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Overexpression of a fusion defensin gene from radish and fenugreek improves resistance against leaf spot diseases caused by *Cercospora arachidicola* and *Phaeoisariopsis personata* in peanut

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Abstract: Peanut (*Arachis hypogaea* L.) is one of the important oilseed crops of the Indian subcontinent and fungal diseases like early leaf spot (ELS) and late leaf spot (LLS) caused by *Cercospora arachidicola* and *Phaeoisariopsis personata*, respectively, are major peanut cultivation constraints. Defensins are basically antimicrobial peptides that have been implicated in plant defense against various microbial attacks. Transgenic peanut plants, developed through *Agrobacterium* mediated transformation of de-embryonated cotyledons and overexpressing a synthetic defensin fusion gene from fenugreek (*Tfgd2*) and radish (*RsAFP2*) linked by a linker peptide, were found to have enhanced resistance to the ELS and LLS infection over the wild type (cv. GG 20). Both transformed and untransformed lines were characterized for leaf spot diseases using a detached leaf assay. PCR and RT-PCR analyses confirmed stable integration and expression of these genes in peanut transgenics. This investigation provides further evidence that a fusion product of two plant defensins can be successfully implemented as a means of imparting resistance to multiple fungal pathogens through genetic engineering in peanut.

Key words: Antimicrobial peptides, *Arachis hypogaea*, biotic-stress resistance, genetic engineering, synthetic fusion-gene

1. Introduction

Globally peanut (*Arachis hypogaea* L.), which is also known as groundnut, is grown in around 109 countries between 40°N and 40°S, encompassing major regions of Asia and Africa (Shoba et al., 2012). It is an important oil seed, food, and feed crop grown on about 20–24 million ha throughout the world (http://www.fas.usda.gov/psdonline/). In India, it is predominantly grown on marginal lands of the semiarid regions under rainfed conditions. This accounts for around 80% of the cultivated area under peanut, mostly with low inputs like fertilizers, insecticides, and fungicides (Government of India, Ministry of Agriculture, 2013).

In countries with tropical and subtropical regions, leaf spot disease, both early and late, caused by *Phaeoisariopsis personata* and *Cercospora arachidicola*, respectively, is a very serious problem of peanut cultivation (Gajjar et al., 2014; Bosamia et al., 2015). Besides having adverse effects on pod quality and yield to an extent of over 50%, these foliar diseases also severely affect fodder yield and quality (Subrahmanyam et al., 1989; Waliyar, 1991). Different sources of resistance to leaf spots have been reported in *A. hypogaea*, but the majority of them belong to the subspecies *fastigiata* and landraces from South America (Mehan et al., 1996; Singh et al., 1997). Until now only moderate levels of resistance have been reported against leaf spot diseases in cultivated species, but many wild species possess resistance to leaf spots (Holbrook and Stalker, 2003). Unfortunately, they often carry undesirable traits like long duration, poor adaptability, and poor yields (Shoba et al., 2012).

Modern crop protection strategies against various pathogens are based on exploiting the natural, intricate plant defense mechanisms through transgenic approaches (Tiwari et al., 2008; Sönmez et al., 2014). The ultimate aim of this is to reduce both the cost of crop protection and the potentially detrimental impact of pesticides on the ecosystems (Holland et al., 2012). In the ongoing pursuit of disease resistant crops, defensins are one of the most extensively studied peptide families and are used as targets for transgenic approaches in a range of plant species (duPlessis, 2012).

Plant defensins are composed of small cysteine-rich proteins of 45–54 amino acids and are closely related to mammalian and insect defensins (Thomma et al., 2002;

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Lay and Anderson, 2005). These play an important role in defense against different fungal pathogens, and are mostly synthesized as precursor proteins, which cleave out the C-terminal mature defensin peptide from the secretory signal peptides via posttranslational processing. The majority of defensins are secreted to the extracellular space, but a few are vacuole specific (Kaur et al., 2011). The latter are divided into two classes based on the absence (class I) or presence (class II) of an acidic C-terminal propeptide (CTPP) of about 33 amino acids (Lay et al., 2014).

Despite the ability of plant defensins to inhibit the growth of pathogenic fungi, these peptides are nontoxic to most animal and plant cells (Osborn et al., 1995). Defensins are generally the products of single genes, allowing the plant to deliver these molecules relatively rapidly and with minimal energetic expense to the plant, upon pathogen attack (Thomma et al., 2002). Defensins are expressed in most but not all plants and are key members of a plant's immune system, which helps in protecting the plants from various pathogens (Feng et al., 2012).

Through bioinformatical studies, it was found that the defensin-encoding genes are overrepresented in some plants species, contributing around 3% of all genetic material in *Arabidopsis* (Silverstein et al., 2007). This accentuates the significance of these peptides in general plant biology, including plant defense (duPlessis, 2012). From peanut, a full-length open reading frame (ORF) of a defensin (*AhDRRP*) has been cloned and was expressed in Rostteta, and homology analysis showed 33%–70% homology with the defensin genes of other species (Li et al., 2009). The defensin family is quite diverse in its biological activity (Lay et al., 2003) and has strong potential to be used for engineering disease resistance in crops (Kaur et al., 2011). In vitro antifungal activity of a defensin from fenugreek (Olli and Kirti, 2006), radish (Terras et al., 1995), and a fusion gene of fenugreek (*Trigonella foenum-graecum defensin* 2; *Tfgd2*) and radish (*Raphanus sativus* antifungal protein 2; *RsAFP2*) (Vasavirama and Kirti, 2011, 2013) was tested against some fungal pathogens.

Here, we have developed and characterized a transgenic peanut overexpressing a fusion gene made up of *Tfgd2* and *RsAFP2* defensins linked by a linker peptide sequence (occurring in the seeds of *Impatiens balsamina*), for its resistance to early (ELS) and late leaf spot (LLS) diseases.

2. Materials and methods

2.1. Plant material and culture conditions

Seeds of peanut (cv. GG 20) were obtained from the genetic resources section of the Directorate of Groundnut Research, Gujarat, and were surface sterilized by treating successively with 70% ethanol for 1 min and 0.1 % (w/v) $HgCl_2$ for 3 min, and rinsed three times with sterile distilled water (Radhakrishnan et al., 2000). The testa was then removed aseptically in a laminar air flow hood. Seeds were split into two cotyledons, embryos were removed, and the deembryonated cotyledons were directly used as explants in a petri plate. The cultures were done in a modified MS medium (Radhakrishnan et al., 2002) and maintained at 26 ± 1 °C, 16 h photoperiod with cool white fluorescent light of 3000 lux illumination.

2.2. Plasmid and transformation vector

A defensin fusion gene (Vasavirama and Kirti, 2013) having *Tfgd2* (GenBank accession number: AY227192) and *RsAFP2* (GenBank accession number: U18556.1) genes, linked by a linker peptide (the fourth internal propeptide from the naturally occurring *IbAMP* polyprotein precursor of *Impatiens balsamina* (as described in Francois et al. (2002)), was cloned at *Hind*III position of the pRD400 vector having the *npt*II gene (GenBank accession number: AAC53708.1; Datla et al., 1992). This fusion gene was mobilized to *Agrobacterium tumefaciens* strain GV2260 (Vasavirama and Kirti, 2011) and used in the genetic transformation of peanut (Figure1). The transgene was under the control of the *CaMV35S* promoter and the *nos* terminator.

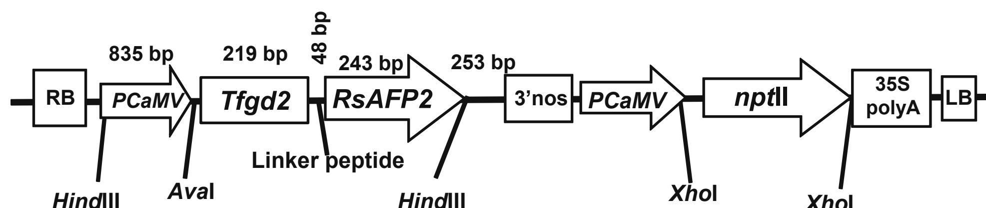

Figure 1. Restriction map of the pRD-400 binary vector carrying the *Tfg*d1 and *RsAFP2* fusion gene expression cassette at *Hind*III position that was used for the transformation of *Arachis hypogaea*. The fusion gene and *npt*II genes are driven by the *CaMV35S* promoter and terminated by the 3′ nontranscribed region of T_{nos}, where LB and RB indicate T-DNA left border and right border; *PCaMV* indicates Cauliflower Mosaic Virus 35S promoter; *npt*II indicates neomycin phosphotransferase gene; *nos* indicates nopaline synthase terminators. The elements are not drawn to scale.

2.3. Plant transformation

The deembryonated cotyledons were infected with the *Agrobacterium tumefaciens* strain GV2260 harboring plasmid vector pRD400 carrying *Tfgd2* and *RsAFP2* fusion genes, by suspending it in half-strength MS medium without hormones for 20 min. It was then incubated for 3 days for cocultivation in MS medium supplemented with 25 mg L^{-1} benzylaminopurine (BAP) for shoot bud induction. The regeneration frequency was calculated on the number of explants regenerated over the number of explants cocultured. The transformation frequency was worked out on the final number of confirmed transgenics produced over the number of explants regenerated.

2.4. Selection and regeneration of transformed tissues

Excess *Agrobacterium* was removed by washing the explants with cefotaxime (Lupin, India) solution (250 mg L^{-1}), which was then transferred to MS medium containing 25 mg L^{-1} BAP and 250 mg L^{-1} cefotaxime for shoot induction. After 15 days the regenerating explants were transferred to a selection medium containing 3 mg L^{-1} BAP, 250 mg L^{-1} cefotaxime, and 100 mg L^{-1} kanamycin to select the putative transgenics. When multiple shoots appeared, the proximal portion of the explants were cut and transferred to MS medium supplemented with 3 mg L^{-1} BAP, 1 mg L^{-1} gibberellic acid (GA), 250 mg L^{-1} cefotaxime, and 100 mg L^{-1} kanamycin for expanding and opening up of the shoot buds (Radhakrishnan et al., 2000). The explants were subcultured at 14-day intervals.

The 1–2 cm long healthy shoots were transferred to MS basal media without hormones and grown for a week, before transferring to a rooting medium. The shoots that survived the kanamycin selection were rooted on MS medium supplemented with 1 mg L^{-1} naphthaleneacetic acid (NAA), 250 mg L^{-1} cefotaxime, and 100 mg L^{-1} kanamycin. The plantlets with well-developed roots were transferred to earthen pots (21 × 23 cm) for hardening. Hardened plants were grown in earthen pots (21 × 23 cm) containing 4.5 kg of soil mix (1 soil:1 sand), under a contained greenhouse (PII) facility developed for the transgenic plants at the Directorate of Groundnut Research, Junagadh, India. The temperature and relative humidity of the PII facility were kept at 30–35 °C and 60%–65% respectively, and pods were harvested at the time of maturity. The explants without agro-infection were used as negative controls.

2.5. Molecular confirmation of putative transgenic plants

2.5.1. PCR analysis

PCR analysis was done on the putative transgenics using gene-specific primers to pick up transgenics carrying the *defensin* fusion gene and the *npt*II genes. The putative transgenics were numbered as DEF.1 to n in this experiment. Genomic DNA was extracted from young leaves of kanamycin-resistant and wild type (WT) plants using the DNAzol kit (Molecular Research Center, Inc., USA) following the manufacturer's instructions. The PCR reaction of a 20 µL volume comprised 2 µL of 10X PCR buffer (Fermentas, USA), 1 µL of genomic DNA (100 ng), 1.6 µL of 2 mM dNTP mix (Fermentas, USA), 1 µL each of forward and reverse primers (25 pM), and 1 U of *Taq* DNA polymerase (Fermentas, USA). The primer pair used for the detection of the *defensin* fusion gene and for the *npt*II gene is given in Table 1. PCR reactions were set up with the following thermal profile: 94 °C for 3 min; followed by 35 cycles of 94 °C for 30 s, 58 °C or 56 °C (depending upon the primers used) for 30 s, 72 °C for 1 min; and a final extension at 72 °C for 7 min. The amplified product was resolved on a 1.2% agarose gel, visualized by ethidium bromide staining and documented using an imaging system (Fuji FLA5200, Japan).

2.5.2. Reverse transcriptase PCR (RT-PCR)

Total RNA was isolated from all five homozygous transformed plants (T_2), obtained from independent transformation events and the WT plants using Trizol LS (Invitrogen, Life Technologies, USA). It was subjected to RNase-free DNase I (Fermentas, USA) digestion and purification. The first strand of cDNA was synthesized from 1 µg of RNA per sample using a first strand cDNA synthesis kit (Fermentas, USA) and the product obtained was further used for second strand amplification using gene specific primers via PCR. The primer pairs used for amplification are given in Table 1.

2.5.3. Real-time quantitative PCR analysis

Real-time PCR was carried out in T_2 generation with the StepOne real-time PCR system (Applied Biosystems, USA). A quantitative PCR mixture for expression of the defensin fusion gene was prepared in a MicroAmp FastOptical 48-well reaction plate. The primer pairs used for quantitative PCR for the expression analysis of the defensin fusion gene and the housekeeping gene, 18S rRNA (GenBank accession number: AF156675.2), used as an internal reference to normalize the initial cDNA content among samples (designed using the Primer Express v3.0, Applied Biosystems), are given in Table 1. All the primers were synthesized from IDT Inc. Ltd (USA).

Each reaction was performed in 25 µL (total volume) and consisted of a SYBR Green Master mix (Qiagen, Netherlands), 10 pmol of each primer, and a 1/10-fold diluted cDNA template. Reactions were set up as follows: 95 °C for 2 min, 40 cycles of 95 °C for 15 s, and 60 °C for 31 s. Each experiment included triplicate reactions with the same cDNA stock. The comparative fold expression of the defensin fusion gene was estimated in terms of the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). The ΔC_T was determined by subtracting 18S rRNA C_T from Def C_T in a given sample. The $\Delta\Delta C_T$ value was determined

Table 1. Details of the primers used for PCR, RT-PCR, and qPCR.

Gene name	Primer sequence (forward)	Primer sequence (reverse)	Annealing temperature (°C)	Product size (bp)
Primers used for PCR				
Defensin fusion gene	Def-Fwd: 5'–GGG GTA CCA TGG AGA AGA AAT CAC TAG CT–3'	Def-Rev: 5'–GGG GGA TCC TTA ACA AGG GAA ATA ACA GAT ACA–3'	58	498
*npt*II gene	*npt*II-Fwd: 5'–GAG GCT ATT CGG CTA TGA CTG–3'	*npt*II-Rev: 5'–ATC GGG AGC GGC GAT ACC GTA–3'	56	750
Primers used for RT-PCR				
Defensin fusion gene	DefRT-Fwd: 5'–ATG GAG AAG AAA TCA CTA GCT–3'	DefRT-Rev: 5'–GGG GGA TCC TTA ACA AGG GAA ATA A–3'	58	498
Primers used for q-PCR				
defensin fusion gene	Def-RT Fwd: 5'–CCA TGC TTC AGT GGC TGT GA–3'	Def-RT Rev: 5'–CCT GCA CCT GCC GCT AA–3'	56	-
housekeeping gene, 18S rRNA	18SRT Fwd: 5'–GGC TCA AGC CGA TGG AAG T–3';	18SRT Rev: 5'–AGC ACG ACA GGG TTT AAC AAG–3'	57	-

by subtracting the lowest expressing transformed plant (calibrator) ΔC_T from the ΔC_T of each transgenic plant.

2.6. Segregation analysis

The plants in T_0 generation were grown to produce a sufficient number of seeds (T_1) for its segregation analysis. The T_1 progenies of all the five transgenic events were grown separately in pots under controlled conditions in a PII containment facility. The plantlets at the 2–4-leaf stage were used for PCR analysis using gene-specific primers to score the amplicons. The χ^2 test was carried out in the progenies from all the events based on expected and observed frequencies.

2.7. Evaluation of transgenic plants by in vitro antifungal activity assay

For resistance studies against *C. arachidicola* and *P. personata*, a detached leaf assay or an in vitro antifungal activity assay was carried out on five homozygous transgenic lines (T_2) and the WT as per Moraes and Salgado (1982). One set of uninoculated WT leaves along with two highly susceptible varieties (TMV2 and JL24) were also used as controls. For each line, 10 leaves were taken and the experiment was repeated three times. Days till the appearance of lesions, number of lesions on a leaflet, and lesion diameter on transgenic, WT, and other control line leaves were recorded.

The *C. arachidicola* and *P. personata* conidia, harvested from a single-lesion culture, were multiplied on inoculated detached leaves (Subrahmanyam et al., 1983) of the susceptible peanut variety TMV2. The fungus was then cultured on a PDA medium for the production of conidia (28 ± 2 °C). Conidial suspensions were obtained by flooding 15- to 20-day-old single conidial cultures in petri plates with sterile distilled water, followed by filtration through four layers of cheesecloth so as to remove most of the mycelial fragments. Tween-20 (v/v 0.5%) was added to the suspension so that the spores remained on the leaves at the time of inoculation. The working conidial suspension (50,000 spores mL^{-1}) was prepared from the stock suspension after counting the number of conidia using a hemocytometer.

For the inoculation with spores, first expanded leaves of both transgenic and WT lines were collected and individual leaf petioles, each supported by a foam plug, were immersed in Hoagland's solution (10 mL) in 1 × 14 cm test tubes. The volume of Hoagland's solution was constantly maintained to keep the leaves alive and turgid for several weeks (Melouk and Banks, 1978). Leaves were inoculated by misting leaf surfaces with a conidial suspension (1 mL) using an atomizer (DeVilbiss 15-RD, USA). Test tubes with inoculated leaves were placed in racks in a clear polyethylene chamber on a greenhouse bench. Temperatures in the chamber averaged 26 ± 2 °C and 31 ± 2 °C during night and day, respectively, and relative humidity (RH) was maintained between 80% and 90%.

2.8. Statistical analysis

For finding the inheritance pattern of the defensin fusion gene, segregation analysis (in T_1) was done using the chi-square test. For the detached leaf experiment, statistical analysis was done with three replicates per analysis and the significance of the treatment effects was determined by one-way ANOVA (at 5% probability level using the Tukey

test) with SPSS 11.0 (Statistical Package for the Social Sciences, SPSS Inc., USA).

3. Results and discussion

Since the identification of the first plant defensins in the early 1990s, research has revealed the presence and antifungal activity of plant defensins in a wide range of plant species (duPlessis, 2012). Defensins are now considered excellent candidates for transgenic approaches, and many defensins that can impart resistance when overexpressed in otherwise susceptible plants were identified (Gao et al., 2000; Jha and Chattoo, 2009; Portieles et al., 2010). There have been reports that simultaneous use of more than one resistance gene, including a defensin fusion gene, might lead to a greater antimicrobial activity compared with a single gene (Bezirganoglu et al., 2013; Vasavirama and Kirti, 2013; Güler et al., 2014). Vasavirama and Kirti (2011, 2013) reported that this fusion gene, expressed as 35-kDa recombinant protein, displayed inhibitory activity against *Rhizoctonia solani*, *Phytopthora* var. *nicotaianae*, and *P. personata* fungal pathogens. Hence, a polyprotein mode of gene expression was chosen with the thought that a fusion gene (made up of *Tfgd2* and *RsAFP2*) might confer improved ELS and LLS disease resistance in transgenic peanut plants.

3.1. Tissue culture and transformation

Transformation efficiency was evaluated as the number of independent transgenic lines with respect to the initial number of explants cultured. In the present investigation, out of 794 explants cultured in 20 batches, 672 (85.0%) regenerated successfully and produced 1386 shoots. After 6 weeks of culture on the selection medium, 635 (45.8%) shoots survived in a selection medium, of which 372 (58.5%) shoots survived under a PII containment facility.

Finally, only 14 shoots (3.76%) were found to be PCR positive when screened with transgene specific primers (Table 2). Of these, five transgenic events, namely DEF.5, DEF.7, DEF.35, DEF.47, and DEF.68, were used for further detail analysis. Though the regeneration frequency recorded was quite high, the number of transgenic plants finally recovered was relatively low. A similar trend was observed by Bhauso et al. (2014) and Sarkar et al. (2014). In addition, the defensin fusion gene transgenic peanut plants appeared identical to untransformed greenhouse-grown plants in terms of morphology, growth habit, and flowering behavior (data not shown). Thus, the integration of the transgene did not impair any main genes contributing to major phenotypic traits.

3.2. Integration of the transgene in the host genome

Kanamycin-resistant clones were subjected to PCR analysis using transgene specific primers so as to confirm the integration of the defensin fusion gene and *npt*II in T_0 transgenic plants. PCR analysis detected the presence of the 498 bp amplicon of the defensin fusion gene and the 750 bp of *npt*II (Figures 2A and 2B) genes, confirming the presence of the transgenes.

3.3. Expression of the transgene

Real time PCR analysis was performed in the representative homozygous transgenic (T_2) plants to quantify the expression of the defensin fusion gene transcript. The result showed that the transgenic lines have expressed the gene, and hence the possibility of silencing the transgene at the transcriptional level was ruled out (Figure 3), which was also observed by Sarkar et al. (2014) and Priya et al. (2015). Using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001), the relative expression of the fusion gene was interpreted and the lowest expressing transgenic line DEF.7 was selected as a calibrator. Three transgenic lines, DEF.5, DEF.35, and DEF.68, showed significantly higher levels of expression (39, 26, and 34 times, respectively), whereas DEF.47 showed only a 2.44-fold increase over the calibrator (Figure 4). A similar kind of gene expression was also observed by Tiwari et al. (2008) while studying the expression of the *cry1EC* gene for resistance against *Spodoptera litura* in transgenic peanut.

3.4. Segregation analysis of the defensin fusion gene in the progenies

All the selected five T_0 plants were fertile and produced seeds. The progenies of five defensin positive transgenic plants (T_1) were tested for segregation by chi-square analysis. The segregation pattern for the defensin fusion gene in T_1 plants showed a ratio of around 3:1, which is expected for a single dominant gene inheritance, for four out of five transformed lines studied (Table 3), which is similar to that reported by Cheng et al. (1997) and Tiwari et al. (2008).

Table 2. Regeneration and transformation frequencies of cotyledonary explants of peanut cultivar GG 20 transformed with *Tfgd2-RsAFP2* defensin fusion gene.

Number of cocultivations	Total explants cocultivated	Shoots produced	Number of regenerated explants	Shoots passed antibiotic selection	Shoots producing roots	Plantlets hardened and survived in glasshouse	Final recovery of putative transgenics
20	794	1386	672 (85%)	645 (46%)	635 (98%)	372 (58%)	14 (3.76%)

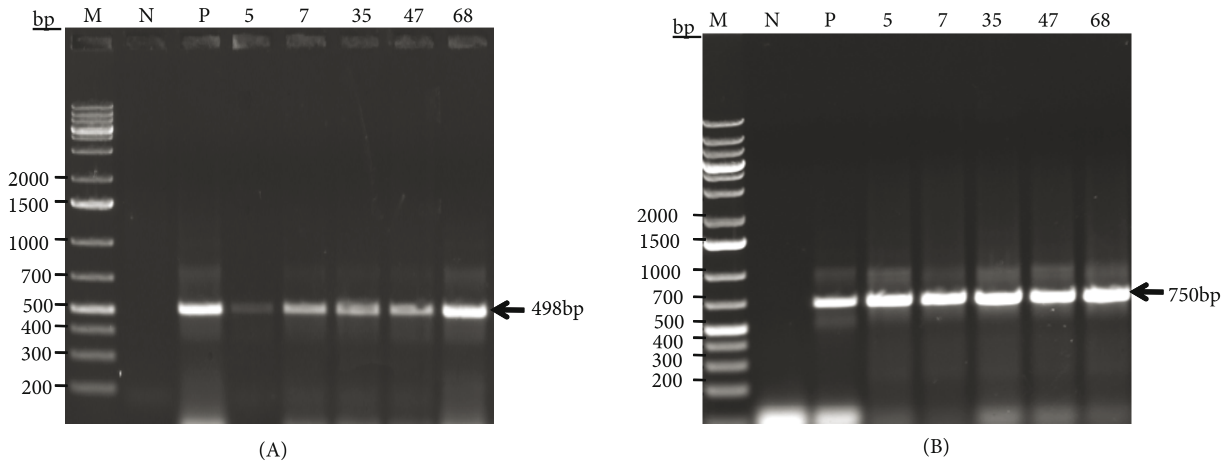

Figure 2. Preliminary confirmation of putative transgenic peanut plants by PCR amplification of: (A) defensin fusion gene (*Tfg*d1 and *RsAFP2*); and (B) *npt*II gene, using gene specific primers. *Lane* M indicates 1kb plus DNA ladder; *Lane* N indicates negative control (nontransformed plant); *Lane* P indicates positive control (pRD-400 vector with the defensin fusion gene); *Lanes* 4–8 indicate five transgenic lines, namely DEF.5, 7, 35, 47, and 68 in T_0 generation showing the presence of the defensin fusion gene and *npt*II gene, respectively.

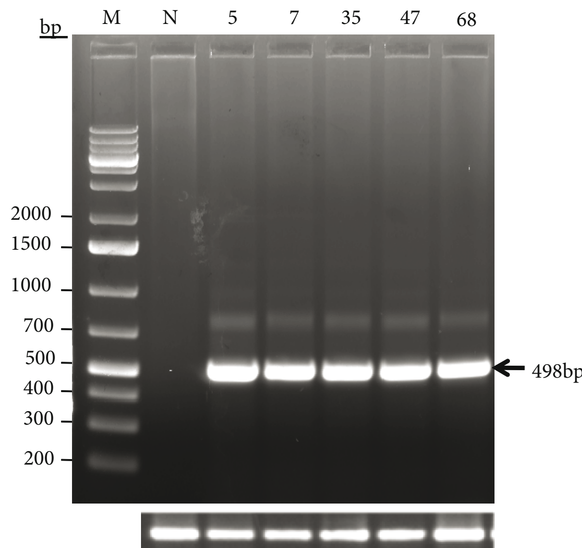

Figure 3. Reverse transcriptase PCR (RT-PCR) analysis for the defensin fusion gene transcript in transgenic and wild-type peanut plants. *Lane* M indicates 1kb plus DNA ladder; *Lane* N indicates wild-type plant; *Lanes* 3–7 indicate five transgenic lines, namely DEF.5, 7, 35, 47, and 68; *Bottom gel row* indicates the amplified product of *18SrRNA* gene (housekeeping gene) that was used as an internal control for both transgenic and wild type plants. All the transgenic lines (*lanes* 3–7) amplified a clear gene-specific band of a 498 bp size, which was absent in the nontransformed (cv. GG 20) line (*lane* N).

3.5. Characterization of transgenics by a detached leaf assay for ELS and LLS diseases

A detached leaf assay was conducted for ELS and LLS diseases in five transgenic (DEF.5, DEF.7, DEF.35, DEF.47, and DEF.68) and three control lines (TMV 2, JL 24, and GG 20). The disease symptoms appeared 15 days after inoculation as brown specks with a yellow halo (Figures 5A and 5B, top row) on the leaves. In the present investigation, the average number of lesions and lesion size were significantly less in transgenic peanut lines as compared with the control lines (P > 0.05) in the detached leaf assay for both LLS and ELS diseases. All the transgenic lines showed a significantly elevated resistance to *C. arachidicola* and *P. personata* when measured for the

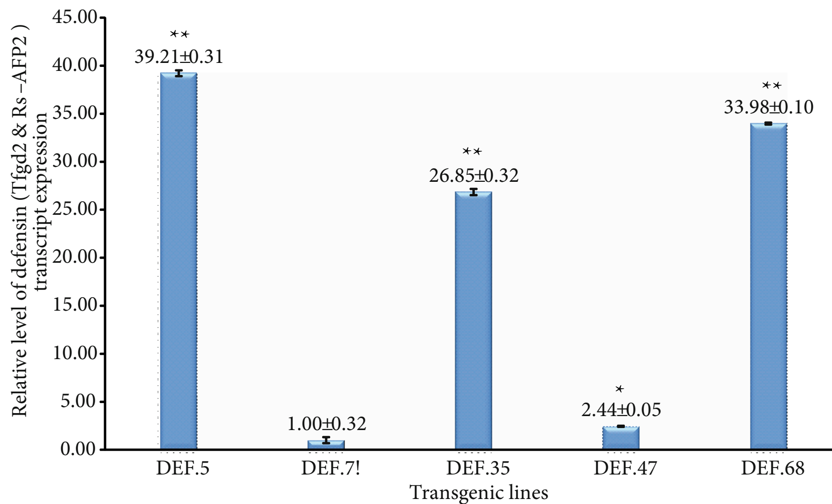

Figure 4. Quantitative expression of the defensin fusion gene (*Tfgd2-RsAFP2*) transcript in transgenic peanut plants. The relative expression of the fusion gene transcript in transgenic (T_2) peanut plant was analyzed by real-time quantitative PCR using $2^{-\Delta\Delta C_T}$ method. Amplicon abundance was monitored in real time by measuring SYBR Green fluorescence. Bars denote fold expression as compared with the lowest expressing transgenic plant, which was taken as a calibrator (!DEF.7 ±SD). The level of the defensin fusion gene transcript in T_2 plants was normalized with reference to 18S rRNA, which was taken as an internal control. Two tail Student's t-test was used to determine significant (*P < 0.005) and highly significant (**P < 0.001) difference. The control (nontransformed plant) showed no transcript.

Table 3. Segregation analysis in the selfed transgenic peanut progenies (T_1) for deciphering the inheritance of the defensin fusion gene.

Transgenic lines	Number of seeds			Observed ratio	Test ratio	χ^2	P
	Total	PCR (+ve)	PCR (–ve)				
DEF.5	7	5	2	2.5 : 1	3 : 1	0.048	0.8265
DEF.7	9	7	2	3.5 : 1	3 : 1	0.037	0.8474
DEF.35	8	5	3	1.66 : 1	3 : 1	0.667	0.4140
DEF.47	9	6	3	2 : 1	3 : 1	0.333	0.5638
DEF.68	10	7	3	2.33 : 1	3 : 1	0.133	0.7153

Here the fusion gene-specific amplification is represented as PCR (+ve) and the absence of amplification as PCR (–ve).

number of lesions and lesion size (Table 4; Figures 5A and 5B, bottom row).

Even for the appearance of ELS and LLS disease lesions (days after inoculation), all the transgenics were found to be significantly better than both nontransformed WT (cv. GG 20) and two other susceptible controls (TMV 2 and JL 24) (Table 4). Such a delay has also been observed by Liu et al. (1994) in transgenic potato as a result of overexpression of the osmotin gene. duPlessis (2012) also recorded improved resistance in transgenic grapevine (for *Vv-AMP1* gene) to powdery mildew (*Erysiphe necator*) infection based on lesion number and size, in a detached leaf assay. Similarly, delayed onset of the damping-off (*Alternaria brassicae*) disease was reported in brassica transgenics as compared with nontransformed lines (Mondal et al., 2003).

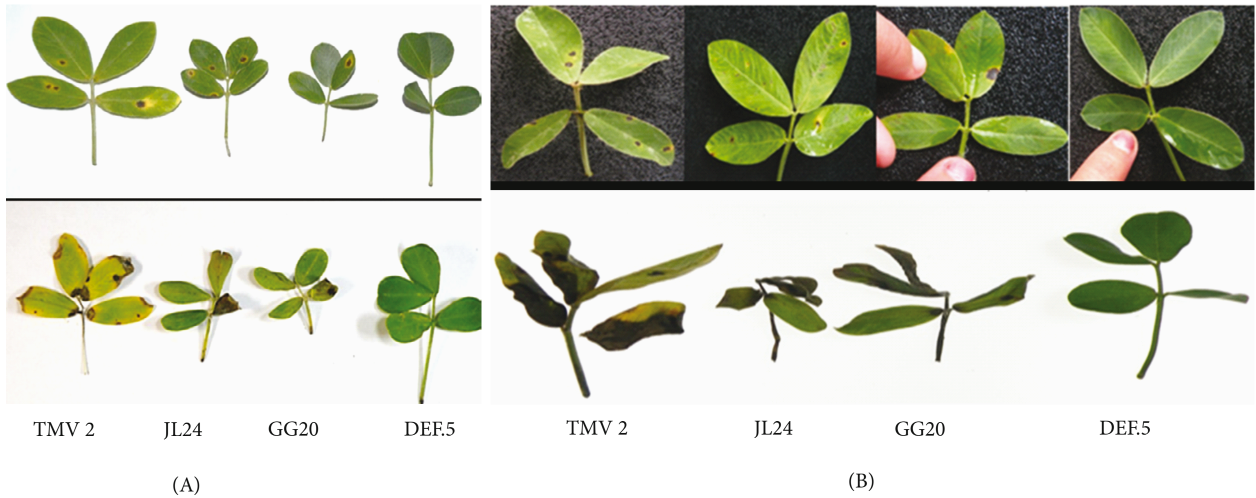

Figure 5. Bioassay analysis of control and defensin fusion gene transgenic (T_2) peanut plants for early and late leaf spot diseases. (A) The top and bottom rows indicate the *Cercospora arachidicola* disease development 10 and 21 days after inoculation, respectively. (B) The top and bottom rows indicate the *Phaeoisariopsis personata* disease development 10 and 28 days after inoculation, respectively. Cultivar GG 20 was used as a nontransformed control, whereas TMV 2 and JL 24 were used as susceptible controls. There was a clear difference in the disease development on control and fusion gene transgenic peanut leaves inoculated with *Cercospora arachidicola* and *Phaeoisariopsis personata*.

Table 4. Mean performance of transgenic, wild type, and other control lines for the appearance of lesions, number of lesions, and lesion size in detached leaf experiments for early leaf spot (ELS) and late leaf spot (LLS) diseases.

Plant no.	Appearance of lesions (days after inoculation)		Number of lesions		Lesion size (mm)	
	ELS	LLS	ELS	LLS	ELS	LLS
DEF.5	14.67 ± 0.33 b[z]	15.33 ± 0.33 b	1.33 ± 0.33 b	1.67 ± 0.33 b	2.27 ± 0.34 c	2.27 ± 0.62 b
DEF.7	16.33 ± 0.33 a	16.67 ± 0.67 a	1.67 ± 0.33 b	3.67 ± 0.88 b	2.07 ± 0.22 c	2.70 ± 0.21 b
DEF.35	16.33 ± 0.33 a	16.33 ± 0.33 ab	1.33 ± 0.33 b	3.00 ± 0.58 b	2.37 ± 0.15 c	2.63 ± 0.27b
DEF.47	16.67 ± 0.33 a	16.33 ± 0.33 ab	2.00 ± 0.58 b	3.33 ± 0.88 b	2.60 ± 0.23 c	2.77 ± 0.77 b
DEF.68	16.00 ± 0.58 a	17.00 ± 0.58 a	2.33 ± 0.67 b	3.67 ± 0.88 b	2.57 ± 0.22 c	2.90 ± 0.49 b
TMV 2	8.33 ± 0.33 d	8.67 ± 0.33 c	6.00 ± 0.58 a	11.00 ± 0.58 a	7.50 ± 0.17 a	7.33 ± 0.15 a
JL 24	9.33 ± 0.33 d	8.33 ± 0.33 c	5.67 ± 0.33 a	12.67 ± 0.33 a	6.67 ± 0.33 b	7.37 ± 0.09 a
GG 20	10.67 ± 0.33 c	8.33 ± 0.33 c	6.33 ± 0.67 a	12.33 ± 0.88 a	7.13 ± 0.26 ab	7.47 ± 0.12 a
LSD (0.05)	1.12	1.27	1.50	2.12	0.74	1.24

The data are mean of three replicates ± SE. [z]Means followed by the same lower case letters within a column are not significantly different at P ≤ 0.05, by Tukey's studentized comparison test.

To our knowledge, this is the first report of transgenic peanut development using a fusion defensin gene imparting resistance to both ELS and LLS diseases. Similar to these observations, potatoes (*Solanum tuberosum*) transformed with the alfalfa antifungal peptide defensin (*alfAFP*) and *Nicotiana megalosiphon* defensin (*NmDef02*) were found to have elevated resistance to *Verticillium dahliae* (Gao et al., 2000) and *Phytophthora infestans* (Portieles et al., 2010) as compared with the untransformed lines. A similarly constitutive expression of *Dahlia mercki* defensin (*DmAMP1*) in rice and Chinese cabbage defensin (*BSD1*) in tobacco exhibited improved

resistance against *Magnoporthae oryzae* and *Rhizoctonia solani* (Jha et al., 2009) and *Phytopthora parasitica* (Park et al., 2002) pathogens, respectively, as compared with controls. These observations indicate that the fusion gene was functional and capable of imparting a higher level of resistance to a wide host range of fungal pathogens. These results reiterate the fact that, through genetic engineering strategies, defensin peptides can enhance plants' resistance to different pathogens.

Defensins, like *NaD1* and *TvD1*, isolated from *Nicotiana alata* and *Tephrosia villosa*, respectively, have been shown to enhance both disease and insect resistance in plants (Lay et al., 2003; Vijayan et al., 2013). On a similar note, transgenic tobacco plants expressing *Tfgd2* and *RsAFP2* defensin fusion gene also exhibited resistance against both an insect pest (*Spodoptera litura*) and fungal diseases (*Rhizoctonia solani, Phytopthora parasitica* var. *nicotianae*) (Vasavirama and Kirti, 2013). Therefore, future studies focusing on the defensin fusion gene transgenic peanut should evaluate the effect of its peptide combination on a range of biotic stresses (including insect pests).

As reported for various plant species (Gao et al., 2000; Portieles et al., 2010), our results also confirmed the potential implementation of the defensin fusion gene, through transgenic approaches, for imparting *C. arachidicola* and *P. personata* fungal disease resistance in peanut. This also reconfirms the findings reported by Vasavirama and Kirti (2011) that a fusion gene for defensins can be successfully used for imparting multiple disease resistance in peanut.

Most plant defensins do possess some antimicrobial activity, although the exact mode of action remains unconfirmed. However, we know that defensins show little homology in their amino acid sequences and are considered as part of a superfamily of similarly folded peptides possessing antifungal activity (Thomma et al., 2002). Moreover, defensins have been shown to have peptide promiscuity whereby different conditions (protein concentrations or pH) can change the function of any peptide (Franco, 2011), which can be linked to the evolution of peptides with several functions related to plant defenses. However, it is reported that defensins bind to the sphingolipids, which are fungal membrane specific receptors, and facilitate fungal inhibition via fungal membrane permeabilization (Thevissen et al., 1999). These findings would pave the way towards further investigations into the various possible biological activities of the fusion protein that would provide insight into the exact mode of action of these defensins in peanut.

In years to come, when whole genome sequence data become available, other high end molecular profiling tools and datasets will be available for cultivated peanut, and it will be possible to evaluate the presence and function of various defensin peptides in this species. All of the above cited factors might be reasons for getting multiple disease resistance in peanut when defensin fusion gene transgenic lines were analyzed for resistance against ELS and LLS diseases. However, the exact mechanism of action of the defensin fusion gene product in imparting resistance against a range of fungal pathogens needs further studies, which is beyond the purview of this investigation.

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