

Development of a GFP-Expressing *Aspergillus flavus* Strain to Study Fungal Invasion, Colonization, and Resistance in Cottonseed

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Abstract Cotton bolls were inoculated with a green fluorescent protein (GFP)-expressing *Aspergillus flavus* (strain 70) to monitor fungal growth, mode of entry, colonization of cottonseeds, and production of aflatoxins. The GFP strain and the wild-type did not differ significantly in pathogen aggressiveness as indicated by similar reductions in inoculated locule weight. GFP fluorescence was at least 10 times higher than the blue green yellow fluorescence (BGYF) produced in response to infection by *A. flavus*. The GFP produced by the strain made it possible to identify and monitor specific plant tissues colonized by the fungus. For example, the inner seed coat and cotyledon were colonized by the fungus within 72 h of inoculation and the mode of entry was invariably through the porous chalazal cap in intact seeds. The amount of GFP fluorescence was shown to be an indicator of fungal growth, colonization and, to some extent, aflatoxin production. The *A. flavus* strain expressing GFP should

be very useful for rapidly identifying cotton lines with enhanced resistance to *A. flavus* colonization developed through genetic engineering or traditional plant breeding. In addition, development of GFP expressing *A. flavus* strain provides an easy and rapid assay procedure for studying the ecology, etiology, and epidemiology of cotton boll rot caused by *A. flavus* resulting in aflatoxin contamination.

Keywords Aflatoxin · Antifungal genes ·
Aspergillus flavus · Cotton ·
Green fluorescent protein · Transgenic plants

Introduction

The saprophytic soil-borne fungus *Aspergillus flavus* produces the carcinogenic aflatoxins in lipid-rich seeds of corn, cotton, peanuts, and tree nuts. Aflatoxins frequently contaminate these agricultural commodities during plant growth, storage, and handling. Several monographs have been published on fungal growth, colonization, mycotoxin production, and contamination of food and feed supplies by *Aspergillus* species [1, 2]. Aflatoxin contamination of cottonseed is thought to occur in two phases. The first phase of infection and aflatoxin contamination occurs prior to seed maturity and is often associated with insect damage to the boll, particularly by pink boll worm [1, 3–5]. The second phase in aflatoxin contamination of cottonseeds occurs after seed maturity and boll opening, and involves direct

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infection of seed by *A. flavus* [4, 5]. This process is favored by moist, warm conditions during boll maturation, harvesting, and storage.

After analyzing cotton plants inoculated at cotyledonary leaf scars, unopened flower buds, and involucrel nectaries or the peduncle of newly opened flowers for aflatoxin contamination in cotton bolls, Klich and her colleagues [6–8] concluded that the fungus might gain entry through the vascular bundles, primarily through xylem vessels. Hamsa and Ayres [9] observed that hyphal invasion in stored cottonseeds proceeded via chalaza, micropyle, and cracks that developed in the seed coat during ginning. Likewise, after microscopic analyses of contaminated cottonseeds, Lee et al. [10] and Huizar et al. [8] hypothesized that the fungus may enter through non-lignified layers of the seed coat. However, clear evidence regarding entry of the fungus and the infection process during pre- or post-harvest phases of contamination has not been available from these studies. In this study, we were interested in determining if the fungus interacts with the cotton fiber and to what extent; point of penetration of the fungus into the seed; and the time course and extent of invasion as well as toxin formation in the seed.

The green fluorescent protein (GFP) requires only UV or blue light and oxygen and does not require destructive sampling or substrates or cofactors for localization of activity as required for other reporter genes such as β -glucuronidase. *Gfp*-expressing fungi, including that of *A. flavus*, have been used to track fungal growth, localization, distribution, and colonization in plant tissues [11]. Recent examples include screening of corn genotypes for resistance to *A. flavus* and aflatoxin accumulation [12] and tracking infection patterns by *gfp*-expressing *Fusarium graminearum* in barley and *Arabidopsis* [13]. In the present study, an

aflatoxigenic *A. flavus* strain was engineered to express the GFP from the jellyfish *Aequorea victoria*. We utilized this strain as a visual marker to understand the mode of entry, fungal growth, and colonization by *A. flavus* in cottonseed during boll and seed development. We also utilized this *A. flavus* strain to successfully evaluate, both qualitatively and quantitatively, resistance to the fungus by cottonseed from transgenic plants expressing the antifungal protein CPO-P [14, 15] or the synthetic peptide D4E1 [16–18].

Materials and Methods

Fungal Cultures

Aspergillus flavus 70 (ATCC # MYA-384) and *A. flavus* 70-GFP were grown for 7 d at 30°C on maltose extract agar (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. The conidial suspension (Fig. 1a–c) was adjusted to the appropriate concentration with the aid of a hemocytometer. Bright fluorescence was observed from both conidiophores and mycelia of the *A. flavus* 70-GFP strain with even more fluorescence from conidiophores (Fig. 1a–c).

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 (*gpdA*) gene

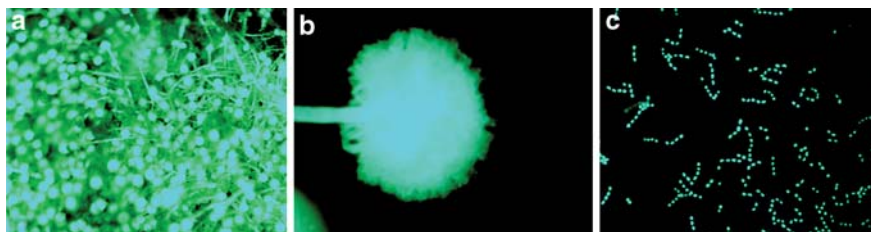


Fig. 1 Seven-day-old cultures of *Aspergillus flavus* 70-GFP with highly fluorescent mycelia and conidiophores (a, b). Conidial suspension (c) obtained from the cultures was used for

inoculation of cotton bolls and seeds. Photographs were taken in a dark room with a 1-s exposure time



Fig. 2 Vector for expressing green fluorescent protein in *Aspergillus flavus*

promoter [19] and the *A. parasiticus nmt-1* gene transcriptional terminator [20]. All of these elements were subcloned into the plasmid vector pBlueScript-SK (Stratagene) to produce the vector *gpd-EGFP*. Plasmid *gpd-EGFP* was cotransformed with the vector pSL82 harboring the *A. parasiticus niaD* gene into the *niaD* mutant of *A. flavus* 70 (Fig. 2). One isolate stably expressing high levels of GFP, designated *A. flavus* 70-GFP, was used in all experiments.

Inoculation of Cottonseed and Bolls

Detached Mature Boll Inoculation

In order to obtain bolls with lint not previously colonized by microbes, unopened bolls of *G. hirsutum* var. Coker 312 approaching maturity (40 days post anthesis, dpa) were harvested from the field prior to opening and placed in a forced-air drying oven (40°C) in individual cotton bags. Bolls opening within 7 d were used in tests. Intact open bolls were misted until run-off with a spore suspension containing 3.7×10^5 spores per ml of the *A. flavus* 70-EGFP. Bolls were incubated in dark at 100% humidity, 31°C for 0–240 h. Upon completion of the incubation period, bolls were placed in individual cotton bags and dried in a forced-air oven at 50°C for 3–5 d for aflatoxin analysis. Boll locules were stored in sealed plastic bags at 4°C until analyzed for GFP activity.

Boll Inoculation in Planta

Twenty cotton bolls (28 dpa) in each of greenhouse-grown cotton plants (var. Coker 312) were wounded in the center of one of the locules to a depth of 5–10 mm with a 3 mm diameter cork borer to mimic bollworm exit holes. A small aliquot (10 µl) of the *A. flavus* 70-GFP suspension (10^4 conidia/ml) was pipetted 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 8,200g with a swing bucket rotor. One hundred microliters of supernatant was removed and used for fluorescence measurement as described below.

Inoculation In situ of Immature Seeds

Immature cottonseeds (28 dpa) from greenhouse-grown bolls from transgenic plants expressing the bacterial chloroperoxidase gene (*cpo-p*) [15] or a gene encoding the synthetic peptide D4E1 [18] and controls (transformed with *uidA* only) were prepared by removing fibers 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 11 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 d. Each seed was sliced longitudinally and the cotyledons were removed for fluorescence measurement as described below.

GFP Analysis of Fungal Colonization

Infected bolls, locules, seeds, and tissues were analyzed for GFP fluorescence under UV light in a dark room or blue light with an Olympus SZH10 GFP-stereomicroscope (excitation 480 nm; emission 535 nm). Digital photographs were taken with a Nikon DXM1200 camera operated with the software ACT-1 (Nikon, USA). The amount of the inner seed coat colonized by *A. flavus* 70-GFP was estimated (fungal incidence or severity of fungal infection spread) 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. Seeds were observed under the stereomicroscope and the fungal incidence was recorded from each seed.

Fungal colonization in cotyledons was also quantitatively assessed by measuring GFP fluorescence from the fungus as follows: Cotyledons were separated and ground in liquid nitrogen. Five hundred

microliters 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 $8,200 \times g$ for 10 min in a swing bucket rotor. One hundred microliters 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 HTS 7000 fluorometer (Perkin-Elmer). Average fluorescence values ($n = 5$) were obtained for each sample and were subjected to non-parametric ANOVA using the GraphPad Prism software.

Aflatoxin Analysis

Aflatoxin B1 + B2 concentrations in inoculated locules were determined as previously described [21]. Briefly, intact locules were pulverized and extracted with an 85% aqueous acetone solution. Extracts were purified, partitioned against methylene chloride, and concentrated. Concentrates and aflatoxin standards were separated on TLC plates (silica gel 60, 250 μm) by development with diethyl ether–methanol–water (96:3:1). Extracts were either concentrated or diluted to permit accurate densitometry and aflatoxin B1 + B2 was quantified [22] with a scanning densitometer (Model CS-390, Shimadzu Scientific Instruments, Inc., Tokyo).

Results

Infection of Cotton Bolls

Bolls of greenhouse-grown plants were wound-inoculated with the wild-type *A. flavus* 70 and its derivative 70-GFP to study the infection process in planta. Bolls were collected after 3 weeks (50 dpa) and analyzed for fungal infection and aggressiveness and toxin production. Detached mature bolls sprayed with *A. flavus* spores were also analyzed for fungal spread and colonization. Regardless of the mode of inoculation (wound- or open boll-inoculation), fungal spread and colonization was rapid and was observed in cotton locules, intercarpellary membranes, seed coat, and cotyledons. Results indicated that *A. flavus* 70 and 70-GFP did not differ significantly ($P < 0.05$)

in pathogenic aggressiveness as determined by the fresh weight of inoculated locules normalized to gram/seed (Fig. 3).

Fluorometric output was also measured from inoculated locules (seeds and fiber) to distinguish GFP-related fluorescence from the blue green yellow fluorescence (BGYF) emanating from cotton bolls infected with *A. flavus* [23] or any other autofluorescence emanating from tissues such as cotyledons. Locules inoculated with *A. flavus* GFP fluoresced at least 10 times greater than locules inoculated with the wild-type *A. flavus* 70, indicating that fluorescence caused by GFP could be easily distinguished from autofluorescence caused by BGYF using the measurement parameters reported in this study (Fig. 4). Similarly, weak autofluorescence from seed cotyledon tissue did not pose any problem in differentiating the bright fluorescence from *A. flavus* 70-GFP infected cotyledons. Moreover, fluorescence from *A. flavus* 70-GFP could be detected in a reproducible manner from a minimum hyphal mass of less than 5 mg (Fig. 5).

Fungal Growth, Colonization and Mode of Entry into Cottonseeds

More than 400 mature seeds from either lab-inoculated open bolls (Fig. 6a, b) or wound-inoculated, greenhouse-grown mature bolls (Fig. 6c) were analyzed under UV light or using the Olympus stereomicroscope fitted with GFP filters. Inoculated bolls were colonized by the fungus within 4–7 d.

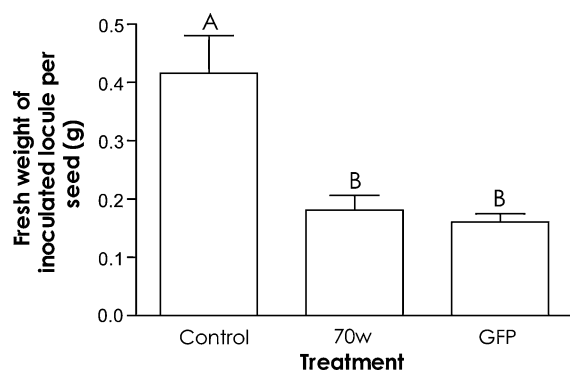


Fig. 3 Fresh weight of inoculated bolls as affected by *Aspergillus*. *A. flavus* strain 70 and strain 70-GFP did not differ significantly ($P < 0.05$) in pathogenic aggressiveness

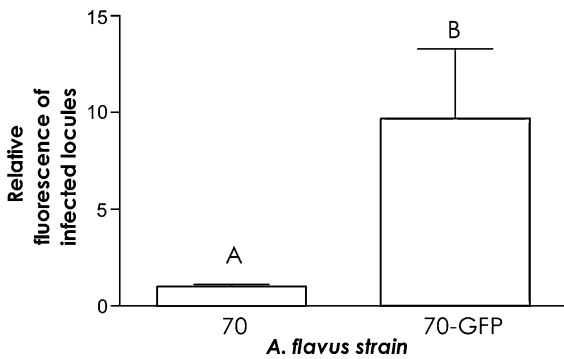


Fig. 4 Locules (seed and fiber) inoculated with *A. flavus* 70-GFP fluoresced 9.7 times greater than locules infected with the parental strain *A. flavus* 70, indicating that fluorescence caused by GFP could be distinguished from fluorescence caused by BGYP using the measurement parameters reported in this study ($P < 0.01$)

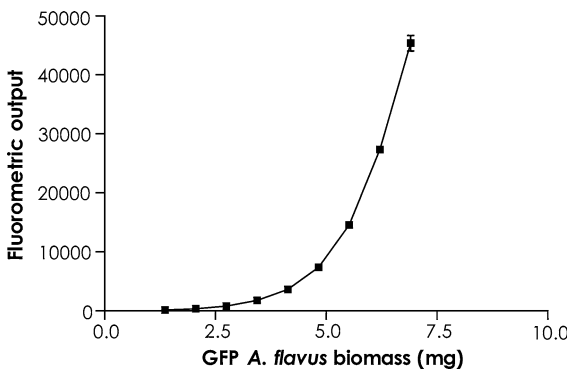


Fig. 5 Relationship between fungal mass and fluorescence output from 7-day-old cultures

Formation of abundant conidiophores and spores were observed on locule surfaces and on infected seeds (Fig. 6d, e). Growth of the fungus on the intercarpellary membrane separating the locules was also observed within 2–3 days of boll inoculation (Fig. 6f). Although dispersed mycelial growth and conidiation was observed among cotton fibers (Fig. 6g), none was observed inside the lumen of the cotton fiber. Sclerotia formation was observed on cotton locule surface (Fig. 6h) similar to formation on potato dextrose agar media (not shown). High levels of GFP were detected in immature sclerotia that form on the surface of locule fibers at about 96 h post infection. Immature sclerotia were highly fluorescent and melanization of maturing sclerotia was observed to proceed in a polar fashion from basal end to the growing end. GFP activity was quenched until no

further fluorescence could be detected in mature, fully melanized sclerotia (Fig. 6h). No fungal growth was observed inside the placenta, the source of vascular connection for seed nourishment and growth. In wound-inoculated bolls, the fungal spread was absent in locules opposite to the inoculated locule. The locules adjacent to the inoculated locule showed signs of fungal spread such as BGYP yellow fibers, infected seeds, and cotyledons (Fig. 6i).

The progression of *A. flavus* 70-GFP into cottonseed was monitored under the GFP microscope. Weak autofluorescence from uninfected cotyledons (Fig. 7b) did not interfere with fluorescence measurements in *A. flavus* 70-GFP infected cotyledons (Fig. 4). Compared to uninfected control cotyledons (Fig. 7a, b), infected cotyledons (Fig. 7c–f) were highly fluorescent and in severe cases of infection often resembled a spongy, almost liquid mass due to the action of hydrolytic enzymes produced during fungal growth and were later replaced completely by fungal conidia and sclerotia (not shown). Penetration into the intact seed coat invariably occurred at the chalazal end within 48 h after inoculation (Fig. 7c) and the colonization progressed toward the micropylar end within 2–3 d after entry through chalazal cap (Fig. 7d, e) leading to total colonization within 7 d resulting in highly fluorescent (300-fold higher compared to uninfected cotyledon control), watery cotyledons (Figs. 7f and 8). GFP in the cotyledon could be detected at about 48 h (Fig. 7d) followed closely by aflatoxin production beginning at about 72 h and reaching high levels (200 ppm) by 168 h (Fig. 8). A positive correlation was observed between GFP fluorescence from the fungal strain and aflatoxin levels in cottonseed ($R^2 = 0.85$).

Evaluation of Antifungal Activity in Transgenic Cottonseed

Isolated immature seeds from non-transgenic control and transgenic cotton plants expressing the antifungal genes, *cpo-p* or D4E1, were inoculated with *A. flavus* 70-GFP and assayed for fluorescence as a function of fungal growth in situ and the disease incidence scored. Seeds from transgenic plants expressing the antifungal genes showed more than 50% reduction in GFP fluorescence indicating reduced fungal growth in cotyledons of transgenic seeds compared to controls

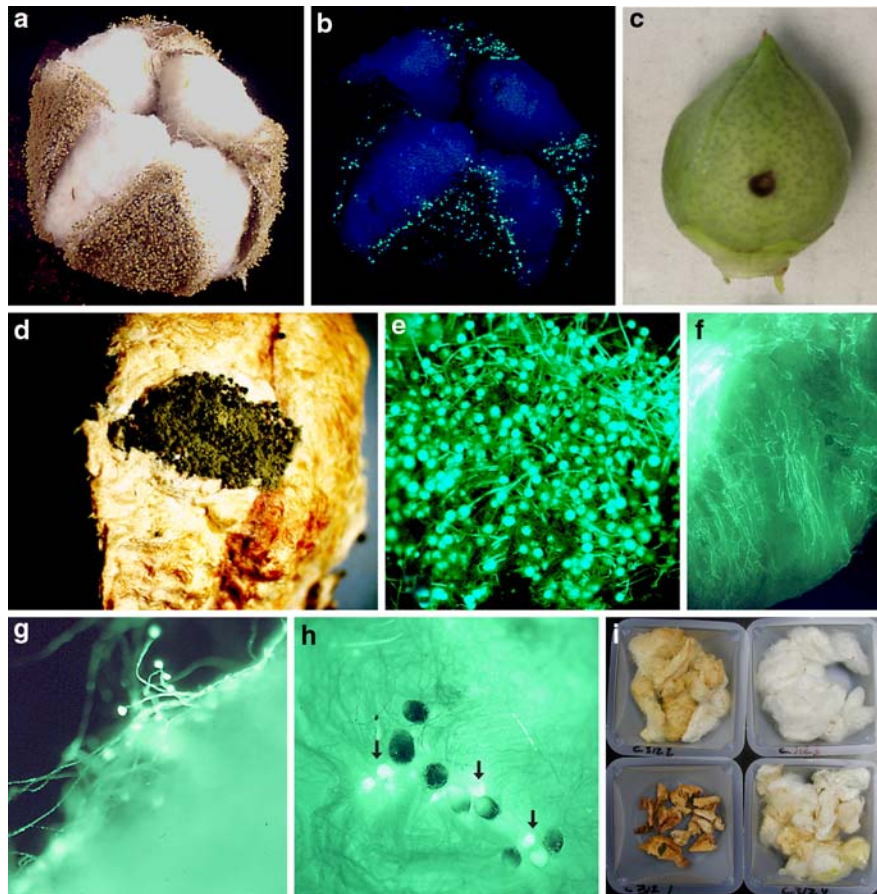


Fig. 6 Cotton boll inoculated with *A. flavus* 70-GFP under white light (**a**) and ultraviolet light (**b**). Note green fluorescence of fungal conidia on carpels. Inoculation of 28 dpa cotton bolls in planta mimicking boll worm exit hole (**c**); inoculated locule showing fungal contamination within a week (**d**); fluorescence of *A. flavus* 70-GFP at the inoculated site on the locule (**e**); growth of the fungus on the intercarpellary membrane separating the locules (**f**); formation of conidiophores on the locules by the fungus (**g**); formation of sclerotia on cotton locules (**h**) similar to formation on potato dextrose agar plates

(not shown). Note progression of melanization from bright fluorescence of immature sclerotia (arrows) to none in melanized, mature sclerotia. The fluorescence in the background is due to BGYF from fibers due to *Aspergillus* infection and also from the GFP-expressing fungus, not in focus. Spread of infection in wound-inoculated cotton boll showing the spread from the inoculated locule (bottom left) to adjacent locules (**i**). Note the yellow-stained fiber from the adjacent locules, the source of BGYF. GFP-fluorescence photographs (**e–h**) were taken in a dark room with a 1-s exposure time

(Table 1). The incidence of fungal spread was also significantly lower in transgenic cottonseeds compared to controls (Table 1).

Discussion

Using an *A. flavus* strain expressing the GFP gene we have demonstrated the infection process by the fungus in cottonseed and bolls. GFP production in *A. flavus* did not interfere with pathogen aggressiveness as determined in boll inoculation experiments

(Fig. 3) or its ability to produce aflatoxins (Fig. 8). Boll inoculation experiments also indicated that fluorescence due to GFP could be distinguished from fluorescence due to BGYF using the correct GFP filter combination in the fluorometer. The detached seed colonization assay indicated that the GFP-expressing *A. flavus* should be very useful for rapidly identifying cotton lines with enhanced resistance to *A. flavus* colonization developed through genetic engineering or traditional plant breeding. In addition, it is also possible to identify plant parts that are naturally resistant or susceptible to fungal invasion by

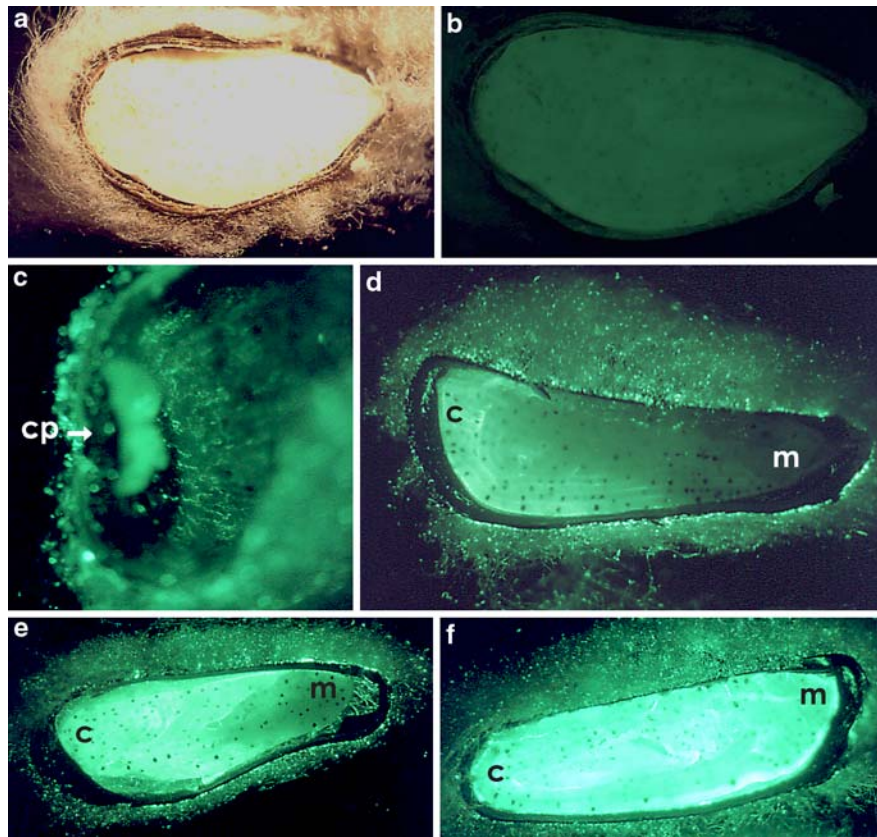


Fig. 7 Examination of infection and progression of the GFP-expressing fungus into seed cotyledon. Longitudinal sections of uninfected control cottonseed under white light (**a**) and GFP fluorescence (**b**); note the autofluorescence from cotyledon. Penetration of the fungus into intact seeds occurs through the chalazal plug (cp) and the infection spreads from the chalazal end (**c**) to the micropylar (m) end of the cottonseed. Gradual

progression of fungal infection and colonization of cotyledons after 48 h (**c, d**), 96 h (**e**), and 168 h (**f**) of boll inoculation. Autofluorescence (BGYF) from cotton fibers due to *Aspergillus* infection dotted with fluorescent conidia from the GFP-expressing *A. flavus* is also visible in **c–f**. All photographs were taken in a dark room with a 1-s exposure time

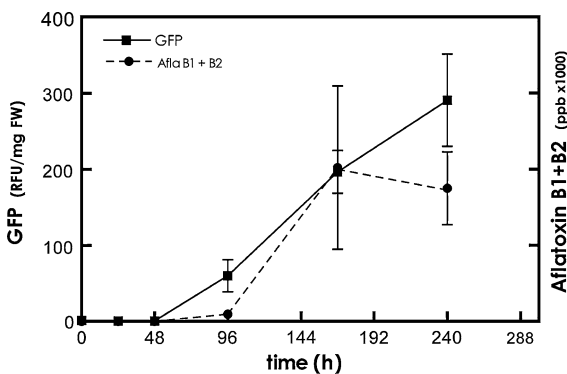


Fig. 8 GFP fluorescence and aflatoxin B1 + B2 concentrations in cottonseed inoculated with the GFP-expressing *A. flavus* after 0–240 h of inoculation

analyzing the growth and colonization pattern of the fungus and this would be useful in our research on tissue-specific expression of transgenes. The availability of the *A. flavus*-GFP strain allowed us to examine the infection process in real time without destructive sampling.

Preharvest aflatoxin contamination of cottonseed due to *A. flavus* infection occurs either through the insect exit holes in cotton bolls or after the boll opening [4, 5]. Regardless of the mode of entry, the fungal infection inside the boll and cottonseed could be observed within 48 h after inoculation (Fig. 6f–i and 7d). In addition, we have established, using fluorescence microscopy of cottonseeds inoculated with the *gfp*-expressing *A. flavus*, that the fungus

Table 1 Colonization of *A. flavus* 70-GFP in control and transgenic cottonseed expressing the antifungal peptide D4E1 or chloroperoxidase (cpo-p) as measured by the GFP fluorescence

Plant ID ^a	Incidence of severely infected seeds ^b	Cotyledon fluorescence ^c
Control (GUS gene)	0.49 ± 0.05	100 ± 0.75
Transformed with D4E1 gene	0.07 ± 0.07**	28.75 ± 12.26**
Transformed with cpo-p gene	0.09 ± 0.09**	55.42 ± 14.85**

**Significant difference ($P < 0.05$) in disease incidence or cotyledon fluorescence compared to the control as determined by the Wilcoxin Rank-Sum test

^a Each plant tested least three times. Thirty-three immature seeds (28 dpa)/plant were assayed in each experiment

^b Severely infected seeds classified as those seeds scored as 2 or higher in the fungal infection spread scale

^c Cotyledon fluorescence (relative to control) determined using Perkin-Elmer HTS 7000 fluorometer with excitation at 485 nm and emission at 535 nm

gained entry in intact seeds via the chalazal cap under preharvest conditions. Cottonseed imbibes water during germination through the chalazal plug and this region of the seed coat is anatomically different from the rest of the seed coat in that it is comprised of loosely packed, branched parenchyma cells and abundant intercellular spaces [24–27]. We have shown that *A. flavus* enters through the porous chalazal cap cells first and the infection proceeds rapidly from the chalazal end to the micropylar end (Fig. 7c–f) leading to total colonization of seed cotyledons within 7 days after the fungal entry into cotton bolls.

Using the *A. flavus* 70-GFP we have also successfully demonstrated both in situ and in planta the antifungal efficacy of transgenic cotton plants (Table 1) expressing the bacterial chloroperoxidase [15] or the synthetic peptide D4E1 gene [18]. Often the level of inhibition of fungal growth is difficult to determine by plate counts of fungal colonies following exposure to extracts of transgenic plant tissues expressing antifungal proteins or peptides. The sensitivity of GFP fluorescence detection makes it possible to measure even small changes in the amount of fungal growth both in vitro and in planta and to evaluate inhibitory activities of unknown antifungal proteins or peptides [28, 29]. We and our

collaborators continue to use this rapid assay using the *gfp*-expressing *Aspergillus* strain in evaluating transgenic cottonseed, corn, and peanut for resistance to preharvest contamination of food and feed products with aflatoxins.

In summary, we have developed an easy and rapid assay using a *gfp*-expressing *A. flavus* to study the ecology, etiology, epidemiology of cotton boll rot caused by *A. flavus*, and aflatoxin contamination in cottonseed. This assay is equally useful in understanding the different modes of fungal entry and aflatoxin production in other susceptible crops as diverse as peanut, corn, and tree nuts from soil or airborne *Aspergillus*. In addition, we have shown a close relationship between GFP fluorescence emanating from the fungus to fungal growth and aflatoxin levels (Fig. 8). Our current analysis indicates a linear correlation ($R^2 = 0.855$) between the function of the *gpd* promoter driving expression of the *gfp* gene and aflatoxin production. We are currently in the process of evaluating other strains of *A. flavus* transformed with *gfp* driven by aflatoxin gene promoters such as *omtA* or *ver-1* [30, 31] so that aflatoxin levels can be directly correlated to GFP values.

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