tcchitinase-I*, M: molecular weight marker

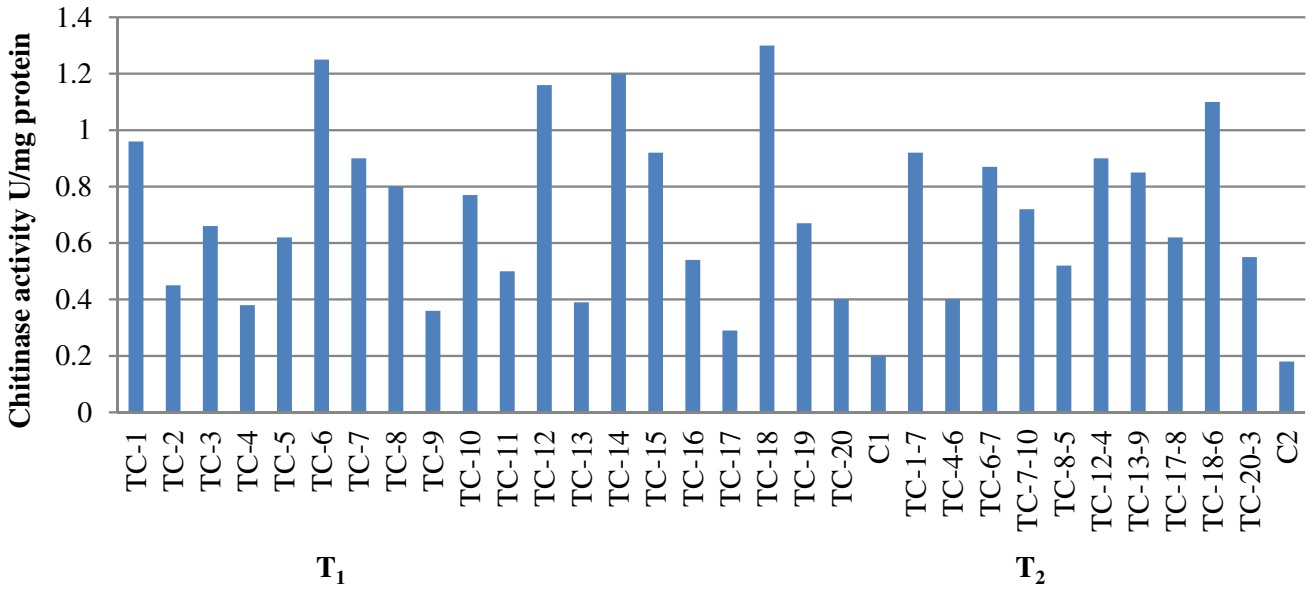

Fig. 5 Chitinase activity in the peanut transgenic plants. Graph bars represent the mean ± SD values of two replicates

Chitinase activity in the transgenic plants

The chitinase activity varied among the transgenic events expressing *Tcchitinase-I* gene, where 6.5-fold increase in the chitinase activity (0.29–1.30 Umg^{-1} protein) was recorded as compared to the non-transformed control plants (0.20 Umg^{-1} protein) (Fig. 5). Of the 20 T_1 transgenic events tested, 6 transgenic events (TC-1, TC-6, TC-14, TC-15 and

Table 1 Performance of peanut transgenic plants (T_1) carrying *Tcchitinase-I* gene against ELS, LLS and rust diseases, respectively, in peanut cv ICG 13942

Event no	Incubation period (IP) (days)			Latent period (LP) (days)			No. of Lesion(s)/Pustule(s) per leaf			Leaf area damage (%) (days)			Infection frequency (cm^2) (days)		
	ELS	LLS	Rust	ELS	LLS	Rust	ELS	LLS	Rust	ELS	LLS	Rust	ELS	LLS	Rust
TC-01	14b	20ab	12 cd	15 cd	14d	25abc	31bd	24d	34ab	10.26 cd	20.12a	10.34c	3.81bc	3.84acd	5.72acd
TC-06	15ab	17bc	19abc	20ab	20c	22c	30d	25 cd	25d	8.23d	16.13abc	10.87bc	2.70d	3.25 cd	4.13 cd
TC-07	10bd	12 cd	23a	12d	19bd	31a	31 cd	37ab	22de	11.18acd	10.56d	3.23de	4.05abc	4.23c	3.68de
TC-09	13bc	8 cd	18bc	10 cd	21abc	14d	34acd	27acd	35a	10.78bd	14.67bc	9.18acd	4.63b	6.12a	9.15a
TC-10	6de	10d	8de	17c	23ab	21acd	43a	31abc	30abc	14.76b	19.14ab	15.21ab	3.77c	5.91ab	6.42bc
TC-12	8d	19b	20ab	18abc	21bc	26b	40b	26bd	29c	12.65c	11.34 cd	17.34a	3.03bd	3.56bd	6.23 cd
TC-14	12c	21a	13bd	19b	26a	27ab	37bc	22de	30bc	16.10a	6.20de	6.12 cd	2.85 cd	2.50de	4.61bd
TC-17	14abc	16c	16c	16bd	9de	18 cd	35c	40a	26 cd	15.03ab	12bd	5.09d	3.25acd	4.62bc	7.54abc
TC-18	18a	19abc	20b	21a	22b	25bc	27de	30bc	27bd	5.62de	13.10c	11.10abc	2.13de	2.82d	3.95d
TC-20	12acd	14acd	10d	18bc	20acd	12de	42ab	30c	29acd	13.56bc	12.34acd	8.15bd	6.12a	4.84abc	7.84b
Non-Transformed	10 cd	13bd	15acd	16acd	19 cd	21bd	40abc	35b	31b	14.32abc	18.10b	12.08b	4.84ab	5.82b	8.16ab
SE ±	0.865	0.421	0.712	2.674	0.940	1.225	3.146	5.401	2.210	0.332	0.450	0.692	1.023	0.562	0.210
LSD 5%	2.390	1.163	1.967	7.390	2.598	3.385	8.695	14.928	6.108	0.917	1.243	1.912	2.827	1.553	0.580
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 means of ten plants per event. Mean followed by the same letter are not significantly different at 5% level

Fig. 6 **a–c** Showing the infected groundnut cv ICG 13942 WT (ELS, LLS and Rust diseases, respectively) plants in the field. **d** Transformed TC-18 (T_1) plants showing healthy leaves

TC-18) had significantly higher chitinase activity than the rest, which sustained in the T_2 progeny of four of these six events (TC-1-7, TC-6-7, TC-12-4 and TC-18-6).

Evaluation of peanut transgenics for resistance against early leaf spots (ELS), late leaf spot (LLS) and rust diseases

The progenies of twenty T_1 transgenic events were tested and they showed significant differences for all the components of resistance to ELS, LLS and rust diseases in detached leaf bioassay (Table 1).

For evaluation of ELS, the event TC-18 and TC-06 showed longer incubation period (18 and 15 days), longer latent period (21 and 20 days) and less number of lesions per leaf (27 and 30) in T_1 transformed plants compared to non-transformed (control) plants (10 days IP, 16 days LP and 40 lesions). Most of the transgenic events (TC-1, TC-06, TC-07, TC-09 and TC-18) showed less LAD (5.62–11.18%), less number of lesions per leaf (27–34) and less IF (2.13–4.63 cm^2) compared to non-transformed counter parts (LAD 14.32%, lesions 40, IF 4.84 cm^2). According to our observations, the event TC-18 showed better performance in all the recorded resistance parameters for ELS disease. They are longer IP (18 days), longer LP (21 days), lesser number of lesions per leaf (27), less LAD (5.62%) and lower IF (2.13 cm^2) in comparison to other events in T_1 plants (Table 1).

T_1 transgenic events evaluated for LLS showed significant genotypic difference for all the components. Most of the transgenic events 01,06,10,12 and 18 showed longer IP (17 to 23 days) and the events 9,10,12,14 and 18 showed longer LP (21 to 24 days) than the non-transformed plants (13 days IP, 19 days LP). The event no. 14 showed lesser number of lesions per leaf (22 lesions), less leaf area damage (6.20%) and lower IF (2.50 cm^2) in comparison to the control plants (35 lesions, 18.10% LAD, 5.43 cm^2 IF). Thus, the event TC-14 was found to be performed better in all the parameters (longer IP-21 days, longer LP-26 days, lesser number of lesions per leaf-22, less LAD-6.20% and lower IF-2.50 cm^2) tested compared to other events screened for LLS disease resistance (Table 1).

For evaluation of rust, the event no. 7 showed longer incubation (23 days), longer latent periods (31 days) and less no. of lesions (22 lesions) than their control plants (15 days IP, 21 days LP and 31 lesions). All transgenic events except event 10, 12 showed less LAD (3.23–11.10%) than the control plants (12.08%). Similarly, most of the transgenic events showed lower Infection frequencies (3.68 to 7.84 cm^2) except transgenic event TC-9 (9.15 cm^2) than the control plants (8.16 cm^2). Thus, the event TC-7 showed the best results for all the resistance parameters tested for rust disease even compared to all other T_1 transgenic plants (Table 1).

According to our observation, the three transgenic events of TC-18, TC-14 and TC-7 displayed significantly higher resistance to *C. arachidicola, C. personatum* and *P. arachidis* pathogens in T_1 plants, respectively (Fig. 6a–d).

Correlation between chitinase activity and disease resistance

Disease severity correlated well with the chitinase activity and the infection frequency of ELS, LLS and rust in the T1 transgenic plants with the Pearson correlation coefficients ranging from − 0.7226 ($P = 0.05$), − 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.

Discussion

Fungal diseases constitute a major challenge to the millions of peanut growing farmers throughout the tropical regions. A large proportion of the potential peanut crop is lost yearly to several major stresses despite the efforts at transgenics for resistance. Analysis of transgenic plants provides a powerful tool for functional studies of defense genes in peanut.

As there are no reports on *Tcchitinase-I* gene expression in peanut, we report the transgenic peanut events expressing the *Tcchitinase-I* gene and also evaluated for their tolerance to leaf spot (ELS, LLS) and rust diseases.

The *T. cacao* chitinase gene used in the present study is a class I chitinase and belongs to PR3 family, having high chitinase activity due to the presence of chitin binding domain (CBD) (Sela-Buurlage et al. 1993). Deletion of chitin binding domain (ChBDTob) from tobacco class I chitinase has been reported to cause a threefold reduction of activation energy and antifungal activity due to lack of its binding capacity to chitin. While a CBD is not required for chitinolytic or antifungal activities, it increases both, perhaps by anchoring to the substrate and increasing its effective concentration for hydrolysis (Iseli et al. 1993). Interestingly, all other classes of chitinase have either no or lower antifungal activity as compared to class I chitinases (Sela-Buurlage et al. 1993).

Engineering for disease resistance in legumes has been considered important in the recent years. However, very little progress is seen in the improvement of legumes through the transgenic approach and more so with peanut because of the recalcitrancy in regeneration of the crop. There are reports of regenerability and *Agrobacterium*-mediated genetic transformation efficiency in the peanut, but less frequency of transformation 55%, 31% in JL 24 (Sharma and Anjaiah 2000; Anuradha et al. 2006), 55% in ICGV 86031 (Prasad et al. 2013), 40% in ICGV 89104 (Prasad et al. 2013) and 34% in K6 (Mehta et al. 2013). Survival rate of the in vitro regenerated plantlets was over 75% in cv. Golden and 49% in cv. Bari-2000, while healthy putatively transgenic (T_0) plants with over 41% transformation frequency in cv. Golden and 32% in cv. Bari-2000 were produced through *Agrobacterium*-mediated gene transfer of the rice chitinase gene and all the plants flowered and set seed normally (Iqbal et al. 2012). Whereas in the present investigations, we have developed the transgenic T_0 peanut plants with high transformation efficiency of 63.63% when compared to earlier reports.

The integration of the transgene was confirmed by PCR and Southern blot analyses. Segregation studies showed Mendelian ratio of 3:1 of the *Tcchitinase-I* gene in T_1 and T_2 generation transgenic peanut plants. Similarly, Anuradha et al. (2006) have reported that the inheritance of a promoter less *gus:nptII* bifunctional fusion gene in groundnut through Chi-square analysis showed that the segregation of fusion gene followed the Mendelian 3:1 ratio. Tiwari et al. (2008) obtained 3:1 segregation ratio for *cry1 EC* gene in transgenic groundnut as we have observed in the present study. Thus, the inheritance and stable expression of transgenes is important in crop improvement through gene manipulations. In the present study, the expression of the transgenes was also confirmed by RT-PCR.

In the present study, 6.5-fold increase in the chitinase activity (0.29–1.30 Umg^{-1} protein) was recorded in transgenic plants as compared to 0.20 Umg^{-1} protein in the non-transformed control plants. The enhanced chitinase activity in the transgenic plants compared to their non-transformed controls confirmed the expression of *Tcchitinase-I.* Several reports on intensified chitinase activity have been observed in the transgenic plants expressing the other type of chitinase genes (Lin et al. 1995; Datta et al. 2000; Nandakumar et al. 2007). Over 14-fold increase in chitinase activity over controls was reported in the leaves of peanut transformants (Prasad et al. 2013), while in transgenic rice it has been reported up to 14 times (Lin et al. 1995; Nandakumar et al. 2007). A 5-fold increase in chitinase activity in transgenic peanut plants transformed with rice chitinase gene was observed (Iqbalet al. 2012).

Although in our study some of the transgenic events showed increased *Tc-chitinase-I* activity, 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 (Grison et al. 1996; Datta et al. 2001; Pasonen et al. 2004). Nevertheless, most of the transgenic plants showed reduced infection than their non-transformed control plants confirming the antimicrobial property of the expressed *Tcchitinase-I* against these pathogens.

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 the presence of over expressed *Tcchitinase-I* protein. A positive correlation between increased chitinase activity and resistance to ELS has also been shown earlier (Rohini and Rao 2001). Similar correlations have also been observed in various studies on different crop species (Lin et al. 1995; Tabei et al. 1998; Zhu et al. 1998; Carstens et al. 2003; Itoh et al. 2003; Liang et al. 2005; Nandakumar et al. 2007).

For the evaluation of transgenic resistance against ELS, LLS and rust diseases, a total of 20 T_1 events were tested. According to our observation, the events of TC-18, TC-14 and TC-7 performed better in all the parameters tested

compared to other events screened for ELS, LLS and rust disease resistance in T_1 transgenic plants, respectively. The level of resistance to ELS, LLS and rust in these transgenic peanut plants was comparable or higher than that identified in the cultivated peanut showing 2–5 disease scores on a 1–9 scale (Reddy et al. 1992, 1996; Pensuk et al. 2003; Hossain et al. 2007; Badigannavar et al. 2005).

Recent development of transgenic plants using bacterial chitinase (*Bchit*) and rice chitinase (*RCG-3*) genes showed higher expression of enzyme activity conjoined with varied levels of resistance to *C. Arachidicola* (Iqbal et al. 2011, 2012). The variability of pathogen resistance between transgenic events may be due to the localization of chitinase enzymes at the tissue and cellular levels (Leeuwenet al.2001). 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 (Prasad et al. 2013) as we have observed in the present investigations.

Thus, we conclude that the resistance against *C. arachidicola C. Personatum* and *P. arachidis* appeared to be enhanced in those lines which were exhibiting 6.5-fold increase in chitinase enzyme activity by using *Tcchitinase-I* gene for the first time in peanut cv ICG 13942. The lines showing less enzyme activity did not played a role in resistance against pathogen. The resistant lines will go in breeding cycle in next generations to fix the character in character-deficient cultivar. Once this character proved to be inheritable, these lines could be used as good genetic source of disease resistance breeding material. We expect that the combination of this transgenic strategy based on the use of *Tcchitinase-I* gene and traditional breeding will provide durable fungal disease-resistant peanut lines with good agronomic phenotypes.

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Author contributions RM, the first author, has performed experiments and wrote the manuscript. RSN has designed the experiments and also corrected the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

References

Anuradha TS, Divya K, Jami SK, Kirti PB (2008) Transgenic tobacco and peanut plants expressing a mustard defensin show resistance to fungal pathogens. Plant Cell Rep 27:1777–1786

Anuradha TS, Jami SK, Dalta RS, Kirti PB (2006) Genetic transformation of peanut (*Arachis hypogaea* L.) using cotyledonary node as explant and a promoterlessgus: nptII fusion gene based vector. J Biosci 31(2):235–246

Badigannavar AM, Kale DM, Mondal S, Murty GSS (2005) Trombay groundnut recombinants resistant to foliar diseases. Mutat Breed Newsl Rev 1:11–12

Broglie K, Chet I, Holliday M, Cressman R, Biddle P, Knowlton S (1991) Transgenic plants with enhanced resistance to the fungal pathogen *Rizoctoniasolani*. Science 254:1194–1197

Carstens M, Vivier MA, Pretorius IS (2003) The *Saccharomyces cerevisiae*chitinase, encoded by the CTS1-2 gene, confers antifungal activity against *Botrytis cinerea* to transgenic tobacco. Transgenic Res 2:497–508

Chu Y, Bhattacharya A, Wu C, Knoll JE, Ozias-Akins P (2013) Improvement of peanut (*Arachis hypogaea* L.) transformation efficiency and determination of transgene copy number by relative uantitative real-time PCR. Vitro Cell Dev Biol Plant 49:266–275

Chu Y, Deng XY, Faustinelli P, Ozias-Akins P (2008) Bcl-xL transformed peanut (*Arachis hypogaea* L.) exhibits paraquat tolerance. Plant Cell Rep 27:85–92

Datta K, Koukolikova-Nicola Z, Baisakh N, Oliva N, Datta SK (2000) *Agrobacterium*-mediated engineering for sheath blight resistance of indica rice cultivars from different ecosystems. Theor Appl Genet 100:832–839

Datta K, Tu J, Oliva N, Ona I, Velazhahan R, Mew TW, Muthukrishnan S, Datta SK (2001) Enhanced resistance to sheath blight by constitutive expression of infection-related rice chitinase in transgenic elite indica rice cultivars. Plant Sci 160:405–414

Ganesan M, Bhanumathi P, Ganesh Kumar K, Lakshmi Prabha A, Pill-Soon S, Jayabalan N (2009) Transgenic Indian cotton (*Gossypiumhirutum*) harboring rice chitinase gene (*Chi II*) confers resistance to two pathogens. Am J Biochem Biotechnol 5:63–74

Grison R, Grezes-Besset B, Schneider M, Lucante N, Olsen L, Leguay JJ (1996) Field tolerance to fungal pathogens of *Brassicanapus* constitutively expressing a chimeric chitinase gene. Nat Biotechnol 14:643–646

Hoagland DR, Arnon DI (1950) The water culture method for growing plant without soil. California AgrExp Sta. Cir No 347. University of California Berkley Press, California, pp. 1–32.

Hossain MD, Rahman MZ, Abeda K, Rahman MM (2007) Screening of groundnut genotypes for leaf spots and rust resistance. Int J Sustain Crop Prod 2:7–10

Iqbal MM, Nazir F, Ali S, Asif MA, Zafar Y, Iqbal J, Ali GM (2012) Over expression of rice chitinase gene in transgenic peanut (*Arachis hypogaea* L.) improves resistance against leaf spot. Mol Biotechnol 50:129–136

Iqbal MM, Zafar Y, Zazir F, Ali S, Iqbal J, Asif MA, Rasid O, Ali GM (2011) Over expression of bacterial chitinase gene in Pakistani peanut (*Arachis hypogaea* L.) cultivar golden. Afr J Biotechnol 10(31):5838–5844

Iseli B, Boller T, Neuhaus JM (1993) The N-terminal cysteine-rich domain of tobacco class I chitinase is essential for chitin binding but not for catalytic or antifungal activity. Plant Physiol 103(1):221–226

Itoh Y, Takahashi K, Takizawa H, Nikaidou N, Tanaka H, Nishihashi H, Watanabe T, Nishizawa Y (2003) Family 19 chitinase of

Streptomyces griseus HUT6037 increases plant resistance to the fungal disease. Biosci Biotechnol Biochem 67:847–855

Kishimoto K, Nishizawa Y, Tabei Y, Hibi T, Nakajima A, Akutsu K (2002) Detailed analysis of rice chitinase gene expression in transgenic cucumber plants showing different levels of disease resistance to graymold (*Botrytis cinerea*). Plant Sci 162:655–662

Kumar S, Kumar S, Nagi SP, Kanwar JK (2008) *In vitro* selection and regeneration of chrysanthemum (*Dendranthema grandiflorum* Tzelev) plants resistant to culture filtrate of *Septoriaobese*Syd. Vitro Cell Dev Biol Plant 44:474–479

Leeuwen VW, Ruttink T, Borst-Vrenssen AW, Van der Plas LH, Van der Krol AR (2001) Characterization of position induced spatial and temporal regulation of transgene promoter activity in plants. J Exp Bot 52:949–959

Liang XQ, Holbmok CC, Lynch RE, Guo BZ (2005) β -1, 3- Glucanase activity in peanut seed (*Arachis hypognea*) is induced by inoculation with *Aspergillus flavus* and copurifies with a conglutin-like protein. Phytopathol 95:506–511

Lin W, Anuratha CS, Datta K, Potrykus I, Muthukrishnan S, Datta SK (1995) Genetic engineering of rice for resistance to sheath blight. Nat Biotechnol 13:686–691

Livingstone DM, Hampton JL, Phipps PM, Grabau EA (2005) Enhancing resistance to *Sclerotinia minor* in peanut by expressing a barely oxalate gene. Plant Physiol 137:1354–1362

Mauch F, Hadwiger LA, Boller T (1984) Ethylene: symptom, not signal for the induction of chitinase and β- 1,3 glucanase in pea pods by pathogens and elicitors. Plant Physiol 76:607–611

Mehta R, Radhakrishnan T, Kumar A, Yadav R, Dobaria JR, Thirumalaisamy PP, Jain RK, Chigurupati P (2013) Coat protein-mediated transgenic resistance of peanut (*Arachis hypogaea* L.) to peanut stem necrosis disease through *Agrobacterium*-mediated genetic transformation. Indian J Virol 24:205–213

Moham BR, Sajeena A, Setharaman K, Reddy MS (2003) Advances in genetically engineered (transgenic) plants in pest management-an overview. Crop Prot 22:1071–1086

Murashige T, Skoog I (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol 15:473–497

Nandakumar R, Babu S, Kalpana K, Raguchander T, Balasubramanian P, Samiyappan R (2007) *Agrobacterium*- mediated transformation of indica rice with chitinase gene for enhanced sheath blight resistance. Biol Plant 51:142–148

Pasonen HL, Seppanen SK, Degefu Y, Rytkonen A, Weissenberg K, Pappinen A (2004) Field performance of chitinase transgenic silver birches (*Betulapendula*): resistance to fungal diseases. Theor Appl Genet 109:562–570

Patil RS, Ghormade V, Deshpande MV (2000) Chitinolytic enzymes: an exploration. Enzyme Microb Technol 26:473–483

Pensuk V, Patanothai A, Jogloy S, Wongkaew S, Akkasaeng C, Vorasoot N (2003) Reaction of peanut cultivars to late leaf spot and rust. Songklanakarin J Sci Technol 25:289–295

Prasad K, Bhatnagar-Mathur P, Waliyar F, Sharma KK (2013) Over expression of a chitinase gene in transgenic peanut confers enhanced resistance to major soil borne and foliar fungal pathogens. J Plant Biochem Biotechnol 22:222–233

Rajinikanth M, Rama Swamy N (2018) Optimization of factors affecting Agrobacterium-mediated genetic transformation in groundnut (*Arachis hypogaea* L.). Adv Plants Agric Res 8(3):275–282

Reddy LJ, Nigam SN, Moss JP, Singh AK, Subrahmanyam P, McDonald D, Reddy AGS (1996) Registration of ICGV 86699 peanut germplasm line with multiple disease and insect resistance. Crop Sci 36:821

Reddy LJ, Nigam SN, Subrahmanyam P, Reddy AGS, McDonald D, Gibbons RW, Pentaiah V (1992) Registration of 'ICGV 87160' peanut cultivar. Crop Sci 32:1075

Rohini VK, Rao KS (2001) Transformation of peanut (*Arachis hypogaea* L.) with tobacco chitinase gene: variable response of transformants to leaf spot disease. Plant Sci 160:889–898

Sela-Buurlage MB, Ponstein AS, Bres-Vloemans SA, Melchers LS, Van Den Elzen PJM, Cornelissen BJC (1993) Only specific tobacco (*Nicotianatobacum*) chitinases and β-1, 3-glucanases exhibit antifungal activity. Plant physiol 101:857–863

Sharma KK, Anjaiah V (2000) An efficient method for the production of transgenic plants of peanut (*Arachis hypogaea* L.) through *Agrobacterium tumefaciens* mediated genetic transformation. Plant Sci 159:7–19

Sharma KK, Lavanya M, Anjaiah V (2000) Method for the isolation and purification of genomic DNA from peanut that is suitable for analytical applications. Plant Mol Biol Rep 18:393–400

Sreeramanan S, Maziah M, Xavier R (2009) A protocol for *Agrobacterim*-mediated transformation of banana with a rice chitinase gene. Emirates J Food Agric 21:18–33

Subrahmanyam P, Williams JH, McDonald D, Gibbons RW (1984) The influence of foliar disease and their control by selective fungicide on a range of groundnut (*Arachis hypogaea* L.) genotypes. Ann Appl Biol 104:467–476

Sundaresha S, Manoj Kumar A, Rohini S, Math SA, Keshsamma E, Chadrashekar SC, Udayakumar M (2009) Enhanced protection against two major fungal pathogens of groundnut, *Cercospora arachidicola* and *Aspergillus flavus* in transgenic groundnut over-expressing a tobacco β 1–3 glucanase. Eur J Plant Pathol 126:497–508

Tabei Y, Kitade S, Nishizawa Y, Kikuchi N, Kayano T, Hibi T, Akutsu K (1998) Transgenic cucumber plants harboring a rice chitinase gene exhibit enhanced resistance to graymold (*Botrytis cinerea*). Plant Cell Rep 17:159–164

Takahashi W, Fujimori M, Miura Y, Komatsu T, Nishizawa Y, Hibi T, Takamizo T (2005) Increased resistance to crown rust disease in transgenic Italian ryegrass (*Lolium multiflorum* Lam) expressing the rice chitinase gene. Plant Cell Rep 23:811–818

Tiwari S, Mishra DK, Singh A, Singh PK, Tuli R (2008) Expression of asynthetic cry1EC gene for resistance against *Spodoptera litura* in transgenic peanut (*Arachis hypogeae* L.). Plant Cell Rep 27:1017–1025

Zhu H, Muthukrishnan S, Krishnaveni S, Wilde G, Jeoung JM, Liang GH (1998) Biolistic transformation of sorghum using a rice chitinase gene. J Genet Plant Breed 52:243–252

Zhu Q, Maher EA, Masoud S, Dixon RA, Lamb CJ (1994) Enhanced protection against fungal attack by constitutive co-expression of chitinase and glucanase genes in transgenic tobacco. Nat Biotechnol 12:807–812