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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

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Abstract Groundnut is an important oilseed crop of the Indian subcontinent. Yield losses due to fungal diseases are enormous in the cultivation of this crop. Over-expression of PR proteins leads to increased resistance to pathogenic fungi in several crops. The PR protein glucanase hydrolyses a major cell-wall component, glucan, of pathogenic fungi and acts as a plant defense barrier. We report in this paper, over-expression of a tobacco glucanase in transgenic groundnut and its resistance towards *Cercospora arachidicola* and *Aspergillus flavus*. PCR, Southern and Northern hybridization confirmed stable integration and expression of the glucanase gene in groundnut transgenics. When screened for resistance against *Cercospora arachidicola* the transgenics showed not only reduction in the number of spots but also delay in the onset of disease. Resistance was also demonstrated against one another important pathogen of groundnut, *Aspergillus flavus.* The transgenics not only resisted hyphal spread but also did not accumulate aflatoxin in the seeds. The results demonstrate the potential of a PR protein from a heterologous source in developing fungal disease resistant groundnut.

Keywords Glucanases · Disease resistance · Groundnut · *Cercospora arachidicola* · *Aspergillus flavus*

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Introduction

Groundnut, an important oil seed crop has received attention for improvement. Fungal and viral diseases are the most common diseases of groundnut worldwide. Although variation in disease resistance among commercial groundnut cultivars does exist, the development of resistant cultivars is considered the most effective and economic means of controlling diseases. Genetically engineered resistance has been actively investigated in recent years as a complement to traditional breeding and selection. The simplest means for genetic engineering of resistance to fungal diseases entails the constitutive expression of one or more defense proteins in transgenic plants. The approaches followed to obtain disease resistant plants have been: (1) expression of gene products that are directly toxic to pathogens or that reduce their growth which include PR proteins such as hydrolytic

enzymes (chitinases, glucanases), antifungal proteins (osmotin and thaumatin-like), antimicrobial proteins (thionins, defensins, lectin), ribosome-inactivating proteins (RIP) and phytoalexins; (2) expression of gene products that destroy or neutralize a component of the pathogen arsenal such as polygalactouranase, oxalic acid and lipase; (3) expression of gene products like the *npr*1 releasing signals that regulate plant defense which includes the production of specific elicitors, hydrogen peroxide (H_2O_2), salicylic acid and ethylene; and (4) expression of resistance (R) products involved in the hypersensitive response (HR) and in interactions with avirulence (Avr) factors.

Defense proteins with clearly demonstrated antifungal activities have been effective in several cases. Constitutive expression of bean chitinase protects transgenic tobacco and canola seedlings from *Rhizoctonia solani* (Broglie et al. 1991). Another defense protein, osmotin, on constitutive expression in potato delayed the onset of potato late blight (*Phytophthora infestans*) (Liu et al. 1994). Terras et al. (1995) demonstrated that constitutive expression of raddish defensin protects transgenic tobacco from the foliar pathogen *Alternaria longipes*.

The defense protein glucanase catalyses the hydrolysis of glucan, a major cell-wall component of most filamentous fungi and leads to increased resistance to fungal pathogens when over-expressed in various plants (Broglie et al. 1991; Mondal et al. 2007). Adoption of glucanase-mediated resistance against fungal pathogens in economically important crop like groundnut can become an important aspect of crop improvement programmes. In this direction, it was envisaged to prove the efficacy of a tobacco glucanase against selected important pathogens of groundnut using transgenic approach.

Many reports of transformation and development of groundnut transgenics using a variety of genes such as the *bar* gene for herbicide tolerance (Brar et al. 1994), *cry*1A (Singsit et al. 1997), a gene encoding the nucleocapsid protein of tomato spotted wilt virus (Yang et al. 1998), and chitinase (Rohini and Rao 2001) have been reported.

However, the reported successes appear to revolve around the low *in vitro* culture responses seen in groundnut cultivars. As a result, transformation successes tend to be poor and call for intensive experimentation. However, to tackle the problems pertaining to regeneration in groundnut and certain other recalcitrant crops, alternate methods to minimize or eliminate the steps of regeneration are being standardized. Research with *Arabidopsis* has benefited from the development of high throughput transformation methods that are referred to as *in planta* transformation protocols, these avoid plant tissue culture (Azipiroz-Leehan and Feldmann 1997). In particular, the development of the *Agrobacterium tumefaciens*-mediated vacuum infiltration method (Bechtold et al. 1993) has had a major impact on *Arabidopsis* research. *In planta* transformation methods have also been standardized for rice (Supartana et al. 2005), wheat (Supartana et al. 2006), and maize (Chumakov et al. 2006). In all these crops, *Agrobacterium* is directed towards either the apical meristem or the meristems of axillary buds. One such viable *in planta* transformation protocol has also been standardized by our group in several crops (Rohini and Rao 2001). The strategy essentially involves *in planta* inoculation of embryo axes of germinating seeds and allowing them to grow into seedlings *ex vitro*. Since the *in planta* transformation protocols do not involve regeneration procedures, the tissue culture-induced somaclonal variations are avoided. The embryo transformation protocol thus standardized has been exploited in the present study with an objective to prove the efficacy of the tobacco glucanase gene against major fungal pathogens of groundnut. This is the first study of its kind to show the efficacy of a single PR protein gene against two very important fungal pathogens of groundnut.

Therefore, using a genotype-independent, tissue culture-based method of generation of transgenic plants, we have expressed tobacco glucanase gene in groundnut and demonstrated the efficacy of the transformants against two major fungi, *Cercospora arachidicola* and *Aspergillus flavus*.

Material and methods

Plant material

Seeds of groundnut variety TMV-2 were soaked overnight in distilled water and were surface sterilized first with 1% Bavistin for 10 mins and later with 0.1% $HgCl_2$ for few seconds. After treatment with each sterilant, the seeds were washed thoroughly with distilled water. They were later put for germination

in petri plates at 30°C. Two-day old seedlings were taken as *ex plants* for *Agrobacterium* infection.

Bacterial strain and vector

Agrobacterium tumefaciens strain LBA4404, harbouring the binary vector, pKVD4 (Fig. 1), was used for transformation. The vector harbours glucanase, *uidA* and *npt* II as the gene of interest, screenable and selectable markers, respectively. All the genes were under the 35SCaMV promoter and *nos* terminator. *Agrobacterium* strain LBA4404/pKVD4 was grown overnight at 28°C in LB medium (pH 7.0) containing 50 $\mu g ml^{-1}$ kanamycin. The bacterial culture was later resuspended in 100 ml of Winans' AB medium (pH 5.2: Winans et al. 1988) and grown for 18 h. For *vir* gene induction treatments, wounded tobacco leaf extract (2 g in 2 ml sterile water) was added separately to the *Agrobacterium* suspension in Winans' AB medium, 5 h before infection (Cheng et al. 1996).

Transformation and recovery of transformants

Transformation of groundnut and generation of the primary transformants was accomplished using the tissue culture-independent *in planta* transformation procedure as standardized earlier (Rohini and Rao 2001). The seedlings with just emerging plumules were infected by wounding at the meristem with a sterile needle and subsequent immersion in the culture of *Agrobacterium* for 16 hr. Following infection, the seedlings were

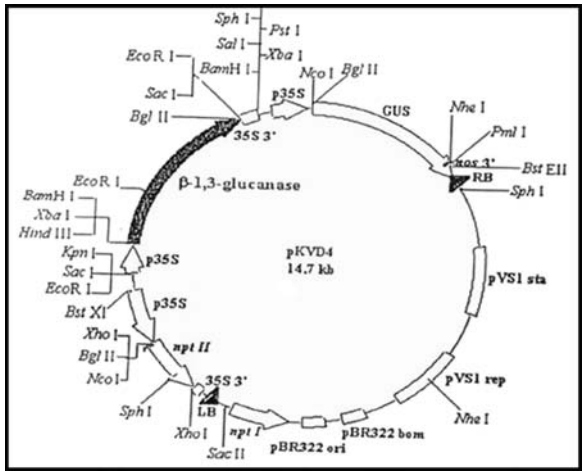

Fig. 1 T-DNA map of the binary vector pKVD4 (14.7 kb). LB, left border; RB, right border; enclosed within the T-DNA are *npt*II, glucanase and *uid*A genes

washed briefly with sterile water and later transferred to autoclaved soilrite (vermiculite equivalent) moistened with water for germination under aseptic conditions in the growth room in wide mouth capped glass jars of 300 ml capacity, 5 seedlings per jar. After 5–6 days, the seedlings were transferred to soilrite in pots and were allowed to grow under growth room conditions for 10 days before they were transferred to the greenhouse.

Molecular analyses of the putative transgenic plants

Tissues from the progeny plants were analyzed for the presence of the introduced genes. Genomic DNA was isolated following the procedure of Dellaporta et al. (1983) from fresh leaf tissue of the greenhouse-grown plants.

PCR analyses

Putative groundnut transformants were grown in green house following the recommended package of practices (Anonymous 2000). Preliminary analysis was made using the grid PCR technique to select the putative transformants. For this, the plants were divided into different grids containing 100 plants each. Samples from each 10 plants either along the row or along the column formed a composite sample. As a result, from each grid of 100 plants numbered from 1 to 100, a total of 20 composite samples originated. Genomic DNA from composite samples was isolated by C-TAB method as mentioned earlier. PCR analysis of composite samples was done using specific primers under standardized PCR conditions. PCR was also performed to confirm the presence of the gene in the plants that were selected to be advanced further. Three sets of primers to amplify the *uid*A gene, 35S promoter-*uid*A gene and *npt* II gene were used. In order to amplify the *uid* A gene fragment, PCR was initiated by a hot start at 94°C for 7 min followed by 32 cycles of 1 min at 94°C, 1 min 30 s at 60°C and 1 min at 72°C. The annealing temperature was 60°C to amplify a 750 bp 35S promoter-*uid*A gene and 58°C for a 750 bp *npt* II gene. The products were run on a 1% agarose gel.

Southern analysis

In order to analyze the total genomic DNA for integration of the glucanase gene, 15 μg of total

genomic DNA was digested with the appropriate restriction enzyme. The digested DNA samples were electrophoresed on a 0.8% agarose gel. The separated fragments along with the uncut DNA were transferred onto a nylon membrane and hybridized with a labeled 2 kb *Hind*III-*Eco*R1 glucanase gene fragment. Hybridization was performed at 65°C in Church buffer for 18 h. Membranes were washed for 30 min each in 2X SSC, 0.1 % SDS; 0.1X SSC, 0.1% SDS at 65°C (Sambrook et al. 1989). The blots were later exposed to a phosphorimager.

Western blot analysis

Leaf tissue (100 mg) of plants was harvested and ground in 1 ml of ice-cold extraction buffer (phosphate buffer [pH 6.8], 50 mM; 0.1% Triton). The homogenate was centrifuged at 13,000×*g* for 10 min at 4°C. The supernatant was used as a crude enzyme sample. The protein amount was determined using a spectrophotometer. The crude protein (50 µg) samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins blotted onto nitrocellulose membrane (Millipore (India) Pvt. Ltd.) from SDS-PAGE gel were probed with antibody against nptII at a 1:1000 dilution. Membranes were incubated with a goat anti-rabbit IgG ALP conjugate as a secondary antibody and reaction detected according to the manufacturer's instructions using BCIP/NBT as substrate (GeNei, Bangalore, India).

Analysis for the efficacy of the transformants

Enzyme assays

Total proteins from the leaves of the wild type and transformed plants were extracted with 200 mM Acetate buffer (pH 5.2). A spectrophotometric method was used for estimation of the reducing sugars on digestion of the glucose polymer laminarin following the method of Nelson (1944). Protein quantification was carried out using the Bradford method (Bradford 1976). The assay was repeated to ensure reproducibility.

Screening for disease resistance against important fungal pathogens of groundnut

(1) *Cercospora arachidicola*

Isolation of *Cercospora arachidicola*

Diseased groundnut leaves were collected separately and placed in clean, dried polythene covers. The leaves were kept overnight under dark conditions at room temperature and relative humidity of 60–70%. Spotted portion of the infected leaves were cut and surface sterilized by using 0.1% mercuric chloride or 0.1% sodium hypochlorite for 1–2 min in sterilized petri plates, transferred to another sterilized petriplate, and washed by using sterile distilled water 2–3 times in order to remove the traces of the sterilant. The sterilized bits of infected leaves were inoculated on the plates containing *Cercospora*-specific Richard's media. The plates were incubated at room temperature for 10–12 days after which the plates were fully covered with white mycelial growth which were used to inoculate the plants.

Fungal bioassay

The conidial spore suspension was prepared as mentioned in our earlier paper (Rohini and Rao 2001) and sprayed onto 5–6 week old groundnut plants. Observations were recorded on number of spots on each branch and the disease was scored at different scale/grades at 10-day intervals during the crop period. Percent disease index (PDI) was calculated. The plants were scored as resistant if 0–25% of the leaf area was covered by spots, moderately resistant if 25–30% of the leaf area was covered by spots and susceptible if 40–50% of the leaf area was covered.

(2) *Aspergillus flavus*

A hypervirulent isolate of *Aspergillus flavus* (Acc. No. EFO30718) was obtained from RARS, Tirupati, A. P, India. In the present experiment, a spore suspension of 1×10^6/ml was inoculated onto sterilized filter paper in a petri dish, which contained the surface sterilized groundnut transgenic seeds and incubated at 32°C; 70–80%RH. Wild type seeds were used as a check. The seeds were scored after 9 days for the growth of mycelia and germination and estimated for the aflatoxin content through reverse phase HPLC (Arthur et al.

2006). The peak at 30 min retention time in transgenic and wild type was compared to the peak generated by the purified aflatoxin (Sigma, USA) used as standard.

Statistical procedures

The experiment was conducted in a completely randomized design (CRD). The glucanase activity was expressed in units/mg protein. Percent Disease Index was calculated according to Wheeler 1969 and the data was subjected to analysis of variance (one-way ANOVA). All analyses were performed using the SAS (1996) package.

Results

In planta transformation of groundnut variety TMV-2 with glucanase gene

Approximately 50 seedlings were subjected to *in planta* transformation. Of these, 30 plants survived after being transferred to the pots in the greenhouse. Under the greenhouse conditions, the plants grew normally, flowered and set pods. These plants were designated as the T0 plants. Seeds were harvested and used for raising the T1 generation plants.

Molecular analysis of the T_1 generation plants

There were 225 T_1 generation plants that could be established from the 30 primary transformants in the greenhouse. These plants were divided into groups of 100 as grids to form 50 composite samples for PCR analysis. PCR analysis with *npt* II specific primers of the 50 grid samples (Fig. 2a) revealed the possibility of presence of the gene in 90 plants out of the 225 analysed. Among these 35 plants which amplified the nptII gene fragment repeatedly were taken further into next generation (Fig. 2b).

Analysis of the T_2 generation plants

Molecular characterization of T2 plants for the inheritance of the glucanase

Randomly selected seeds of all the 35 T_1 plants were allowed to grow and establish in the greenhouse. Amplification of 450 bp *uid*A gene fragment (Fig. 3a) was seen in the DNA of all the established 110 T_2 generation plants. These plants were later analyzed for the efficacy of the glucanase gene.

Bioassay of the T_2 generation glucanase transformed plants against Cercospora sp.

There was a varying response of the T_2 plants when inoculated with the spores of *Cercospora*. Based on the number of spots produced on the plants or percentage of leaf covered by the spots, the Percent disease index (PDI) was calculated and the plants were categorized as susceptible, intermediary resistant and resistant (Fig. 3b). It was observed that 16, 81 and 13 lines, respectively, were resistant, moderately resistant and susceptible to *Cercospora* (Fig. 3c). Though 16 plants performed well against *Cercospora* infection, 7 plants (15-10-3, 15-10-5, 19-218-34, 19-218-7, 29-300-152, 29-300-153, 31-166-35) that showed the lowest PDI in the bioassay against *Cercospora* were selected for analysis into the next generation.

Analysis of the T_3 generation plants

Efficacy analysis In the T_3 generation, the main emphasis was on the stability, integration and expression of the transgenes in the selected plants. In this direction, 35 plants from 7 T_2 plants were screened for resistance against *Cercospora* to begin with. Though all the plants performed well, 5 plants with lowest PDI (Fig. 4a) were subsequently selected for further confirmatory molecular and expression analysis.

The disease intensity was recorded at 10 day intervals starting from 7 DAI and percent disease index was calculated as presented in Fig. 4b. No disease was observed on 7th DAI in all the transgenic lines, however, wild type plants recorded 5 PDI. The disease symptoms were delayed even after 17th DAI in all transgenics except in T-9 which recorded PDI of 5 as against 15 in wild type plants. On 27th day, T-2, T-7 and T-11 lines showed further delayed symptoms recording no disease. This was followed by 5 PDI in T-1, T-3, T-4, T-5, T-6, T-8 and the maximum 10 PDI was observed in T-9. On the contrary, the wild type recorded 50 PDI. Maximum PDI of 15 was recorded in T-9 and T-11 as against 75 in the wild type on 37th

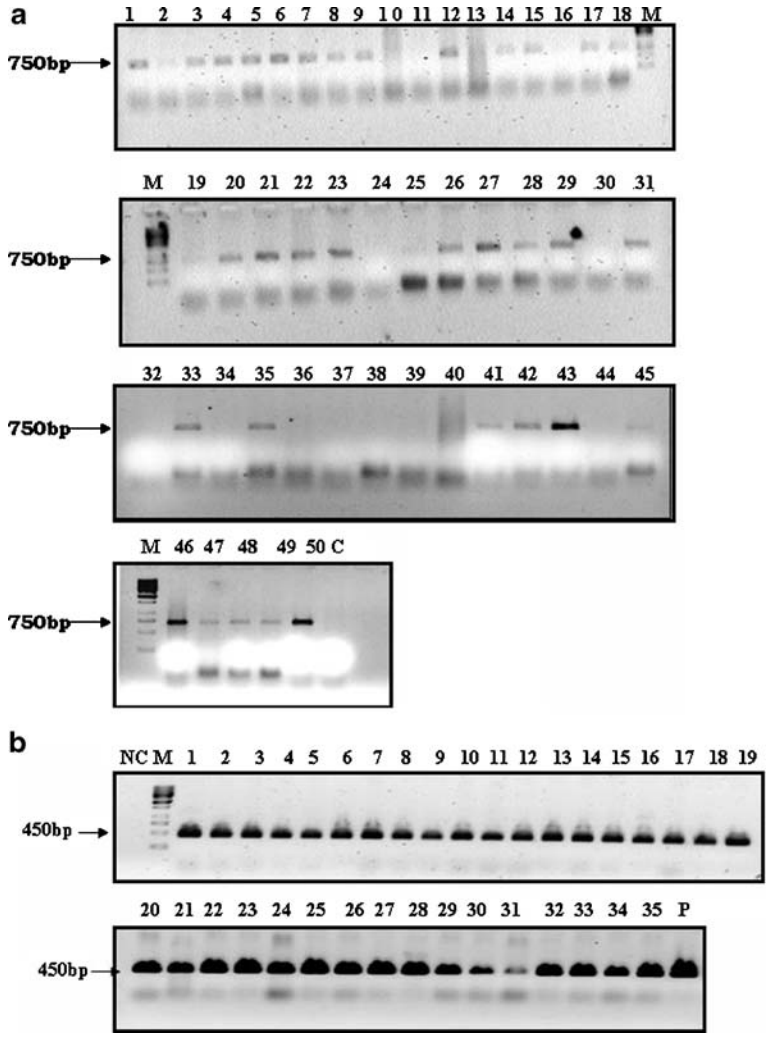

Fig. 2 Analysis of the T1 generation groundnut plants by PCR: **a** Grid PCR with *npt*II gene specific primers. Lane M: DNA ladder (1 kb); Lane C: negative control (DNA from untransformed plants); Lanes 1–50: DNA from grid composite samples of putative transformants. **b** PCR analysis of the grid PCR positives with primers for *uid*A gene. Lane M: DNA ladder (1 kb); Lane NC: negative control (DNA from untransformed plants); Lane P: positive control (plasmid DNA); Lanes 1–35: DNA from putative transformants

DAI and minimum of 5 in T-2 (29-300-152-153), T-3 (29-300-152-45), T-5 (29-300-152-36), T-7 (31-166-35-26) and T-8 (31-166-35-10) which were finally selected for further confirmation.

Expression analysis Glucanase activity was assayed in 5 of the best plants selected based on the performance in the *Cercospora* bioassay. The results were found to be statistically significant at $p=0.05\%$ and the graphical data are presented in Fig. 4c. The selected transgenic plants expressing glucanase ranged from 2.16 to 6.67 U/ mg protein as against 1.03 U/ mg protein in wild type plants (Fig. 4c). This shows that the transgenics had higher glucanase activity and lower PDI. Three plants (29-300-152-45, 31-166-35-26 and 31-166-35-10) showed more than a 5 fold increase in the glucanase activity.

A strong correlation was seen between the two parameters, glucanase activity and PDI (Fig. 4d). It was seen that the plants that expressed high levels of glucanase also showed low PDI indicating that the resistance to the fungal diseases was due to the over-expressed tobacco glucanase in groundnut plants.

As corroboration, a protein of ~35 kda (NPTII) was identified by the Western blot analysis in the plants with high glucanase activity (Fig. 4e). The absence of this band in the total protein extract of the non-transgenic plants confirmed not only integration of the transgene but also its expression.

Molecular analysis Analysis was carried out with respect to the integration and copy number in the best performing plants. Firstly, the 5 resistant plants selected were confirmed for the stability of the

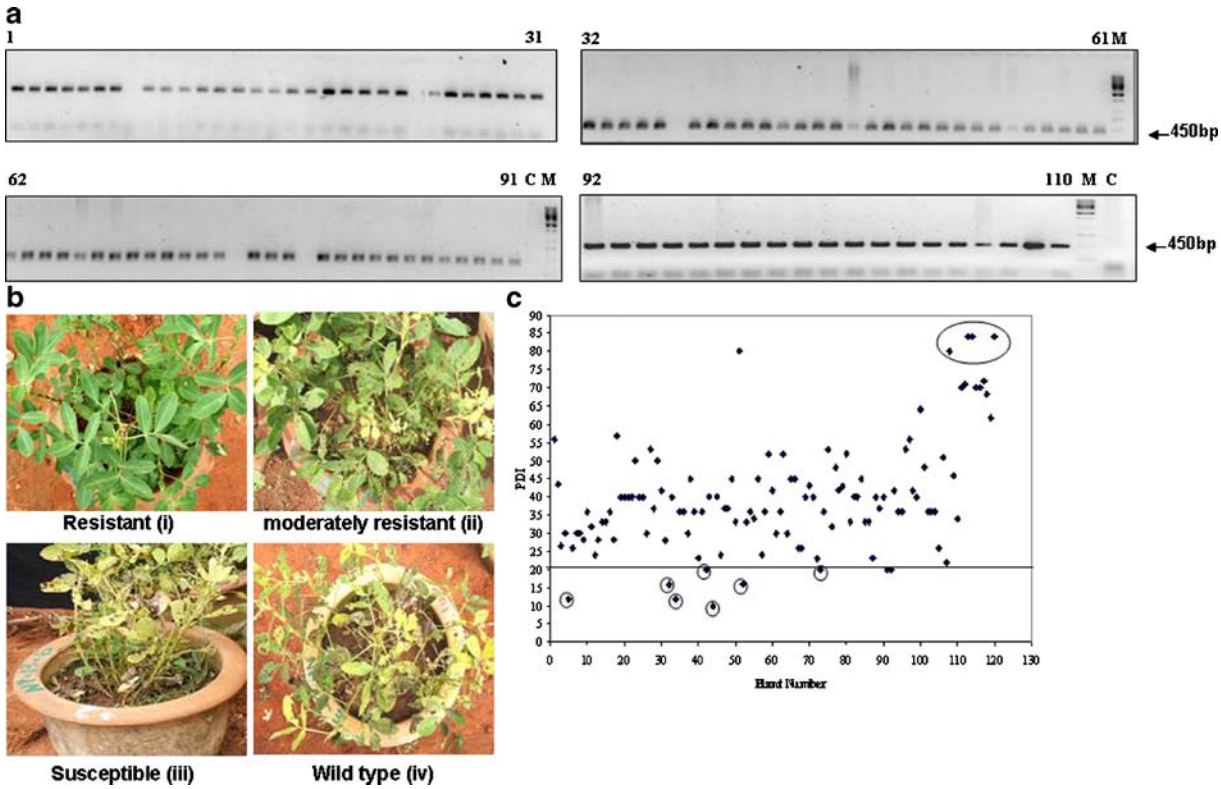

Fig. 3 Analysis of T2 generation groundnut plants harboring the glucanase gene. **a** PCR analysis of the T2 generation plants with primers for *uid*A gene. Lane M: DNA ladder (1 kb); Lane NC: negative control (DNA from untransformed plants); Lane P: positive control (plasmid DNA); Lanes 1–35: DNA from putative transformants. **b** Response of the transgenics of T2 generation against *Cercospora arachidicola* under greenhouse conditions. Based on the spots the plants were divided as, (i) resistant (ii) moderately resistant (iii) susceptible, along with the (iv) wild type. **c** Percent disease index (PDI) of *Cercospora arachidicola* in T2 generation transgenic plants as against the wild type plants. Individually circled plants are those 7 transgenic plants (15-10-3, 15-10-5, 19-218-34, 19-218-7, 29-300-152, 29-300-153, 31-166-35) with minimum PDI selected into further generation. The plants in that larger circle with maximum PDI belong to the wild type

transgenes by PCR using primers for *uid*A, *npt* II genes and also 35S promoter and the *uidA* gene (Fig. 4f). The integration was confirmed by Genomic Southern analysis, which was carried out with both uncut and restrict digested DNA from two randomly selected plants. Genomic DNA was digested with *Hind* III as the enzyme cuts once in the T-DNA region and probed with a 2 kb *Hind*III-*Eco*RI glucanase gene fragment. A strong signal shown by the uncut DNA of transgenic plants (Fig. 4g(i)) and the difference in the hybridization pattern of the two selected transgenic plants revealed the single copy integration in these plants (Fig. 4g(ii)), whereas no signal was observed with the DNA of untransformed plants. In an attempt to corroborate between the expression, efficacy and transcript accumulation, 3 selected plants (29-300-152-45, 31-166-35-26 and 31-166-35-10) with more than 5 fold increase in glucanase activity were further assessed for transcript accumulation by Northern analysis (Fig. 4h(i)) using the same glucanase gene fragment as a probe. Strong hybridization signals at approximately 2 kb in the transgenic plants revealed transcript accumulation supporting the increase in the expression of glucanase in these plants.

Performance of the transgenic plants against *Aspergillus flavus*

The efficacy of glucanase gene was checked against *Aspergillus flavus*. Spores of the seed-borne storage pathogen were inoculated directly onto seeds, incubated and scored for infection and mycelial growth. Subsequently, the accumulation of the toxin was

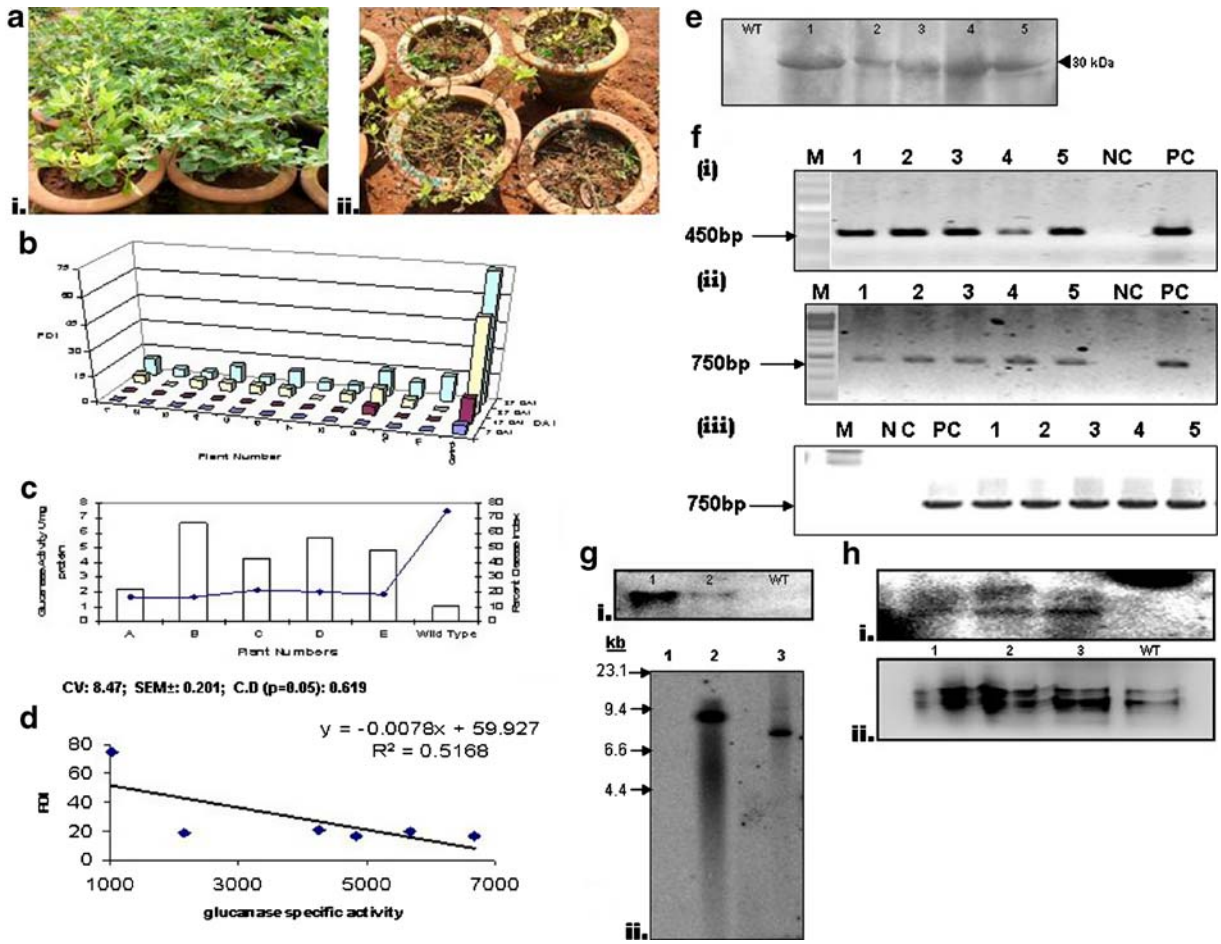

Fig. 4 Analysis of T3 generation plants. **a** Response of the transgenics of T3 generation groundnut plants against *Cercospora arachidicola*. (i). transgenic plants (ii). wild type plants. **b** Percent disease index (PDI) at different time interval after deliberate infection with *Cercospora arachidicola*. PDI was recorded on 7th, 17th, 27th and 37th day after infection. Line 1–11 (on x-axis): transgenics plants. Control: wild type plant. **c** Glucanase activity: Varied levels of glucanase expression in transgenic and wild type plants in relation with PDI of *Cercospora arachidicola*. Bar A: 29-300-152-153, B: 29-300-152-45, C: 29-300-152-36, D: 31-166-35-26 and E: 31-166-35-10. **d** Graph showing the correlation between the two parameters, PDI and glucanase activity in the transgenic plants against the wild type. **e** Western blot analysis of *npt*II in T3 plants. Lane WT: total protein extract from wild type plants; Lanes 1–5: total protein extract from transgenic plants (lane 1: 29-300-152-153, 2: 29-300-152-45, 3: 29-300-152-36, 4: 31-166-35-26 and 5: 31-166-5-10). **f** PCR analysis of the selected T3 generation plants using (i) *uid*A gene specific primers (ii) 35 S promoter-*uid*A gene specific primers and (iii) *npt*II gene specific primers. Lane M: 1 kb ladder; lanes 1–5: transgenics (lane 1: 29-300-152-153, 2: 29-300-152-45, 3: 29-300-152-36, 4: 31-166-35-26 and 5: 31-166-5-10); lane NC: negative control (untransformed plant); lane PC: positive control. **g** Genomic Southern analysis. i) Uncut DNA probed with *glucanase* gene fragment. Lane 1 & 2: transgenic plants; Lane 3: untransformed wild type plant. ii) 15 µg of genomic DNA was digested with *Hind*III and probed with radiolabelled *glucanase* gene fragment. Lane 1: untransformed wild type plant. Lane 2 & 3: transgenic plants (lane 2: 29-300-152-153 and lane 3: 31-166-35-26). **h** Northern hybridization: i) Transcripts of glucanase probed with radiolabelled *glucanase* gene fragment. ii) Total RNA isolated from transgenic and wild type plants as loading control. Lane 1–3: Transgenic plants (lane 1: 29-300-152-45, lane 2: 31-166-35-26 and lane 3: 31-166-35-10); WT: wild type plant

checked by HPLC. An aliquot of seeds from wild type and 5 T3 plants were subjected to *Aspergillus flavus* inoculation. The wild type seeds (Fig. 5a(i)) were completely covered by mycelial growth whereas the transgenics showed a survival rate of 100% (Fig. 5a(ii–vi)) and had no accumulating aflatoxin as detected by HPLC (Fig. 5b(iii) and 5b (iv)) at 30 min retention time when compared to the wild type seeds which accumulated aflatoxin at 6,00,000 µg/kg of seed (Fig. 5b(i)). The peak in the

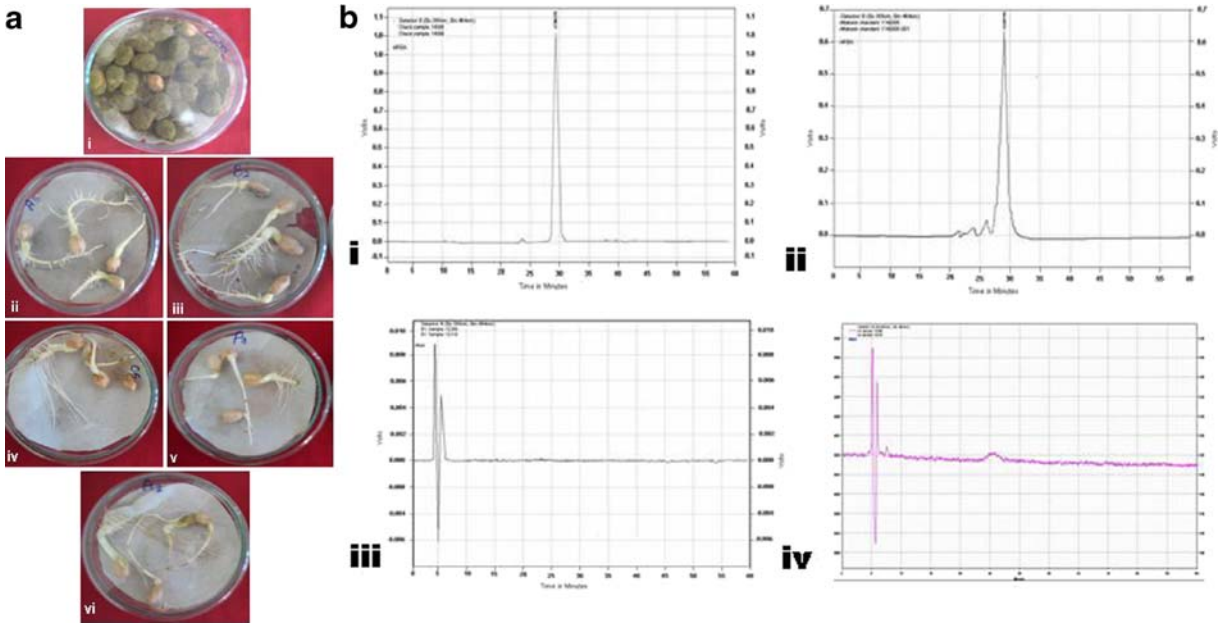

Fig. 5 Analysis of the transformants for tolerance against *Aspergillus flavus*. **a** The seeds of transgenic plants and the wild type plants were inoculated with the spore suspension of *Aspergillus flavus* and observed for mycelial growth after 9 days. (i). seeds from wild type plants; (ii– vi): seeds from transgenic plants (ii: 29-300-152-153, iii: 29-300-152-45, iv: 29-300-152-36, v: 31-166-35-26 and vi: 31-166-5-10). **b** HPLC chromatograms of the aflatoxin from, (i). wild type groundnut seeds; (ii). Purified aflatoxin; (iii) & (iv). Seeds of two representative transgenic plants

wild type seeds was comparable with that of the standard (Fig. 5b(ii)).

Discussion

Genetic engineering of disease-resistance through transfer of plant defense-related genes into crops is valuable in terms of cost, efficacy and reduction of pesticide usage (Lin et al. 2004). In this study, we explored the possibility of the use of a glucanase gene from tobacco against two important fungal pathogens of groundnut viz., *Cercospora arachidicola* and *Aspergillus flavus*. The study confirmed that the inhibition of the hyphal growth of these two fungi was due to the transgenic protein extracts containing glucanase, which is known to hydrolyse newly-formed glucan and disrupt cell walls of the growing hyphae (Mondal et al. 2007).

The transgenic plant recovery achieved in this study was by a tissue culture-independent method (Rohini and Rao 2001), i.e. by germination of the *Agrobacterium*-treated embryo from a mature seed. *In planta* transformation protocols have been developed in several other crops like rice (Supartana et al. 2005), wheat (Supartana et al. 2006), and Maize (Chumakov et al. 2006), apart from *Arabidopsis thaliana* (Feldmann and Marks 1987) and soybean (Chee et al. 1989).

This method is based on the report of Graves and Goldman (1986) who observed that transformed plant tissues could be obtained via *Agrobacterium*-mediated infection of the mesocotyl region of germinating seeds. In the present study, *Agrobacterium* infection was directed at the plumule, cotyledonary node and surrounding areas of young seedlings of groundnut. Following co-cultivation, seedling axes were allowed to germinate on soilrite and were later shifted to the greenhouse. In this method, *Agrobacterium* is targeted to the wounded apical meristem of the differentiated seed embryo. Therefore, *Agrobacterium tumefaciens* transfers the gene into the genome of diverse cells which are already destined to develop into specific organs and the meristematic cells still to be differentiated. This results in the primary transformants (T_0) being chimeric in nature. The chimeric plants producing the stable transformants in the T_1 generation depends on the type of cells that were transformed in the T_0 plants. Because of the reason that only a sector

of T_0 transformants may contain T-DNA resulting in chimeric shoots, T_1 generation plants were subjected to analysis.

The preliminary experiment to choose the transformants was based on grid PCR which predominantly identifies the non transformants. Nevertheless, PCR with the DNA of individual plants is necessary to carry them forward to the next generation. In the T_1 generation, 35 such plants which were confirmed as transgenics by PCR were selected to be taken ahead into T_2 generation.

The performance of these plants was checked in the T_2 generation when the plants that were confirmed PCR positives were challenged with the spores of *Cercospora arachidicola*. *C. arachidicola* is a major pathogen of groundnut and is highly virulent in the Indian cultivars and causes local necrotic lesions on leaves called the leaf spot disease or the tikka disease. A stringent selection of the best performing plants was based on the percent disease index. The plants were divided as susceptible, intermediary resistant and resistant based on PDI and in the T_2 generation, 16 plants that showed 5–10% of the leaves infected were grouped as resistant plants as against >50% of the leaves infected in the wild type. Though all the 16 plants were PCR positives, 7 plants with very low PDI were nevertheless decided to be taken further.

In the T_3 generation, the transgenics were not only analysed for their fungal efficacy but also at the molecular level for integration and expression of the glucanase gene. The main aim was to carry out molecular analysis of promising transgenic lines. The plants that performed well against the *Cercospora* infection were checked for the copy number. Genomic Southern analysis using *Hind*III, a single cutter indicated that the T-DNA is stably integrated as a single copy event in two different locations in the two selected lines from two representative T1 lines. The selection of two plants for genomic Southern analysis was based on the fact that the selected lines basically originated from two T1 backgrounds, 29–300 and 31–166. Further, transcript accumulation of the glucanase gene was confirmed by the Northern blot analysis and prolonged exposure of the hybridized RNA revealed high levels of the transcript accumulation in all the three plants that showed more than five fold increase in glucanase activity. Co-integration of the *npt* II gene in the transgenic plants was confirmed by Western blot analysis using antibodies against the NPTII protein.

Variation in the specific activity of the glucanase in the transgenic plants and the increase in the expression of the protein proved the fact that the resistance behavior of the transgenic plants was because of the over-expression of the glucanase enzyme in these transgenic plants. Further evidence for the efficacy of the introduced glucanase gene was by the strong correlation seen between the glucanase activity and PDI. The plants that had low PDI and were tolerant to the target fungus also showed fold increase in the glucanase activity confirming the improved efficacy of the transgenic plants.

Further, the increase in the glucanase activity in the plants correlated with their behavior against another pathogen of groundnut, *Aspergillus flavus. Asergillus flavus* and the closely related subspecies *parasiticus* have a worldwide distribution and normally occur as saprophytes in soil and on many kinds of decaying organic matter. Groundnut is one of the crops that are readily colonized by these fungi. Most *A. flavus* and *A. parasiticus* can produce a polypeptide-derived secondary metabolite called aflatoxin, which are highly toxic, mutagenic and carcinogenic. Host-plant resistance to *A. flavus* infection is considered the most effective method for reducing aflatoxins, but resistant germplasm is limited. The stability and the efficacy of the transgenic lines was evident by the performance in the assay against *Aspergillus flavus*. Aflatoxin quantified by HPLC revealed that the transgenics did not accumulate any aflatoxin even at the end of 9 days after inoculation by contrast with 6,00,000 µg/kg of aflatoxin that accumulated in the seeds of wild type that eventually failed to germinate.

The absence of the spread or initiation of infection in both the *Cercospora* and *Aspergillus* bioassay, gives evidence to the over-expression and efficacy of the expressed glucanase gene product. The restricted growth of pathogens leading to reduction in disease symptoms in transgenic plants expressing glucanase has been well documented in different host-pathogen systems (Zhu et al. 1994). The hydrolytic enzyme glucanase is lytic on the cell wall of necrotrophic fungal pathogens whose major cell wall component is glucan. In the present study, disease progression in the transgenics when compared to that in the wild type revealed a delay in the onset of the disease in transgenics. Such a delay has been observed in earlier studies (Mondal et al. 2003; Broglie et al. 1991) and it was attributed to the antifungal activity. It is well-

accepted that coordinated expression of many PR proteins may provide requisite levels of tolerance against the pathogen (Anand et al. 2003). The present study gains significance in that the glucanase gene has conferred fairly good resistance to two fungal pathogens of groundnut. On pathogen infection the defense signaling cascade is up-regulated bringing about enhanced expression of PR proteins besides triggering the hypersensitive response. It is likely that in the background of expression of defense responsive proteins, enhanced expression of glucanase might have increased the tolerance levels of the transgenic plants.

In our study, 15% of the T_1 transformants were found to harbour the transgene, with the best performing plants coming from 2 independent T_1 lines eventually. The identification of transgenic groundnut lines with enhanced fungal resistance has provided necessary information for determining the usefulness of such plants as parents in traditional breeding programmes for fungal disease resistance.

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