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Disease resistance conferred by the expression of a gene encoding a synthetic peptide in transgenic cotton (*Gossypium hirsutum* L.) plants

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Summary

Fertile, transgenic cotton plants expressing the synthetic antimicrobial peptide, D4E1, were produced through *Agrobacterium*-mediated transformation. PCR products and Southern blots confirmed integration of the *D4E1* gene, while RT-PCR of cotton RNA confirmed the presence of *D4E1* transcripts. *In vitro* assays with crude leaf protein extracts from T0 and T1 plants confirmed that *D4E1* was expressed at sufficient levels to inhibit the growth of *Fusarium verticillioides* and *Verticillium dahliae* compared to extracts from negative control plants transformed with pBI-d35SΩ-*uid*A-nos (CGUS). Although *in vitro* assays did not show control of pre-germinated spores of *Aspergillus flavus*, bioassays with cotton seeds *in situ* or *in planta*, inoculated with a GFP-expressing *A. flavus*, indicated that the transgenic cotton seeds inhibited extensive colonization and spread by the fungus in cotyledons and seed coats. *In planta* assays with the fungal pathogen, *Thielaviopsis basicola,* which causes black root rot in cotton, showed typical symptoms such as black discoloration and constriction on hypocotyls, reduced branching of roots in CGUS negative control T1 seedlings, while transgenic T1 seedlings showed a significant reduction in disease symptoms and increased seedling fresh weight, demonstrating tolerance to the fungal pathogen. Significant advantages of synthetic peptides in developing transgenic crop plants that are resistant to diseases and mycotoxin-causing fungal pathogens are highlighted in this report.

Introduction

The average annual cotton production loss as a result of diseases in the United States is about 12% and it is higher in other cotton-growing areas of the world. The seedling disease complex, fungal wilt pathogens and boll rots are the major cotton diseases worldwide. Cottonseed is also attacked by the saprophytic fungus, *Aspergillus flavus* that produces aflatoxin. Aflatoxin, one of the deadliest mycotoxins known, is produced by the fungus on other crops as well such as corn, peanuts and tree nuts (Sinha and Bhatnagar, 1998). The presence of aflatoxin in cottonseed endangers the health of livestock consuming cottonseed meal used in animal feeds and the health of humans consuming milk products from the affected livestock. The objective of our research is to transform cotton with a gene encoding a synthetic antimicrobial peptide (D4E1) in order to prevent or minimize pre-harvest contamination of cottonseed by the aflatoxigenic fungus, *A. flavus* and provide resistance or tolerance to other phytopathogens. We chose this antimicrobial peptide because synthetic analogues such as *D4E1* (FKLRAKIKVRLRAKIKL), compared to naturally occurring antimicrobial peptides, offer more target specificity

and increased efficacy at lower concentrations (Cary *et al*., 2000a). This synthetic peptide has also been shown to inhibit further development of pre-germinated conidia of *A. flavus*, *Fusarium* and other phytopathogens, including bacterial pathogens, at low concentrations (Rajasekaran *et al*., 2001) and to be more resistant to degradation by fungal and plant proteases than natural peptides (De Lucca *et al*., 1998). Moreover, we have demonstrated that crude protein extracts from leaf tissue of transgenic tobacco plants expressing the synthetic peptide *D4E1* gene significantly reduced *in vitro* the number of fungal colonies arising from germinated conidia of *A. flavus* and *Verticillium dahliae*, and showed greater levels of disease resistance *in planta* to the fungal pathogen, *Colletotrichum destructivum*, which causes anthracnose (Cary *et al*., 2000a). In this study, we report the development of transgenic cotton lines with enhanced resistance *in vitro* and *in planta* to several fungal pathogens including *A. flavus* as a result of expression of a synthetic peptide gene.

Results

Transformation of cotton

More than 50 independent, putatively transformed lines were produced on sub-lethal levels of the antibiotic G418 (10 μg/mL) and they were subjected to selection at higher levels of G418 (40 μg/mL) to reduce the number of escapes after embryogenic callus was obtained from each colony. Transgenic plants were produced from PCR-positive embryogenic callus lines 10–15 months after culture initiation (data not shown). All of the transgenic cotton plants carrying the *D4E1* gene were morphologically similar to non-transformed or pBI-d35SΩ-*uid*A-nos-transformed negative controls (CGUS) with respect to growth, leaf shape and flowering. There was no difference between controls and the transgenic cotton plants with regard to seed set. Four T0 plants transformed with the *D4E1* gene, labelled C357, C358, C373 and C374, and their T1 progenies were assayed for disease resistance along with the CGUS negative control plants. Analyses of T1 progeny plants are provided in this report.

Southern blot analysis

*Eco*RI digested genomic DNA from four transgenic T1 plants, C357, C358, C373 and C374, that had exhibited antifungal activity *in vitro* and a non-transformed Coker 312 plant was probed with a radiolabelled *Hin*dIII-*Sac*I fragment of about 900 bp representing the d35SΩ-D4E1 DNA sequences. All four transgenics exhibited hybridization signals with the probe while the non-transformed control did not (Figure 1). Plants C373 and C374 each had one band of hybridization indicating one integration event, while C358 and C357 showed three and two hybridization signals respectively, indicating multiple integration events.

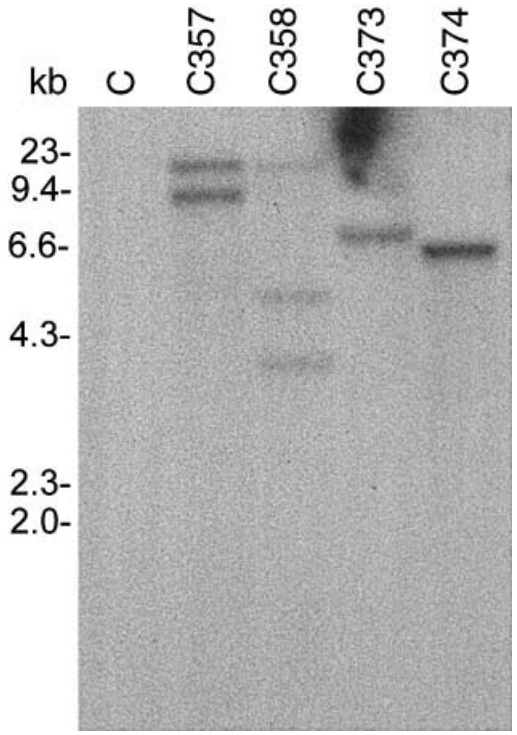

Figure 1 Southern hybridization of d35SΩ-D4E1 transgenic T1 cotton genomic DNA – Southern hybridization of genomic DNA of four cotton T1 plants transformed with pBI-d35SΩ-D4E1-nos and a non-transformed cotton plant (Lane C) was performed to demonstrate the successful integration of the transgene. The membrane was probed with a radiolabelled *Hin*dIII-*Sac*I fragment representing the d35SΩ-D4E1 region of approximately 900 bp.

PCR and RT-PCR analysis

The presence of the integrated T-DNA region in the four T1 transgenics containing the d35SΩ-D4E1 region was also confirmed by PCR amplification of transgenic plant genomic DNA (Figure 2). Using a primer internal to the d35SΩ promoter and to the 3′ end of the nopaline synthase terminator, all four T1 transgenic samples demonstrated a PCR product of the expected size of 460 bp. This was identical to the size of the product obtained upon amplification of the pBI-d35SΩ-D4E1-nos binary vector. No PCR products were obtained from amplification of genomic DNA from a CGUS negative control as expected. Subsequent subcloning and sequencing of the PCR products confirmed that the promoter

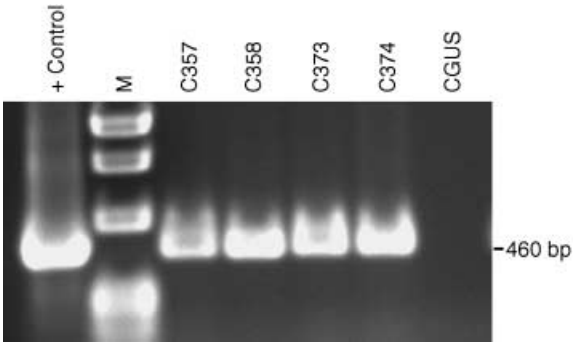

Figure 2 PCR of transgenic cotton genomic DNA – total DNA from transgenic cotton was amplified with primers designed to an approximate 460-bp region spanning from within the d35S promoter to the 3′ end of the nos terminator. Lane CGUS represents PCR amplification using pBI-d35SΩ-*uid*A-nos genomic template DNA (negative control). Lane 'M' represents *Hae*III digested *phi*X-174 marker DNA; +-control denotes pBI-d35SΩ-D4E1-nos plasmid DNA-positive control.

and *D4E1* gene regions were authentic and properly fused (data not shown). Because of the small size of the expected *D4E1* transcripts, RT-PCR of isolated total RNA from the four T1 transgenics and control plants was performed to demonstrate expression of the *D4E1* gene (Figure 3). Primers were based on the 5′ end of the *D4E1* gene and the 3′ end of *D4E1* including 20 bp of the nopaline synthase terminator region. PCR amplification of cDNAs from all four transgenic plants and the plasmid control template yielded the expected product of 79 bp while the CGUS negative control plant did not generate a product. DNA sequence analysis showed that the sequence of the PCR inserts from the four transgenics was identical to that of the region amplified from the pBI-d35SΩ-D4E1-nos binary vector control (data not shown).

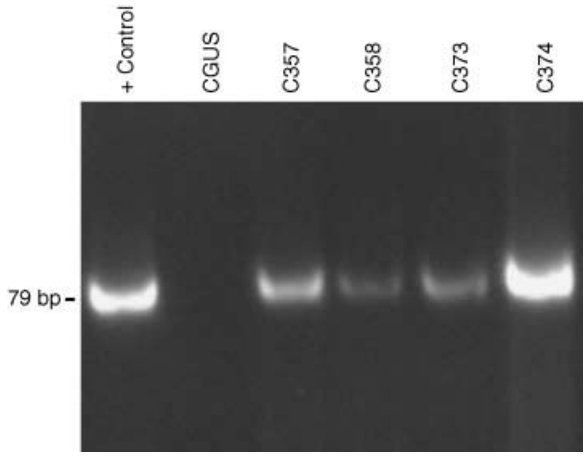

Figure 3 RT-PCR of total RNA from the four T1 transgenic lines and a CGUS negative control plant was performed to demonstrate expression of the *D4E1* gene. Primers were based on the 5′ end of the *D4E1* gene and the 3′ end of *D4E1* including 20 bp of the nopaline synthase terminator region. PCR amplification of cDNAs from all four transgenic lines and the positive plasmid (pBI-d35SΩ-D4E1-nos) control template (+ control) yielded the expected product of 79 bp while the CGUS negative controls did not generate a product.

In vitro assay of antifungal activity

Plant extracts from each transformed young cotton plant significantly reduced ($P < 0.05$) the number of fungal colonies arising from germinating conidia of *Fusarium verticillioides* and *V. dahliae* compared to the extracts from the CGUS negative controls (Figures 4a & b). However, there was no significant difference between controls and transgenics in reducing the number of colonies from germinating conidia of *A. flavus* (Figure 4c). Germinating conidia of *V. dahliae* were more susceptible to extracts from the transformed plants than the other two fungal species. Extracts from the transformed plants reduced the number of germinating conidia of *V. dahliae* by 40% to 75% compared to extracts from the CGUS negative control plants (Figure 4b). Up to 40% reduction in colony forming units of *F. verticillioides* was observed with extracts from transgenic cotton plants as compared to CGUS negative controls (Figure 4a).

In situ inoculation of detached immature cottonseed with *A. flavus* 70-GFP

All immature cotton seeds, inoculated *in situ* with *A. flavus* 70-GFP strain, showed extensive growth of *A. flavus* on the surface of the seed coats within 7 days of incubation (Figure 5a–c). However, upon dissection of seeds, extensive colonization of inner seed coat and cotyledons were observed in controls, but the transgenic seeds showed greatly reduced colonization by the fungus, thus demonstrating that the transgenic cottonseeds are capable of delaying and reducing the pre-harvest contamination by *A. flavus* (Figure 5d–g). Cotyledons from inoculated seeds were collected, and the fluorescence from their extract was measured at excitation 485 nm/emission 535 nm. Cotyledons from transgenic seeds showed significantly less (40–80%) fluorescence than the controls, indicating a reduction in colonization of transgenic seeds by *A. flavus* 70-GFP strain (Figure 6). Significant reduction in the growth and colonization of seed coats by the fungus was also observed, as measured by the fluorescence and visual rating (data not provided).

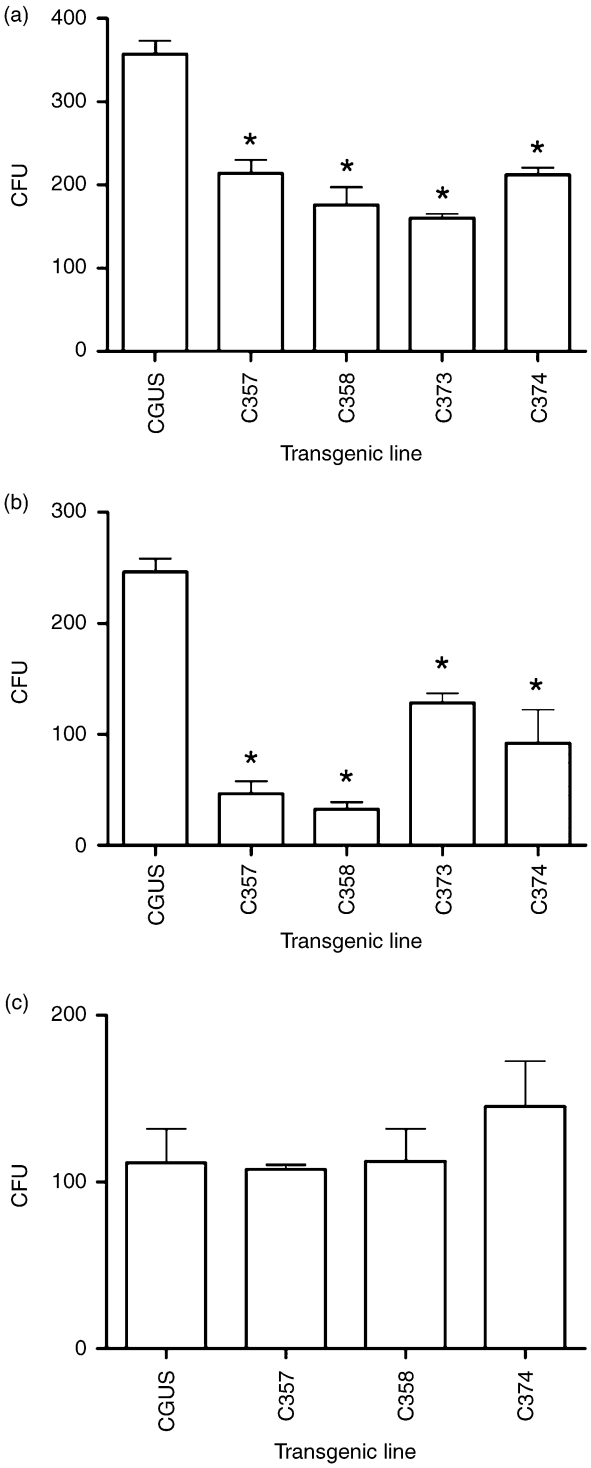

Figure 4 Antifungal assays *in vitro* – (a) Inhibition of growth of pre-germinated conidia of *Fusarium verticillioides* by exposure to leaf extracts from T1 cotton plants expressing the antifungal peptide *D4E1* gene for 1 h (*) denotes a significant reduction ($P < 0.05$) in the number of *F. verticillioides* colonies compared to extracts of CGUS negative control. (b) Inhibition of growth of pre-germinated conidia of *Verticillium dahliae* by exposure to leaf extracts from cotton plants expressing the antifungal peptide *D4E1* gene for 1 h (*) denotes a significant reduction ($P < 0.01$) in the number of *V. dahliae* colonies compared to extracts of CGUS. (c) Lack of significant inhibition ($P < 0.05$) of growth of pre-germinated conidia of *Aspergillus flavus* by exposure to leaf extracts from cotton plants expressing the antifungal peptide *D4E1* gene for 1 h compared to CGUS. C373 was not included in this assay. Error bars indicate standard error of means. Following ANOVA, mean separation was performed using the method of Tukey (Sokal and Rohlf, 2004).

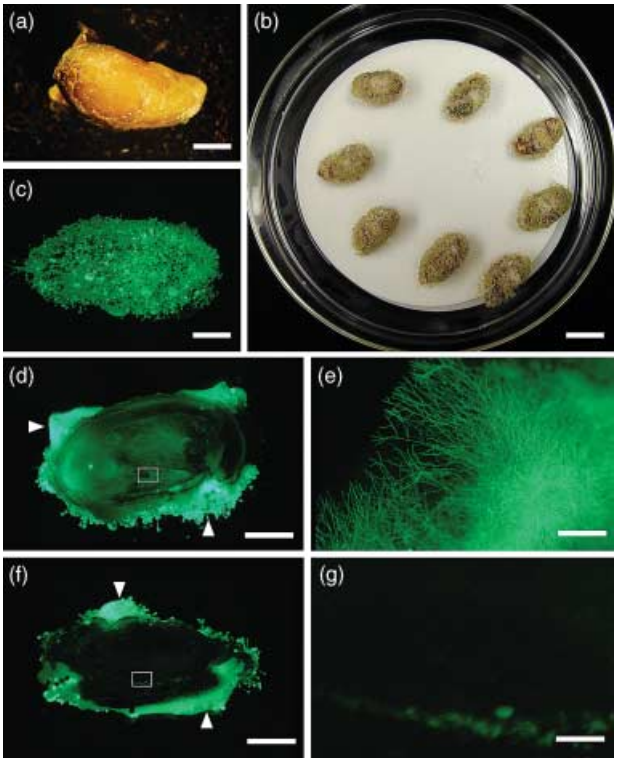

Figure 5 Inhibition assay *in situ* with immature cottonseeds infected with *Aspergillus flavus* 70-GFP. (a) An immature T1 cottonseed 28 dpa devoid of fibers prior to inoculation with *A. flavus* 70-GFP. (b) Growth of *A. flavus* 70-GFP on immature seeds of cotton 7 days after inoculation – picture taken under visible light. (c) Fluorescence due to growth of *A. flavus* 70-GFP on an immature seed. (d) Fluorescence due to *A. flavus* 70-GFP on the inner seed coat of the CGUS negative control; note the rim of bright greenish yellow fluorescence on the outer seed coat (arrows). (e) Small area marked in D is magnified to show the extensive growth of the fungal mycelium growing though the tissue. (f) Lack of abundant fluorescence due to *A. flavus*-GFP on the inner seed coat of the transgenic line C374 although bright fluorescence on the outer seed coat is visible on the outer rim (arrow). (g) Small area marked in F is magnified to show reduction in fungal colonization. Scale bars indicate 40 mm in a, c, d and f; 100 mm in B; 2 mm in E and F.

Boll inoculation *in planta* with *A. flavus* 70-GFP

A. flavus 70-GFP was allowed to colonize T1 cotton bolls for 3 weeks. About 40% of the bolls that were inoculated with *A. flavus* 70-GFP dropped prematurely and were discarded. The remaining bolls (four to six from each transgenic line) with mature seeds (*c.* 50 dpa) were collected after 3 weeks of colonization under greenhouse conditions and analysed for the spread of *A. flavus* 70-GFP locule by locule, and seeds

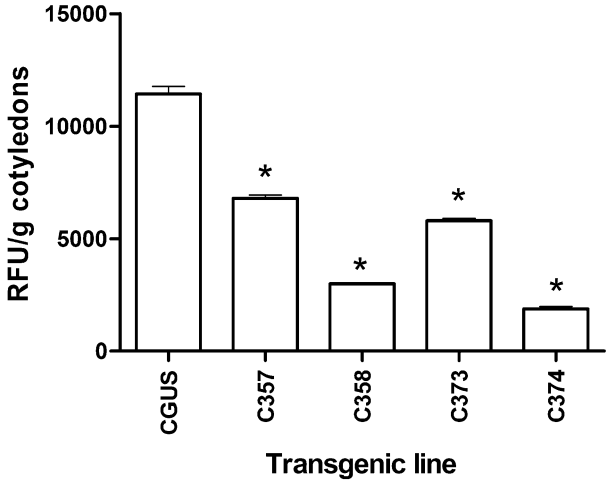

Figure 6 Growth of *Aspergillus flavus* 70-GFP on cotyledons of immature T1 cotton seed 7 days after inoculation as measured by the fluorescence emanating from the fungus. (*) denotes a significant reduction ($P < 0.05$) in the fluorescence in seed cotyledons of transgenic lines compared to that of CGUS negative control seed cotyledons. Error bars indicate standard error of means. Mean separation was performed using the method of Tukey following ANOVA.

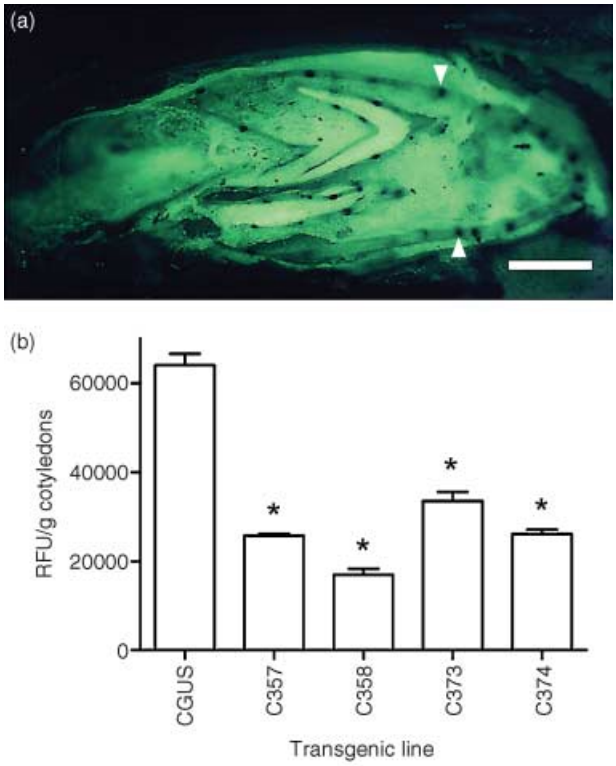

Figure 7 Inhibition assay *in planta* with T1 cotton bolls infected with *Aspergillus flavus* 70-GFP. (a) Fluorescence micrograph showing colonization of mature cottonseed cotyledons by *A. flavus* 70-GFP. Gossypol glands are visible as dark spots (arrows). (b) Growth of *A. flavus* 70-GFP on cotyledons of mature cotton seed 21 days after inoculation as measured by the fluorescence emanating from the fungus. (*) denotes a significant reduction ($P < 0.05$) in the fluorescence in seed cotyledons of transgenic lines compared to that of CGUS negative control seed cotyledons. Error bars indicate standard error of means. Mean separation was performed using the method of Tukey following ANOVA.

were examined under fluorescent microscope (Figure 7a). Diagonally opposite locules with reference to the inoculated locules had minimal infection by *A. flavus*. To determine the extent of damage by the colonization of *A. flavus*, seeds were collected from each boll, bulked and ground in liquid nitrogen. Fluorescence as a result of the *A. flavus* 70-GFP was measured from aliquots in phosphate buffer. A significant 50–75% reduction in fungal growth, as measured by the fluorescence was observed in transgenic seed compared to the CGUS control (Figure 7b).

In planta antifungal assays

In planta assays with the phytopathogen, *Thielaviopsis basicola*, that causes black root rot in emerging T1 seedlings, indicated that transgenic seedlings were more vigorous and less prone to damage by the fungus compared to CGUS negative controls (Figure 8a–c). There was no difference in germination rate in the absence of the fungal pathogen; however, in trays containing *T. basicola*, most of the control seedlings were weak and were 5–7 days behind in germination because of the presence of the fungal inoculum in the soil. All the seedlings were evaluated for black discoloration on hypocotyls and roots, branching of roots and, in severe cases, constricted hypocotyls with loss of cortical tissue and lack of vigour (Wang and Davis, 1997). A greater percentage (40–60%) of transgenic progeny seedlings escaped severe damage (disease severity score > 2) by *T. basicola*, whereas more than 70% of the control seedlings (expressing the GUS gene only) succumbed to the disease (Figure 8c). On average, control seedlings showed a mean severity score of 3.4 (out of 4) and the average severity score for transgenic seedlings was 2.6 (data not provided). The fresh weight of transgenic seedlings was significantly higher than that of controls (Figure 8d).

Discussion

We have demonstrated in this report that genetic transformation of cotton plants with the synthetic peptide *D4E1* gene confers antifungal traits. Crude protein extracts from leaves of transgenic cotton plants inhibited the colony development from germinated spores of *F. verticillioides* and *V. dahliae* (Figures 4a & 4b), two of the most common fungi that cause seedling disease complex and wilt in cotton. In contrast, *A. flavus*, the causal fungus for aflatoxin contamination in

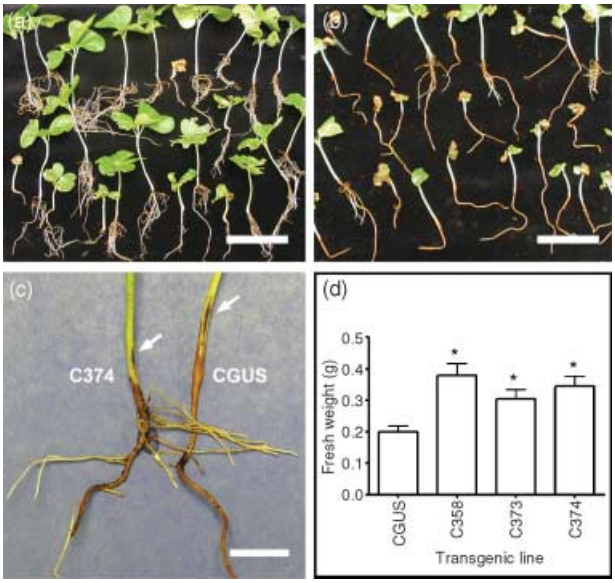

Figure 8 Antifungal assay *in planta* with T1 progeny seedlings of transgenic cotton plants expressing the synthetic peptide *D4E1* gene against a seedling pathogen, *Thielaviopsis basicola*. (a) Seedlings of C374 showing healthy root system and increased vigor. (b) Seedlings of CGUS negative controls – note the reduction in vigor and black discoloration on hypocotyls and roots with reduced or no branching in CGUS negative control seedlings. (c) A close-up of the typical black root rot lesions caused by *T. basicola,* on cotton seedling hypocotyls (arrows) of C374 and CGUS negative control two weeks after planting in soil inoculated with the fungus. Note lack of root branching on discolored primary root of controls compared to C374. (d) Fresh weight of seedlings (n = *c.* 150) grown for two weeks on inoculated soil with *T. basicola*. Transgenic cotton line C357 was not included in this assay. Note significant ($P < 0.05$) increase in fresh weight of seedlings from *D4E1*-transgenic lines compared to CGUS negative controls. Scale bars indicate 60 mm in a, and b; 15 mm in c.

cottonseeds was not controlled by leaf extracts (Figure 4c). However, *in situ* and *in planta* bioassays with *A. flavus*-70 GFP showed that the transgenic cotton plants expressing *D4E1* are capable of controlling the growth and spread of the fungus in cotyledons of cottonseed in a reproducible manner in both immature, detached seeds (Figures 5 & 6) and mature, greenhouse-grown seeds (Figure 7). *Aspergillus* spp. are saprophytes and do not follow the postulates of host–pathogen relationship as many phytopathogens do. Availability of the GFP strain was extremely valuable in our studies to understand the mode of infection and spread of the fungus inside cotton bolls and seeds (Rajasekaran *et al*., 1999; Cary *et al*., 2000b). Transgenic cotton progeny seedlings also showed significant tolerance *in planta* to *T. basicola*, one of the fungal pathogens that causes seedling disease complex and black root rot (Figure 8).

There have been several reports on expression of naturally-occurring peptides and their analogs conferring antimicrobial traits in transgenic plants (Hightower *et al*., 1994; Florack *et al*., 1995; Terras *et al*., 1995; Huang *et al*., 1997; Arce *et al*., 1999; Bi *et al*., 1999; Reynoird *et al*., 1999; Li, Q.S., *et al*., 2001; Zasloff, 2002) including cotton (Emani *et al*., 2003). However, in comparison to synthetic peptides, they do not offer target specificity, increased efficacy at lower concentrations and reduced degradation in the cytoplasmic milieu by plant proteases (Jaynes *et al*., 1993; Rao, 1995; Broekaert *et al*., 1997; Cavallarin *et al*., 1998; Zasloff, 2002). Synthetic peptides such as *D4E1* offer rapid biocontrol or biostatic ability against a wide range of fungal and bacterial pathogens at low concentrations and are non-toxic to mammalian and animal cells (Jaynes *et al*., 1989). Haemolytic activity against mammalian red blood cells was found to be extremely low for the synthetic peptide *D4E1* at levels toxic to phytopathogens (J. M. Jaynes, unpublished). We have demonstrated in our laboratory that the pure synthetic peptide *D4E1* is inhibitory to growth of about 20 bacterial and fungal phytopathogens (Rajasekaran *et al*., 2001). In addition, transgenic tobacco plants expressing *D4E1* demonstrated in bioassays a significant reduction in fungal growth *in vitro* and *in planta* (Cary *et al*., 2000a).

We could not detect *D4E1* peptide via Western or enzyme-linked immunosorbent assay (ELISA) because of the amphipathic characteristics of the peptide that lead to aggregation and inability of the peptide to migrate into an SDS-PAGE gel. In addition, a high level of background as a result of the use of the *D4E1* polyclonal antibody precluded unequivocal ELISA or Western detection, similar to the experience of several other researchers working with small antimicrobial peptides (DeGray *et al*., 2001; Li, Q.S. *et al*., 2001; Li, Z.J. *et al*., 2001).

The modes of action of antifungal peptides have been studied extensively (De Lucca and Walsh, 1999; Zasloff, 2002). Those that interact specifically with the lipid components of cell membranes often cause formation of pores or ion channels that result in leakage of essential cellular minerals or metabolites or dissipate ion gradients in cell membranes. Other peptides have been shown to inhibit chitin synthase or β-D-glucan synthase. The synthetic peptide *D4E1* complexes with ergosterol, a sterol present in germinating conidia of a number of fungal species, suggesting that its mode of action is lytic (De Lucca *et al*., 1998). *D4E1* has been shown to take on a β-sheet conformation in solution or during interaction with cell membranes, which is in contrast to cecropin A, and magainins which assume α-helical structures upon binding to acidic phospholipid bilayers. In addition, *D4E1* was shown to be more resistant to fungal and cotton leaf proteases than cecropin A (De Lucca *et al*., 1998). However, results in our laboratory on the effect of non-transformed control tobacco or cotton plant extracts, spiked

with different levels of *D4E1*, did not correlate with *in vitro* antifungal effects of purified *D4E1*. Moreover, addition of plant protease inhibitors to tobacco plant extracts prior to spiking with *D4E1* did not enhance the antifungal effect of the peptide (Cary *et al*., 2000a). The interaction of amphipathic peptides among themselves and with biological membranes is a complex phenomenon that is not well understood. Further research is needed to elucidate the mode of action *in planta* of this antimicrobial peptide.

Results from transgenic plants were similar to the results from *in vitro* antifungal assays using purified *D4E1* (Rajasekaran *et al*., 2001). We demonstrated that phytopathogens that are sensitive to *D4E1* are inhibited *in vitro* to a greater extent in bioassays than *A. flavus*, which requires a higher IC50 concentration for effective control. For example, IC50 values for sensitive pathogens such as *F. verticillioides*, *V. dahliae*, T. basicola are 0.88, 0.60 and 0.52 μM of *D4E1*, respectively, whereas for *A. flavus* it is 7.8–11.0 μM. Based on our experience with various types of bioassays we concur that *in planta* assays are more meaningful, although *in vitro* assays provide relative efficacy levels of antifungal proteins in a short time frame. In the current study, *in situ* and *in planta* analyses indicated that cotton plants expressing the *D4E1* gene are capable of inhibiting the spread and colonization of *A. flavus* in cottonseed (Figures 5, 6 & 7), although *in vitro* analyses suggested otherwise (Figure 4c). It is conceivable that higher expression levels of the antifungal peptide are required to control saprophytic fungi such as *Aspergillus* spp. Performance of these transgenic cotton plants under field conditions with regard to reducing aflatoxin contamination in cottonseed by *A. flavus* is yet to be evaluated. Increased transgene expression levels are preferred to control a wide range of phytopathogens; for example, we are currently evaluating antifungal gene expression through plastid transformation (DeGray *et al*., 2001; Kumar *et al*., 2004) for effective control of phytopathogens including mycotoxin-producing fungi.

Experimental procedures

Agrobacterium constructs and transformation of cotton seedling explants

The pBI-d35SΩ-D4E1-nos binary vector was introduced into *Agrobacterium tumefaciens* strain LBA 4404 (Gibco-BRL, Bethesda, MD) as described previously (Cary *et al*., 2000a). *Agrobacterium* cultures for transformation experiments were initiated in 50 mL of YEB liquid medium using frozen glycerol stocks (500 μL) as inoculum. These cultures were grown overnight for about 18 h at 26 ± 2 °C on a gyratory shaker. The optical density (A600) values were adjusted to 0.6–0.8 in liquid MS medium prior to use. *Agrobacterium*-mediated transformation of seedling explants of cotton (*Gossypium hirsutum* L. cv. Coker 312) and regeneration of fertile, transgenic plants were carried out according to the published protocols (Rajasekaran *et al*., 1996; Rajasekaran, 2004). Plants transformed with the pBI-d35SΩ-*uid*A-nos construct (CGUS) served as controls. All the primary transformants and their progenies were grown in a greenhouse until maturity.

Analysis of transgenic plant DNA

To determine if the CaMV d35SΩ-D4E1 region was integrated into the cotton genome, a 460-bp segment of DNA encompassing the CaMV d35S promoter-D4E1-nos terminator region was PCR amplified from genomic DNA of transgenic plants. Genomic DNA was isolated from 12 transgenic cotton leaf disks using the REDExtract-N-Amp Plant PCR kit (Sigma, St. Louis, MO). Oligonucleotide primers designed to an internal region of the CaMV d35S promoter, 5′-ATGACGCACAATCCCACTATCCTTC-3′ and to an internal region of the nos terminator, 5′-CTAGTAACATAGATGTCTCCGCGC-3′ were used to amplify cotton plant genomic DNA using Ampli*Taq* Gold polymerase (Applied Biosystems). The following thermocycler (PTC-100, MJ Research, Inc., Watertown, MA) parameters were used: 1 cycle of 95 °C, 10 min; 60 °C, 1 min; 72 °C, 30 s; 34 cycles of 95 °C, 1 min; 65 °C, 1 min; 72 °C, 30 s; and a final extension of 72 °C for 7 min. PCR products obtained were analysed by 1% agarose gel electrophoresis followed by ethidium bromide staining. Validity of PCR products was determined by DNA sequencing.

Genomic DNA for Southern hybridization analysis was isolated and purified using the QIAGEN DNeasy Plant Mini Kit (Qiagen, Valencia, CA). Cotton genomic DNA (20 μg) was completely digested with the restriction endonuclease *Eco*RI and separated by electrophoresis on a 1% agarose gel. DNA fragments were transferred to Nytran Plus nylon membranes (Schleicher and Schuell, Inc., Keene, NH) by vacuum blotting. The nylon membranes were hybridized with a random-primed (Rediprime II Kit; Amersham Biosciences; Piscataway, NJ), ^{32}P-dCTP-labelled d35SΩ promoter-*D4E1 Hin*dIII-*Sac*II fragment. Hybridization was performed overnight at 42 °C in ULTRAhyb Buffer (Ambion Inc., Austin, TX) followed by two 5-min washes in 2 × SSC/0.1% SDS at 42 °C, and a final wash for 15 min in 0.1 × SSC/0.1% SDS at 42 °C. Nylon membranes and Kodak X-OMAT AR autoradiography film (Eastman Kodak, Rochester, NY) were placed between 'intensifying screens' and exposed overnight at −80 °C.

RT-PCR of transgenic cotton RNA

Because of the inherent difficulty of identifying the extremely small *D4E1* transcript using standard Northern hybridization protocols, reverse transcription – polymerase chain reaction (RT-PCR) amplification of total RNA was used to detect the presence of these small transcripts. Total RNA was purified using the QIAGEN RNeasy Plant Mini Kit (Qiagen, Valencia, CA). RNase-free DNase (Qiagen) was used to ensure complete removal of DNA from RNA samples prior to RT-PCR. Reverse transcription and first strand cDNA synthesis of cotton RNA was performed using the Advantage RT- for-PCR Kit (Clontech, Palo Alto, CA). The antisense oligonucleotide primer was designed to the 5′ end of the nos transcriptional terminator sequence including the *Sac*I site (underlined) that linked it with the 3′ end of the *D4E1* coding sequence (5′-ATCGGGGAAATTCGAGCTCTTACAAC-3′). The sense PCR primer represented the 5′ end of the *D4E1* coding region starting with the *Nco*I site (underlined) used to link it with the d35SΩ promoter (5′-CCATGGGATTTAAGTTGAGAGCTAA-GATTA-3′). Using Ampli*Taq* Gold polymerase first strand cDNA representing the *D4E1* transcript was amplified using the following thermocycler parameters: 95 °C, 10 min; 60 °C, 1 min; 72 °C, 30 s; 34 cycles at 95 °C, 1 min; 65 °C, 1 min; 72 °C, 30 s; and a final extension of 72 °C, 7 min. PCR products were separated by electrophoresis in a 10% polyacrylamide gel and their validity was confirmed by DNA sequencing.

DNA sequencing

PCR and RT-PCR generated products were subcloned into plasmid vector pCR 2.1-TOPO (Invitrogen, Carlsbad, CA). The nucleotide sequence of the subcloned PCR products was determined by non-radioactive sequencing using the ABI PRISM 377 Automated DNA Sequencer (Applied Biosystems, Foster City, CA) using standard –21 M13 forward and M13 reverse primers. Sequence information was analysed using DNAMAN (Lynon Biosoft: Quebec, Canada) analysis software.

In vitro analysis of antifungal activity of plant extracts to *A. flavus*, *F. verticillioides* and *V. dahliae*

The inhibitory activity of extracts from cotton plants transformed with *D4E1* gene was assessed *in vitro* following the method of Cary *et al*. (2000a). Briefly, conidial suspensions were prepared from cultures grown on potato dextrose agar (PDA; Difco, Detroit, MI) (PDA) slants for 7 days at 30 °C (*A. flavus*, *F. verticillioides*) or 22 °C (*V. dahliae*). Conidial suspensions in 1% (w/v) potato dextrose broth (PDB, pH 6.0) were adjusted to a density of 10^5 conidia/mL and were germinated in PDB for 8 h at 30 °C (*A. flavus, F. verticillioides*) or overnight at 22 °C (*V. dahliae*) prior to assay.

Plant homogenates were prepared by directly grinding cotton leaves into a fine powder in liquid nitrogen with no buffer added. Ground tissues were then centrifuged at 11 700 ***g*** for 10 min at room temperature and extract collected from each sample. Conidial suspensions (25 μl) were then added to 225 μl of plant extract, mixed, and incubated for 1 h at 30 °C (*A. flavus*, *F. verticillioides*) or 22 °C (*V. dahliae*). Three 50 μl aliquots from each sample were then spread on to PDA plates and incubated at 30 °C or 22 °C for 24–48 h and fungal colonies counted. One-way ANOVA was used to determine the significance of the effect of transgenic plant extracts on germinating conidia. Mean separations were performed using the method of Tukey (Sokal and Rohlf, 1981) using the GRAPHPAD PRISM software.

In situ inoculation of detached cottonseed with *A. flavus* 70-GFP

Construction and transformation of the GFP expression vector

The EGFP gene (ClonTech) was placed under control of the constitutively expressed *A. nidulans* glyceraldehyde phosphate dehydrogenase (*gpd*A) gene promoter and the *Aspergillus parasiticus nmt*-1 gene transcriptional terminator. All of these elements were subcloned into the plasmid vector pBlueScript-SK (Stratagene) to produce the vector *gpd*-EGFP (Rajasekaran *et al*., 1999; Cary *et al*., 2000b). Plasmid *gpd*-EGFP was cotransformed with the vector pSL82 harbouring the *A. parasiticus nia*D gene into the *nia*D mutant of *A. flavus* 70. One isolate stably expressing high levels of GFP, designated *A. flavus* 70-GFP, was used in all experiments.

In situ *inoculation*

A. flavus 70-GFP was grown for 7 days at 30 °C on MEA media before assay. Conidia were harvested by flooding a single plate with 9 mL of 0.01% (v/v) sterile Triton X-100 solution and scraping the surface of mycelium with a sterile pipette, and were used at 2×10^4 conidia/mL for seed inoculation studies. Immature cottonseeds (25–28 days postanthesis, dpa) from greenhouse-grown bolls were prepared by removing fibres from seed coats. About 100 conidia (5 μL) were introduced into immature seed through a needle wound of approximately 3–5 mm depth. A minimum of 10 seeds per plate and three replicates were used per transgenic event. The Petri dishes were sealed with two layers of parafilm to

maintain high humidity, and were incubated at 28 °C for 7 days. Each seed was sliced longitudinally and the cotyledons removed for separate analysis. The seed coat was examined for GFP fluorescence of mycelium using the Olympus SZH10 GFP-stereomicroscope and the amount of the inner seed coat colonized by *A. flavus* 70-GFP was estimated using the following scale: 0 = 0% of inner seed coat colonized with fluorescent mycelium; 1 = 1–25% of inner seed coat colonized; 2 = 26–50% of inner seed coat colonized; 3 = 51–75% of inner seed coat colonized; 4 = >76% of inner seed coat colonized.

Fungal colonization was also quantitatively assessed as follows: Cotyledons were separated and ground in liquid N2. Five hundred μL of phosphate buffer (50 mM, pH = 7.2) was added to 1 g of powdered seed coat tissue or cotyledons and mixed well by vortexing. The samples were centrifuged at 11 700 ***g*** for 10 min in a swing bucket rotor. One hundred μL aliquots of supernatant from each sample were placed in a 96-well HP Viewplate and the fluorescence was measured at an excitation wavelength at 485 nm and an emission wavelength of 535 nm using the Perkin-Elmer HTS 7000 fluorometer. Average fluorescence values were obtained for each sample and were subjected to nonparametric ANOVA using the GRAPHPAD PRISM software.

In planta bioassays

Boll inoculation with A. flavus-*70 GFP strain*

Six to eight cotton bolls (25–28 dpa) in each of greenhouse-grown control and transgenic plants were wounded in the centre of one of the locules to a depth of 5–10 mm with a 3 mm diameter cork borer. A small aliquot (10 μL) of the *A. flavus* 70-GFP suspension (10^4 conidia/mL) was dropped into the hole, and the fungus was allowed to colonize bolls for 3 weeks. Seeds from each boll (50 dpa) were harvested separately and ground in phosphate buffer (pH = 7.2; 50 mM), vortexed and centrifuged for 10 min at 11 700 ***g*** with a swing bucket rotor. One hundred μL of supernatant was removed and placed in a 96-well microtitre plate (black View Plate, HP). GFP fluorescence output because *A. flavus* 70-GFP was expressed per g of seed cotyledon tissue.

Cotton seedling soil inoculation assay with Thielaviopsis basicola

Resistance to *T. basicola*, the causal agent of black root rot in cotton seedlings, was evaluated according to the published protocol (Wang and Davis, 1997). T1 seeds were soaked overnight in water and the seed coats were removed. Seeds were then planted in a nursery tray (30 cm × 30 cm × 9 cm) containing pre-autoclaved soil (Pro-Mix) well-mixed with two PDA plates of *T. basicola* cultures. The noninoculated control treatment consisted of plain PDA plates blended with water and mixed with soil. Twenty-five seeds were planted in each tray and 3–4 trays were made for each of controls and transgenic lines. All tray flats were placed in growth chamber set at 24 °C (day) and 18 °C (night) for 2 weeks and the soil was kept moist. Germination rate and individual seedling weight after 2 weeks of planting were recorded and subjected to statistical analysis (ANOVA). Disease severity ratings scale for *T. basicola* was assigned to each seedling according to the procedures of (Wang and Davis, 1997).

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