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Overexpression of a chitinase gene in transgenic peanut confers enhanced resistance to major soil borne and foliar fungal pathogens

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Abstract A chitinase gene from rice (*Rchit*) was introduced into three varieties of peanut through *Agrobacterium*-mediated genetic transformation resulting in 30 transgenic events harboring the *Rchit* gene. Stable integration and expression of the transgenes were confirmed using PCR, RT-PCR and Southern blot analysis. Progeny derived from selfing of the primary transgenic events revealed a Mendelian inheritance pattern (3:1) for the transgenes. The chitinase activity in the leaves of the transgenic events was 2 to 14-fold greater than that in the non-transformed control plants. Seeds of most transgenic events showed 0-10% *A. flavus* infection during in vitro seed inoculation bioassays. Transgenic peanut plants evaluated for resistance against late leaf spot (LLS) and rust using detached leaf assays showed longer incubation, latent period and lower infection frequencies when compared to their non-transformed counterparts. A significant negative correlation existed between the chitinase activity and the frequency of infection to the three tested pathogens. Three progenies from two transgenic events displayed significantly higher disease resistance for LLS, rust and *A. flavus* infection and are being advanced for further evaluations under confined field conditions to confirm as sources to develop peanut varieties with enhanced resistance to these fungal pathogens.

Keywords: *Arachis hypogaea*, *Aspergillus flavus*, groundnut, rice chitinase, transgenic, peanut, late leaf spot disease, rust

Abbreviations

LLS Late leaf spot disease

Introduction

Peanut or groundnut (*Arachis hypogaea* L.) is an agronomically and economically important oilseed crop grown extensively throughout the tropical and sub-tropical regions of the world. Fungal diseases such as late leaf spot (LLS) caused by *Phaeoisariopsis personata* (Berk. and Curt.) Van. Arx, and rust caused by *Puccinia arachidis* Speg. are the most important foliar diseases, besides *Aspergillus flavus* contamination that affect the peanut production and quality (Waliyar et al. 2000). Foliar diseases damage the plant by reducing the green leaf area available for photosynthesis and by stimulating leaflet abscission leading to extensive defoliation which results in lower seed quality, reduced seed size and oil content besides affecting the haulm production and quality (McDonald et al. 1985). Both, the late leaf spot and rust occur together causing yield losses of over 50% in the semi-arid tropics (SAT). Moreover, the invasion by *A. flavus* that produces aflatoxins (a potent carcinogen) for which no adapted genotypes with sustained resistance are available, adversely affects peanut quality making it unfit for international trade. Contamination of peanut with aflatoxin have assumed significance in SAT regions of the world where currently, over 4.5 billion people are chronically exposed to uncontrolled amounts of these toxins. Although, aflatoxin contamination may not affect crop productivity, it makes the produce unfit for consumption as toxins pose significant health hazards.

Although, the adoption of resistant cultivars by the SAT farmers is the best option to overcome the yield losses, efforts for peanut crop improvement have suffered due to its narrow germplasm base and a lack of durable resistance to these important pathogens. Despite several sources of resistance to LLS and rust that have been reported in *A. hypogaea* (Waliyar et al. 1993; Singh et al. 1997; Pensuk et al. 2003; Hossain et al. 2007), the level of resistance identified is insufficient to withstand epidemic conditions. In addition, a majority of the resistant germplasm belongs to subsp. *fastigiata*, land races from South America that possess poor agronomic traits including low shelling outturns, thick pod shells, heavy pod reticulation and unacceptable seed coat colors (Subramanyam et al. 1989). Besides, crosses involving this species result in transfer of many unwanted traits together with desired resistance gene. This undesirable genetic linkage between disease resistance and poor pod characters has hampered the progress in breeding activities. For reasons of cost, efficacy and environmental concerns, much research is presently aimed at transgenic expression of genes that confer significant levels of disease resistance. The advances in genetic engineering and transformation with chitinase genes from different origins resulted in a quantitatively enhanced fungal disease resistance in several crop species including cotton (Ganesan et al. 2009), banana (Sreeramanan et al. 2009), Italian rye grass (Takahashi et al 2005), cucumber (Kishimoto et al. 2002), grapevine (Yamamoto et al. 2000), rice (Lin et al. 1995), and tobacco (Zhu et al. 1998).

Although, enhanced fungal disease resistance has been reported more recently in peanut by using antifungal genes such as the 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 (Sunderesha et al. 2009), to the best of our knowledge, this is the first report of comprehensive greenhouse evaluation of peanut transgenic plants for sustained resistance to all major soil borne and foliar diseases including *A. flavus* infection, late leaf spot (LLS) and rust.

Materials and Methods

Plant material

Three varieties of peanut, *viz.*, JL 24 (Spanish type), ICGV 89104 (Spanish type), and ICGV 86031 (Virginia type) were used for genetic transformation. Seeds of all the three varieties were obtained from the Groundnut Breeding Unit of ICRISAT. Peanut variety JL 24 is susceptible to LLS, rust and *A. flavus*, while the genotypes ICGV 89104 and ICGV 86031 are high yielding germplasm lines, the former being reported as resistant to *A. flavus* infection (Rao et al. 1995).

Gene constructs

The rice chitinase gene (*RChit*) (class I chitinase) characterized by the presence of an N-terminal cysteine-rich domain, capable of binding to chitin belongs to PR3 family was obtained from a genomic library of rice DNA (Huang et al. 1991) [kindly provided by Dr. S. Muthukrishnan, Kansas State University, USA]. The plasmid pCAMBIA1302:Rchit was constructed by cloning a *Hind*III fragment of *Rchit* gene from pRT99GUS:Rchit into the *Hind*III site of the binary plasmid pCAMBIA1302 which has a kanamycin resistance gene for bacterial selection, a hygromycin coding gene (*hpt*) as a selectable marker under the control of CaMV 35S promoter, and a green fluorescent protein coding gene (GFP) for possible use as a reporter gene (Fig. 1). The binary plasmid pCAMBIA:Rchit was mobilized into *Agrobacterium tumefaciens* strain C58 for the genetic transformation of peanut.

Transformation procedure

Genetic transformation of peanut was performed by *Agrobacterium*-mediated gene transfer using the previously reported protocol (Sharma and Anjaiah 2000; Sharma and Bhatnagar-Mathur 2006). Shoot buds transformed with the plasmid pCAMBIA1302:Rchit were subjected to two step selection in shoot elongation media (SEM) supplemented with hygromycin. Initial two subcultures incorporated 2 µM hygromycin in SEM and then increased to 5 µM hygromycin in subsequent subcultures. The transformed shoots that survived during shoot elongation were placed in a rooting medium. The rooted transgenic plantlets were further transferred to a controlled environment chamber for 10 days followed by their transfer to a containment greenhouse for their hardening and acclimatization. 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 generation. The transgenic plants in T_0, T_1, T_2, and T_3 generations were subjected to molecular analysis using standard procedures.

PCR and RT-PCR analysis of the transformants

The genomic DNA was isolated from the control and the putative transgenic plants of peanut by using the procedure described earlier (Dellaporta et al. 1983). PCR analysis was carried out on the transgenics in T_0 and subsequent generations for amplification of the coding region of the *Rchit* and *hpt* genes. PCR was performed with a 25 µl reaction mixture containing 200 ng of the genomic DNA, 2.5 µl of 10x PCR buffer without Mg^{2+}, 0.5 µl of 10 mM dNTPs, 0.75 µl of 50 mM $MgCl_2$, 0.5 µl (10 µM) of each primer, and 0.25 µl (1 unit) of Taq DNA polymerase (Invitrogen, USA). The 814 bp *Rchit* fragment was amplified using 22-mer oligonucleotide primers (forward primer: 5'CGCTAAGGGCTTCTACACCTAC3' and reverse primer: 5'AGCTTATCGATACCGTCGACCT3'). The cycling conditions comprised an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1.5 min, and final extension at 72°C for 10 min. A 819 bp *hpt* fragment was amplified using 22-mer oligonucleotide primers (forward primer: 5'CGTTATGTTTATCGGCACTTTG3' and reverse primer: 5'GGGGCGTCGGTTTCCACTATCG3'). PCR conditions were similar to *Rchit* primers except for the annealing at a temperature of 58.4°C for 1 min. The amplified products were subjected to electrophoresis on a 1.2% agarose gel and visualized on a UV transilluminator. The PCR-positive transgenic plants of peanut were randomly selected for RT-PCR analysis. Total RNA was isolated from leaf tissue using the TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol, and RT-PCR analysis of the putative transformants was carried out using the Thermoscript RT-PCR system (Invitrogen, USA) for 35-40 cycles using gene specific primers as described for PCR.

Southern blot analysis

Twenty-five to thirty microgram of peanut genomic DNA was completely digested with *Hind*III or *Eco*RI and separated on 0.8% (w/v) agarose gel followed by transfer to positively charged nylon membranes (Roche Molecular Biochemicals) using capillary blotting technique. Upon digestion of the genomic DNA with *Hind*III, it released the 1.57 kb *Rchit* gene, while digestion with *Eco*RI was used to ascertain the copy number of the inserted gene. Pre-hybridization, hybridization, washing, and detection of the membranes were conducted using a non-radioactive DIG-based system (Roche Molecular Biochemicals) following the manufacturer's protocol. The 1.57 kb *Rchit* coding sequence fragment was used as a probe. The blot was exposed to X-Omat film (Eastman Kodak Company, USA) for 15-30 min for autoradiography.

Segregation analysis

Inheritance of the transgenes was studied from the PCR screening of *Rchit* gene in T_1, T_2 and T_3 generation transgenic plants. PCR positive and negative plants were identified and chi-square test performed to validate the data for 3:1 seggregation.

Chitinase assay

A colorimetric chitinase assay was performed with the leaves from 45 days-old transgenic and non-transformed control plants of peanut following the method reported elsewhere (Mauch et al. 1984). The level of chitinase was determined using colloidal chitin as substrate. Colloidal chitin was prepared from crab shell chitin (Sigma). The total protein was estimated (Bradford 1976) and the enzyme activity was expressed as U mg^{-1} protein. One unit of activity is defined as the enzyme activity catalyzing the formation of one µmol of *N*-acetyl-glucosamine min^{-1} mg^{-1} protein.

In vitro seed colonization by *Aspergillus flavus*

Peanut transgenic events in T_1, T_2 and T_3 generation were screened by inoculation for resistance to in vitro seed colonization by *A. flavus* isolate *Af 11-4*, a highly aggressive and toxigenic strain (Thakur et al. 2000). Seeds from the transgenic events were surface sterilized with 0.1% aqueous solution of mercuric chloride for 2 min followed by thorough washings with sterilized distilled water. The seeds were dipped in *A. flavus* spore suspension (adjusted to 4 x 10^6 conidia per ml) for 2-3 min prior to plating them in sterile petri dishes of 9 cm diameter containing pre-moistened filter paper. After incubating for 7 d at 28±1°C in a humid atmosphere (seed germinator, 98 ± 2% relative humidity), percentage rating of seeds with observed fungal growth were recorded (Waliyar et al. 1994). After preliminary screening for *A. flavus*, resistant seeds were advanced to further generations for molecular characterization and further studies.

Detached leaf assay for LLS and Rust

Detached leaf assays were used for evaluation of the transgenic plants in T_1, T_2 and T_3 generation against both late leaf spot and rust as reported earlier. At 40 days after sowing, the quadrifoliate leaves from either the second or third fully expanded leaf from 10 PCR positive transgenic plants from each selected transgenic event were excised from pulvinous region and arranged in randomized block design in plastic trays (39.5 cm x 29 cm x 7 cm) containing sterile river sand that was moistened with Hoagland's nutrient solution. Ten leaves of susceptible control plants were planted in a separate tray. The trays were covered with clear polythene covers and incubated before inoculation for 24 h in a growth chamber for acclimatization. The day and night temperature in the growth chamber was 23°C with relative humidity of 60% and illuminated with white light.

The *Phaeoisariopsis personata* conidia and *Puccinia arachidis* urediospores were produced on inoculated detached leaves of the susceptible peanut var. TMV 2 and harvested with a cyclone

spore collector (ERI Instrument Shop, Iowa State University, Ames, USA). Spores were suspended in sterile distilled water containing Tween-20 and the suspension was adjusted to 50,000 spores ml^{-1} using haemocytometer. Leaves from the test plants were spray inoculated using atomizer with the spore suspension of either LLS or rust, and incubated in a growth chamber. Leaves inoculated with *Puccinia arachidis*, were initially incubated in the dark for 24 h period and were subsequently transferred to the growth chamber until the end of the experiment.

The experimental trays were examined daily beginning 6 days after inoculation (DAI) for incubation period (IP, defined as days from inoculation to appearance of the first symptom) and latent period (LP, defined as days from inoculation to appearance of the sporulating lesion). 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) due to LLS and rust were recorded at 28 days after inoculation.

Statistical analysis

The data collected on chitinase activity, *A. flavus*, late leaf spot 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) using SAS version 7.1. The values were means of ten replicates per event. The correlation analysis was done using Pearson correlation coefficient at 5% and 1% level of significance among the transgenics and non-transformed control plants for late leaf spot (infection frequency), rust (infection frequency) and percentage *A. flavus* infection with chitinase activity.

Results

Transformation of peanut with rice chitinase gene

The chitinase gene from rice (*Rchit*) was introduced into peanut via *A. tumefaciens*-mediated genetic transformation. A total of 65 primary transformants (T_0) comprising of 30 plants of var. JL 24, 25 plants of var. ICGV 89104 and 10 plants of var. ICGV 86031 were regenerated from mature cotyledonary explants following co-cultivation with *A. tumefaciens* harboring the plasmid pCAMBIA1302:Rchit. The regenerated plants exhibited normal growth under greenhouse conditions and produced morphologically normal flowers and pods that contained viable seeds.

Integration and expression of transgenes

The insertion of the *Rchit* gene into the peanut genome was initially verified by PCR analysis. Presence of 814 bp region of the *Rchit* gene was detected in at least 30 of the 65 transgenic plants produced with the plasmid pCAMBIA1302:Rchit (Fig. 2a). The transformation efficiency of these transformants varied from 70% in the var. JL 24 to 40 and 55% in ICGV 89104 and ICGV 86031, respectively. Randomly selected transformants showing amplification of the *Rchit* gene also showed amplification of 819 bp fragment of the *hpt* gene (Fig. 2b). Expression of the introduced genes was

analyzed by RT-PCR from the randomly selected 15 T_1, T_2 and T_3 PCR positive plants. The expected 814 bp amplified fragment corresponding to the *Rchit* gene was detected in all the plants that were selected for analysis (Fig. 2c).

Randomly selected PCR and RT PCR positive events were analyzed by Southern blot hybridization for copy number (*Eco*RI digested DNA) using 1.57 kb *Rchit* fragment as probe. The Southern analysis indicated presence of two copies of the transgene in event number RC-GN-4-1-1 while the event RC-GN-22-3-2 showed single copy of the transgene (Fig. 3). No transgene insertion was detected in non-transformed control plant DNA (Fig. 3). Genetic analysis of PCR tested transgenic plants and their progeny showed that the transgenes were inherited as a single Mendelian trait (3:1 ratio) at $p = 0.05$ in all the events in T_1,T_2, and T_3 progenies (Data not shown).

Chitinase activity in the transgenic plants

The chitinase activity varied amongst the transgenic events expressing *Rchit* gene where, on an average 2- to 14-folds increase in the chitinase activity (0.3 to 2.1 U mg^{-1} protein) was recorded as compared to the non-transformed control plants (0.17 U mg^{-1} protein) (Fig. 4). Of the 30 T_1 transgenic events tested, 5 transgenic events (event RC-GN-1, RC-GN-4, RC-GN-13, RC-GN-22 and RC-GN-30) had significantly higher chitinase activity than the rest, which sustained in the T_2 progeny of three of these five events (RC-GN-13-3, RC-GN-4-1 and RC-GN-22-3), further being stable in T_3 progenies of two of these events (RC-GN-1-2-6, RC-GN-4-1-1 and RC-GN-4-1-3).

Evaluation of peanut transgenics for resistance to *Aspergillus flavus*

Screening of transgenics for *A. flavus* through in vitro seed colonization in 30 T_1 transgenic events showed significantly lower seed infection (0-40%; $p<0.001$) when compared to 65% in the non-transformed controls (Fig. 5). Of these 30 events tested for *A. flavus* infection, 11 were found to be free from any infection in T_1 generation (Fig. 6a). The infection of *A. flavus* in the T_2 progenies of these 11 selected events varied from 5 to 33% . Interestingly, in T_3 generation most of transgenic events were free from infection except for one (RC-GN-4-1-1) that showed 15% *A. flavus* infection.

Evaluation of peanut transgenics for resistance against late leaf spot (LLS)

The progenies of seven T_1 transgenic events tested showed significant ($p<0.001$) genotypic differences for all the components of resistance to LLS in detached leaf bioassays (Table 1). Two events showed longer incubation and latent periods (RC-GN-13 -17 days IP, 23 days LP ; RC-GN-24 -14 days IP, 20 days LP) compared to the non-transformed controls JL 24 (9 days IP, 13 days LP), ICGV 89104 (10 days IP, 12 days LP) and ICGV 86031 (11 days IP, 14 days LP). Most of the transgenic events (RC-GN-1, RC-GN-3, RC-GN-4, RC-GN-13, and RC-GN-24) showed lower leaf area damage (0.23 to 4.5%), lesser lesions per leaf (1 to 23) and lower infection frequency (0.0718 to

0.6689 cm^2) than their non-transformed counterparts (JL 24 -23.9% LAD, 47 LN, 1.639 cm^2 IF; ICGV 89104 -52.5% LAD, 92 LN, 2.407 cm^2 IF and ICGV 86031 -18% LAD, 38 LN, 1.383 cm^2 IF).

The T_2 progeny of 11 transgenic plants from 7 selected T_1 events were further evaluated against LLS (Table 2). Significant genotypic differences were found for all the components of resistance to LLS at $p<0.001$. The transgenic events RC-GN-4-1 and RC-GN-4-4, RC-GN-22-2, RC-GN-22-3 showed longer incubation period (17-19 days) than non-transformed control JL 24 and ICGV 89104 (13 and 14 days respectively). Also, the events RC-GN-1-2, RC-GN-4-1, RC-GN-22-2 showed longer latent period (21-25 days) than the non-transformed controls JL 24 and ICGV 89104 (15 and 17 days respectively). Interestingly, events RC-GN-4-1, RC-GN-13-3, RC-GN-22-2 and RC-GN-22-3 showed lesser number of lesions per leaf (2-8) when compared to the non-transformed control of JL 24 (36) and ICGV 89104 (21). The event RC-GN-13-3, RC-GN-4-1 and RC-GN-22-3 had lower infection frequency when compared to the controls (1.27 and 0.75 cm^2). The events RC-GN-4-1, RC-GN-22-2 and RC-GN-22-3 showed significant reduction in leaf area damage compared to both non-transformed controls. Overall, events RC-GN-4-1, RC-GN-22-3 showed better performance in all the resistance parameters tested for LLS viz., longer incubation period (19; 18 days), longer latent period (25; 28 days), lower leaf area damage (0.3; 0.45%), lesser lesion number per leaf (4; 5), and lower infection frequency (0.129; 0.223 cm^2) when compared to their wild type counterparts.

In T_3 generation plants, progeny of the five selected T_2 transgenic plants from three events were evaluated along with the non-transformed control plants of var. JL 24 and ICGV 89104 against LLS (Table 3). All transgenic events in T_3 showed longer incubation period (15 to 18 days) than the non-transformed control (12 days) and ICGV 89104 (12 days). The event RC-GN-4-1-1 recorded longer incubation and latent period (18 days IP, 23 days LP) than the corresponding non-transformed control JL 24 (12 days IP, 16 days LP) and ICGV 89104 (12 days IP, 18 days LP). All transgenic events recorded lesser leaf area damage (1.675 to 6.15%), reduced lesion number per leaf (1 to 4) and lower infection frequency (0.0450 to 0.148 cm^2) than the non-transformed controls JL 24 and ICGV 89104 which showed higher leaf area damage (19.4; 20.8%), lesion number per leaf (20; 23) and infection frequency (0.871; 1.088 cm^2), respectively (Fig. 6b).

Evaluation of peanut transgenics for resistance against rust

All the T_1 transgenic events evaluated for rust showed significant ($p<0.001$) genotypic differences for all the components of resistance to rust in detached leaf bioassays (Table 1). The event RC-GN-13 and RC-GN-24 showed longer incubation and latent period (17 days IP, 23 days LP; 14 days IP, 20 days LP respectively) than their non-transformed controls (JL 24 -9 days IP, 12 days LP; ICGV 89104 -12 days IP, 14 days LP and ICGV 86031 -11 days IP, 14 days LP). All transgenic events except event RC-GN-1 showed lower leaf area damage (0.35 to 5.7%) than the non-transformed

control JL 24 (21%), ICGV 89104 (19.3%) and ICGV 86031 (18.6%). Similarly, most of the transgenic events showed lower pustule number per leaf (<17) than the non-transformed control JL 24 (257) and ICGV 89104 (119) (Figure 5c; Table 1). The event RC-GN-13 and RC-GN-22 showed significantly lower infection frequency (0.051 and 0.16 respectively) compared to the non-transformed controls JL 24 (9.062 cm^2), ICGV 89104 (3.284 cm^2) and ICGV 86031 (1.212 cm^2).

The selected transgenic events in T_2 generation showed significant genotypic differences for all the components of resistance to rust at $p<0.001$ (Table 2). The events RC-GN-1-2 and RC-GN-3-6 showed longer incubation period and latent period (20 days IP, 23 days LP; 20 days IP, 25 days LP respectively) than the non-transformed controls JL 24 (14 days IP, 16 days LP) and ICGV 89104 (11 days IP, 13 days LP). The event RC-GN-1-2, RC-GN-3-1, RC-GN-3-6, RC-GN-4-4 and RC-GN-22-3 showed significantly lower leaf area damage (0 to 0.1%) than non-transformed controls JL 24 (15%) and ICGV 89104 (17.5%). All transgenic events except events RC-GN-13-3, RC-GN-17-11 and RC-GN-22-12 showed lesser pustule number per leaf (0-7) and lower infection frequency (0 to 0.292 cm^2) than the non-transformed controls JL 24 and ICGV 89104 (28 and 31 pustules per leaf; 1.3 cm^2 infection frequency). Overall, events RC-GN-1-2 and RC-GN-3-6 showed better results for all the resistance parameters for rust viz., longer incubation period (20; 20 days), longer latent period (23; 33 days), lower leaf area damage (0.05; 0.1%), lesser pustule number per leaf (0; 1) and lower infection frequency (0.004; 0.020 cm^2), respectively.

The T_3 progeny of five selected T_2 transgenic plants from three events were evaluated along with the non-transformed control JL 24 and ICGV 89104 against rust (Table 3). The observations indicated that the events RC-GN-4-1-1 and RC-GN-4-1-3 had longest incubation period and latent period during the fungal bioassays. All the transgenic events showed lesser leaf area damage (2.83 to 3.7%), reduced pustules per leaf (125 to 194) than the non-transformed controls of var. JL 24 and ICGV 89104 which recorded significantly higher leaf area damage (31.1; 26.25%) and pustules per leaf (340.5;227.5). The event RC-GN-1-2-6, RC-GN-4-1-1, and RC-GN-4-1-3 showed lower infection frequency (3.471 to 4.12 cm^2) than non-transformed controls JL 24 and ICGV 89104 which showed infection frequencies of 12.03 and 9.16 cm^2 respectively (Fig. 6c).

Correlation between chitinase activity and disease resistance

Disease severity correlated well with the chitinase activity and the infection frequency of LLS, rust and *A. flavus* infection in the T_1 transgenic plants with the Pearson correlation coefficients ranging from -0.381 (P=0.05), -0.416 (P=0.05) and -0.546 (P=0.01), respectively. The T_2 and T_3 progenies of these events also showed significant negative correlation between the chitinase activity and the infection frequencies of LLS, rust and *A. flavus* infection ($P< 0.01$). These results indicated that the transgenic events with high chitinase activity showed lower disease incidence and vice versa (Fig. 7a,b).

Discussion

During the course of this study, transgenic peanut events expressing the rice chitinase gene (*Rchit*) were generated and evaluated for their tolerance to foliar fungal diseases including LLS and rust. Overexpressed chitinases have been reported to impart enhanced protection in two ways; degrading the chitin in hyphae, thereby retarding fungal growth, and by releasing pathogen-borne elicitors that induce defense reaction in plants. Several reports show various transgenic crop species transformed with the *Rchit* gene to significantly increased resistance to fungal diseases. For example, transgenic rice with class I rice chitinase gene *chi11* showed increased resistance to *Rhizoctonia solani*, the rice sheath blight pathogen (Sela-Buurlage et al. 1993) and transgenic grapevine and cucumber with class I rice chitinase gene *RCC2* showed increased disease resistance to *Elisinoe ampeina* and *Botrytis cinerea* that induce anthracnose and gray mold, respectively (Yamamoto et al. 2000; Kishimoto et al. 2002). More recently, expression of the rice chitinase gene *RCC2* in Italian ryegrass showed enhanced resistance to crown rust disease caused by *Puccinia coronata* (Kishimoto et al. 2002).

Apparent from these reports, transformation of various plants with a rice chitinase gene has been shown to be an effective method for enhanced resistance against a broad range of fungal pathogens. The rice chitinase gene used in the present study is a class I chitinase, 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 ($ChBD_{Tob}$) from tobacco class I chitinase has been reported to cause a 3-fold 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). A possible antifungal activity of CBD on its own acting on another substrate has been another explanation for its usefulness. Interestingly, all other classes of chitinase have either no or lower antifungal activity as compared to class I chitinases (Sela-Buurlage et al. 1993).

The integration of the transgenes was confirmed by Southern blot analysis where two events showed low transgene copy number (one event showed one copy and another event showed two copies). Segregation studies showed Mendelian inheritance of the *Rchit* gene in T_1, T_2 and T_3 generation transgenic peanut plants, Since, inheritance and stable expression of transgenes is important in crop improvement through gene manipulations, in the present study the expression of the transgenes were confirmed by RT-PCR.

In the present study, an average of 2 to 14-fold (0.3 to 2.1 U mg^{-1} protein) increase in the chitinase activity was recorded in transgenic plants as compared to 0.17 U mg^{-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 *Rchit*. Several reports on intensified chitinase

activity have been observed in the transgenic plants expressing the chitinase gene (Lin et al. 1995; Datta et al. 2000; Nandakumar et al. 2007). Over 44-folds increase in chitinase activity over controls was reported in the leaves of tobacco transformants, while in transgenic rice it has been reported up to 14-times (Lin et al. 1995; Nandakumar et al. 2007). A 29-fold increase in endochitinase activity in transgenic tobacco plants and 180-fold increase in endochitinase activity in transgenic cotton plants transformed with the endochitinase gene from *Trichoderma virens* has been reported (Emani et al. 2003).

Although, in our study some of the events showed increased chitinase activity, these differed in their level of resistance to LLS, rust and *A. flavus*. 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 chitinase against these pathogens.

Transgenic approaches utilizing chitinase gene for resistance to *A. flavus* are still in experimental stages where studies showed that purified chitinase from peanut, Tex 6 maize kernels and sugar beet inhibits growth of *A. flavus* (Liang et al. 1994; Jwanny et al. 2001; Moore et al. 2004). Since, *A. flavus* is one of the most destructive pathogen in peanut, in vitro seed colonization was carried out to ascertain whether transgenic peanut expressing *Rchit* show any resistance to this pathogen. Encouragingly, transgenic peanut events showed significantly lower *A. flavus* seed infection ($p<0.001$; 0-40%) when compared to their non-transformed control plants (65%), thereby indicating that the levels of resistance might be improved by pyramiding resistance genes from diverse sources to provide transgenic protection to peanut against infection by aflatoxin-producing fungi (Sharma et al. 2006).

Improved resistance against the pathogen is a consequence of enhanced chitinolytic activity and other defense-related mechanism being triggered by the presence of chitinase. In the present study, correlation analysis showed a significant trend toward decreased disease severity in the transgenics with the increasing chitinase activity that confirmed that the inhibition observed was due to the presence of overexpressed rice chitinase protein. A positive correlation between increased chitinase activity and resistance to early leaf spot has also been shown earlier (Rohini and Rao 2001). Similar correlations have also been observed in various studies on different crop species (Liang et al. 1994; Lin et al. 1995; Tabei et al. 1998; Zhu et al. 1998; Carstens et al. 2003; Itoh et al. 2003; Nandakumar et al. 2007).

Clearly we came across four T_3 transgenic plants (RC-GN-4-1-1, RC-GN4-1-3, RC-GN22-3-2 and RC-GN22-3-3) from two independent events (RC-GN-4 and RC-GN-22) which displayed significantly higher disease resistance traits for LLS, rust and *A. flavus* infection. The level of resistance to 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).

In conclusion, we have further confirmed transgenic peanut plants overexpressing the *Rchit* gene for sustained resistance to foliar diseases such as late leaf spot and rust besides, *A. flavus* seed infection. The promising transgenic events identified in this study will undergo further testing for their response to fungal diseases under greenhouse and field conditions and, upon validation may be used as additional source of disease resistance in peanut breeding programs. We expect that the combination of this transgenic strategy based on the use of rice chitinase gene and traditional breeding will provide durable fungal disease resistant peanut lines with good agronomic phenotypes.

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Table 1 Performance of peanut transgenic plants (T_1) carrying rice chitinase gene (*Rchit*) against late leaf spot (LLS) and rust. The values are means of ten plants per event. Mean followed by the same letter are not significantly different at 5% level.

Event #	Incubation Period		Latent Period		Lesion/Pustule number per leaf		Leaf area damage (%)		Infection frequency (cm^2)	
	LLS	**Rust**	**LLS**	**Rust**	**LLS**	**Rust**	**LLS**	**Rust**	**LLS**	**Rust**
RC-GN-1[1]	12cd	10ab	16bc	14a	11a	75bc	2.30a	15.80b	0.430a	3.072b
RC-GN-3[1]	12bc	11abc	20d	19bc	14a	53ab	4.50a	3.35a	0.575a	2.192ab
RC-GN-4[1]	13cd	11abc	19d	14a	13a	45ab	3.7a	2.8a	0.554a	1.93ab
RC-GN-13[1]	17e	17e	23e	23d	1a	1a	0.23a	0.35a	0.072a	0.051a
RC-GN-17[2]	11bc	11abc	17cd	17b	38bc	28ab	19.50cd	5.90a	1.510c	1.248ab
RC-GN-22[2]	12cd	12cd	19cd	19bc	23ab	7a	9.10abc	2.52a	0.752ab	0.16a
RC-GN-24[2]	14d	14d	20d	20c	12a	17a	7.9ab	5.73a	0.668a	1.03ab
Non-transformed var. JL 24	9a	9a	13a	12a	47c	27d	23.90d	21.0b	1.639c	9.062c
Non-transformed var. ICGV 89104	10ab	12bc	12a	14a	92d	19c	52.50e	19.3b	2.407d	3.284b
Non-transformed var. ICGV 86031	11bc	11abc	14ab	14a	38bc	39ab	18.0bcd	18.60b	1.383bc	1.212ab
SE±	0.74	0.686	0.94	0.675	7.17	17.18	3.60	2.801	0.224	0.703
LSD 5%	2.092	1.935	2.657	1.905	20.20	48.39	10.14	7.889	0.632	1.981
Fp	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

1:JL 24 transgenics, 2: ICGV 89104 transgenics.

Table 2 Performance of transgenic plants of peanut (T_2) developed following transformation with the plasmid pCAMBIA1302:Rchit against LLS and rust. The values are means of ten plants per event. Mean followed by the same letter are not significantly different at 5% level.

Event #	Incubation Period		Latent Period		Lesion/Pustule no. per leaf		Leaf area damage (%)		Infection frequency (cm^2)	
	LLS	Rust	LLS	Rust	LLS	Rust	LLS	Rust	LLS	Rust
RC-GN-1-2[1]	17ab	20a	25a	23b	7de	0c	6.30cd	0.05c	0.428cde	0.004c
RC-GN-3-1[1]	14bcd	15bc	24ab	21bc	30ab	0c	15.3ab	0c	1.478b	0c
RC-GN-3-6[1]	13d	20a	20bc	33a	20bc	1c	11.3bc	0.10c	0.799c	0.02c
RC-GN-4-1[1]	19a	16b	25a	25b	3e	4c	0.3d	1.1bc	0.129de	0.202c
RC-GN-4-4[1]	18a	15bc	24ab	21bc	16cd	0c	5.45cd	0c	0.668c	0c
RC-GN-13-3[1]	16bc	13cd	21abc	17cde	2e	13b	3.25cd	1.92bc	0.0709e	0.473ac
RC-GN-17-11[2]	15bcd	12c	22ab	18cd	11cde	8bc	4.35cd	4.7b	0.535cd	0.403c
RC-GN-22-12[2]	13cd	12c	18cd	19cd	17bc	17b	2.54cd	3.45b	1.141b	1.748a
RC-GN-22-2[2]	17a	8e	28a	11f	8e	2c	0.5d	1.6bc	0.563cd	0.247c
RC-GN-22-3[2]	18a	16bc	24ab	22c	5e	0c	0.45d	0c	0.223de	0c
RC-GN-24-9[2]	12d	14bc	17cd	20cd	37a	7c	24.96a	1bc	2.444a	0.292c
Non-transformed var. JL 24	13cd	14bc	15d	16de	36a	28a	19.8a	15a	1.276b	1.308b
Non-transformed var. ICGV 89104	14bcd	11b	17cd	13e	21bc	31a	17.8ab	17.5a	0.759c	1.334b
SE±	0.871	0.853	1.421	1.297	3.84	2.824	2.65	1.231	0.149	0.126
LSD 5%	2.459	2.478	4.01	3.771	10.82	7.962	7.473	3.471	0.421	0.355
Fp	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

1: JL 24 transgenics, 2: ICGV 89104 transgenics

Table 3 Performance of transgenic plants of peanut (T_3) developed following transformation with the plasmid pCAMBIA1302:Rchit against LLS and rust. The values are means of ten plants per event

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Event #	Incubation Period		Latent Period		Lesion/Pustule no. per leaf		Leaf area damage (%)		Infection frequency (cm^2)	
	LLS	**Rust**	**LLS**	**Rust**	**LLS**	**Rust**	**LLS**	**Rust**	**LLS**	**Rust**
RC-GN-1-2-6[1]	17b	12b	22ab	17b	3b	125b	5.95b	2.83b	0.135b	3.94c
RC-GN-4-1-1[1]	18a	14a	23a	18a	3b	138b	4.29b	2.82b	0.105b	4.12c
RC-GN-4-1-3[1]	16b	14a	21bc	18a	1b	141b	1.67b	3.10b	0.050b	3.47c
RC-GN-22-3-2[2]	16b	12b	22ab	17b	2b	162b	3.8b	3.28b	0.119b	5.38bc
RC-GN-22-3-3[2]	15b	12b	20c	17b	4b	194b	6.15b	3.7b	0.148b	5.84bc
Non-transformed var. JL 24	12c	10c	16d	15c	20a	341a	20.8a	31.1a	0.871a	12.04a
Non-transformed var. ICGV 89104	12c	10c	18d	14c	23a	228ab	19.4a	26.25a	1.088a	9.16ab
SE±	0.44	0.21	0.38	0.21	1.32	34.19	1.93	1.74	0.04	1.17
LSD 5%	1.551	0.7320	1.3466	0.7420	4.568	118.31	6.701	6.036	0.142	4.074
Fp	<0.001	<0.001	<0.001	<0.001	<0.001	0.037	0.002	<0.001	<0.001	0.015

1: JL 24 transgenics, 2: ICGV 89104 transgenics

Legends to figures

Fig. 1 Schematic representation of binary plasmid pCAMBIA1302:Rchit used for *Agrobacterium tumefaciens*–mediated genetic transformation in peanut

Fig. 1

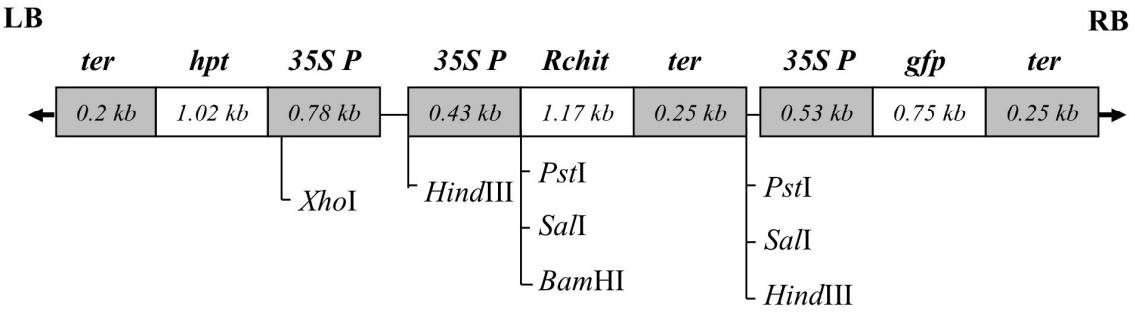


Fig. 2 Molecular analysis of the peanut transformants obtained through *Agrobacterium*-mediated transformation. (a) PCR amplification of genomic DNA showing amplification of a 814 bp fragment of the *Rchit* gene, Lanes 1-16 carry genomic DNA from putative transformants; (b) PCR amplification of genomic DNA showing amplification of a 819 bp fragment of the *hpt* gene, Lanes 1-14 carry genomic DNA from putative transformants; (c) RT-PCR of the cDNA showing amplification of a 814 bp fragment of the *Rchit* gene, Lane 1-5 carry cDNA from putative transformants; B-blank, C-non-transformed control, P-plasmid as positive control, M-mol. wt. marker

Fig. 3 Southern blot analysis of the genomic DNA from leaves of peanut transgenics obtained through *Agrobacterium*-mediated transformation. The genomic DNA of peanut transgenics was digested with *Eco*RI to check the copy number of the integrated gene. Lanes 1 and 2 carry *Eco*RI restricted genomic DNA from events RC-GN-1-2-6 and RC-GN-24-9-3, lanes 3 and 4 carry *Eco*RI restricted genomic DNA from events RC-GN-4-1-1 and RC-GN-22-3-2 respectively, C-*Eco*RI restricted genomic DNA from non-transformed control plants, P-*Eco*RI restricted plasmid pCAMBIA1302:Rchit

Fig. 3

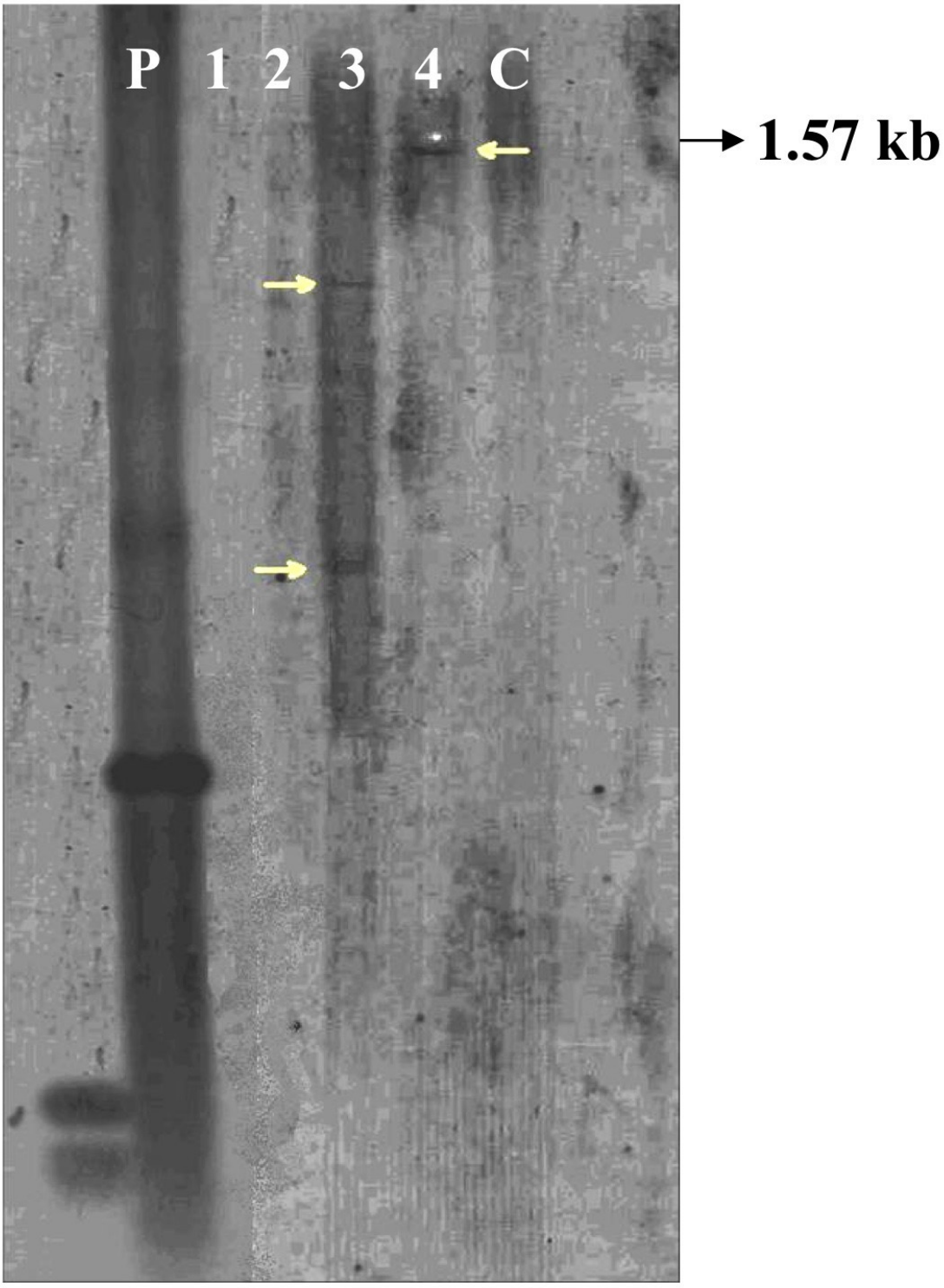


Fig. 4 Chitinase activity in the peanut transgenic plants. Graph bars represent the mean ± SD values of two replicates

Fig. 4

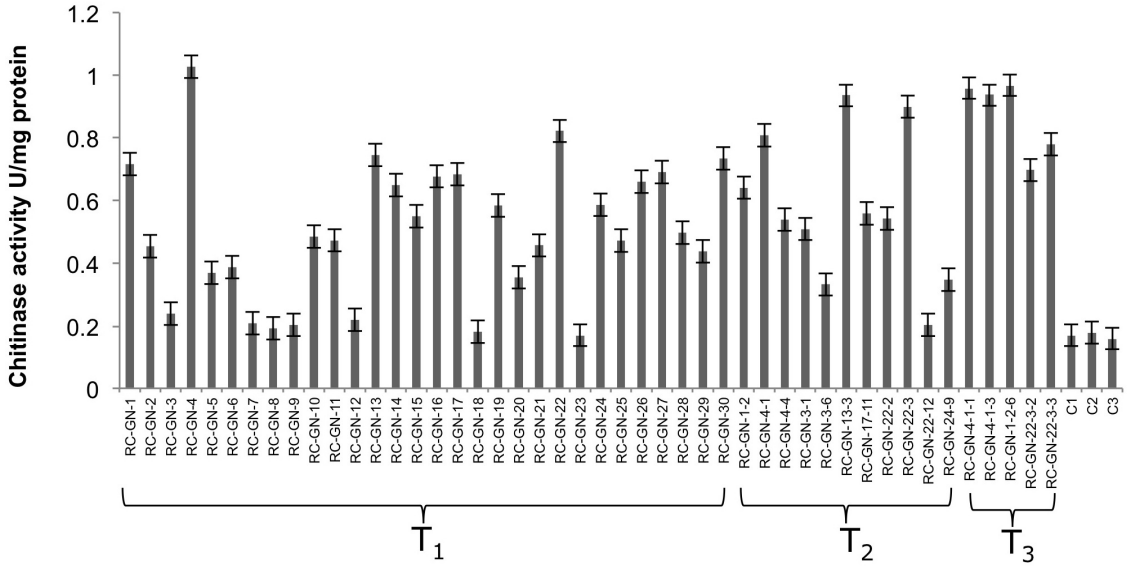
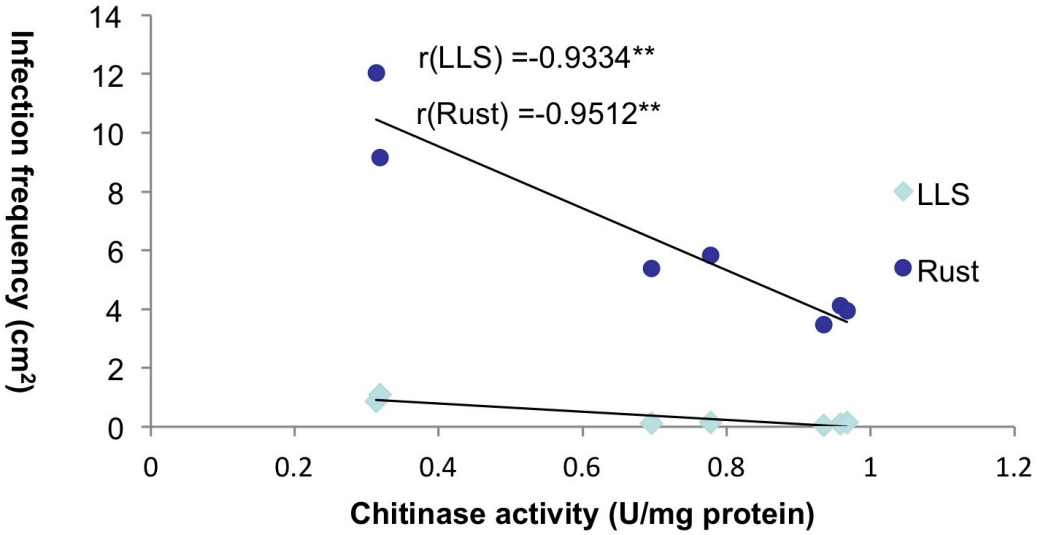


Fig. 5 Percentage *A. flavus* infection in the peanut transgenic plants. Graph bars represent the mean ± SD values of four replicates

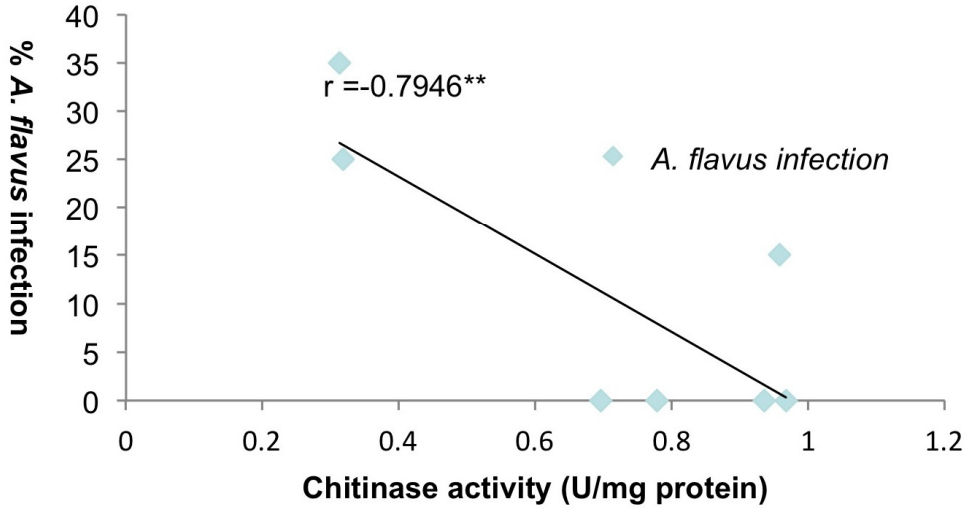
Fig. 6 (a) Extent of *Aspergillus flavus* seed infection in transgenics (left) versus non-transformed controls (right) of peanut; Leaf Bioassays with fungal pathogens. (b) Leaf area damage due to late leaf spot on transgenics (left) and non-transformed control (right); (c) Leaf area damage due to rust on transgenics (left) and non-transformed control (right)

Fig 7 Correlation of the chitinase activity in peanut transgenics with the infection frequency. (a) Correlation between chitinase activity and late leaf spot and rust infection frequency (b) Correlation between chitinase activity and % *A. flavus* infection

Fig. 7a,b


Infection frequency (cm²)
14
12
10
8
6
4
2
0
0
0.2
0.4
0.6
0.8
1
1.2
Chitinase activity (U/mg protein)
r(LLS) =-0.9334**
r(Rust) =-0.9512**
LLS
Rust

a


% A. flavus infection
40
35
30
25
20
15
10
5
0
0
0.2
0.4
0.6
0.8
1
1.2
Chitinase activity (U/mg protein)
r =-0.7946**
A. flavus infection

b