Journal of proteome research

Article

pubs.acs.org/jpr

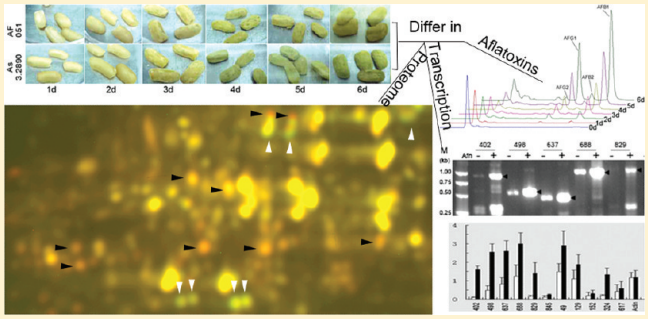
Proteomic Analysis Reveals an Aflatoxin-Triggered Immune Response in Cotyledons of *Arachis hypogaea* Infected with *Aspergillus flavus*

Zizhang Wang, Shijuan Yan, Chunming Liu, Fang Chen, and Tai Wang*

Key Laboratory of Plant Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China

Supporting Information

ABSTRACT: An immune response is triggered in host cells when host receptors recognize conserved molecular motifs, pathogen-associated molecular patterns (PAMPs), such as β-glucans, and chitin at the cell surface of a pathogen. Effector-triggered immunity occurs when pathogens deliver effectors into the host cell to suppress the first immune signaling. Using a differential proteomic approach, we identified an array of proteins responding to aflatoxins in cotyledons of peanut (*Arachis hypogaea*) infected with aflatoxin-producing (toxigenic) but not nonaflatoxin-producing (atoxigenic) strains of *Aspergillus flavus*. These proteins are involved in immune signaling and PAMP perception, DNA and RNA stabilization, induction of defense, innate immunity, hypersensitive response, biosynthesis of phytoalexins, cell wall responses, peptidoglycan assembly, penetration resistance, condensed tannin synthesis, detoxification, and metabolic regulation. Gene expression analysis confirmed the differential abundance of proteins in peanut cotyledons supplemented with aflatoxins, with or without infection with the atoxigenic strain. Similarly, peanut germination and *A. flavus* growth were altered in response to aflatoxin B1. These findings show an additional immunity initiated by aflatoxins. With the PAMP- and effector-triggered immune responses, this immunity constitutes the third immune response of the immune system in peanut cotyledon cells. The system is also a three-grade coevolution of plant−pathogen interaction.



KEYWORDS: *proteomic analysis, Arachis hypogaea, Aspergillus flavus, aflatoxin-triggered immune response*

INTRODUCTION

Fungi such as *Aspergillus flavus* and *Aspergillus parasiticus* produce diverse classes of secondary metabolites that yield aflatoxins.[1] However, the evolutionary source of these metabolites is not known[1,2] and the metabolites are not necessary for fungi growth or development.[1] Recently, trichothecene mycotoxins were found to have a possible advantage in invasive infection as virulence or pathogenicity factors.[3]

Aflatoxin B1 (AFB1) can covalently and readily bind to nucleic acids to form an AFB1−DNA adduct,[4] which results in G to T transversion mutations.[5,6] In a chromosome instability analysis in mouse, AFB1 treatment induced 58% loss of heterozygosity on chromosome 12.[7] Similarly, dietary intake of AFB1 was found associated with chromosome instability of allelic loss at arms of chromosome 13 in human.[8] Therefore, aflatoxins can cause DNA damage and chromosome instability in host cells. In addition, AFB1 is the most cytotoxic agent of the known mycotoxins: a 30 μM dose conferred 50% loss of viability of cultured Vero and BME-UV1 cell lines after 24 h exposure. AFB1 increases the DNA fragmentation and expression of p53 protein but decreases the level of bcl-2 protein, which points to an apoptotic process.[9,10]

Interactions between pathogenic microorganisms and plants have involved coevolution of invasion and defense strategies.[11] When plants perceive pathogens at the cell surface, recognition of pathogen-associated molecular patterns (PAMPs) initiates PAMP-triggered immunity (PTI). However, pathogens such as bacteria have evolved to secrete type III effector proteins into the plant cell via a type III secretion system (TTSS) to suppress PTI, thus allowing for pathogen growth and disease. Furthermore, plant cells have developed effector-triggered immunity (ETI) to survey the pathogen effector proteins by plant resistance (R) proteins and start resistance responses, including localized programmed cell death (PCD) and hypersensitive responses.[12] Otherwise, pathogens colonize plant tissue and cause infection after overcoming the multilayered plant defense response.

Unlike in bacteria, no TTSS has been found in fungal pathogens and no evidence has demonstrated characterized fungal effectors with roles in virulence or PTI suppression.[11] Most of the investigated fungal effectors are functionally unknown small proteins containing a signal peptide for

Received: November 6, 2011
Published: March 19, 2012

dx.doi.org/10.1021/pr201105d | J. Proteome Res. 2012, 11, 2739−2753

secretion into the host apoplast.[11] However, plant innate immunity responds to both bacterial and fungal invasion.[11] For immunity of fungal infections, PAMPs are major cell-wall components including β-glucans, chitin, and mannan.[13] They are mainly recognized by Toll-like receptors, C-type lectin receptors, galectin family proteins and receptors for advanced glycation end-products in mammals,[13] but not many receptors have been identified in plants. For the plant fungus *Magnaporthe grisea,* Avr-Pi-ta in rice functions as an effector to stimulate the ETI.[14]

In addition to producing effectors, many fungi produce a group of toxic perylenequinones for pathogenesis in plants.[15] One of these toxins, cercosporin, generates reactive oxygen species to cause peroxidation of plant cell membranes.[16,17] Disruption of fungal genes for cercosporin biosynthesis markedly reduced necrotic lesions in tobacco leaves.[18] Thus, at least some pathogenic metabolites are related to virulence in plants. However, distinguishing the effects of metabolites on biotic invasion is difficult during infection because when the metabolite is accumulating, the pathogen is always flourishing. Experiments designed to separate both pathways are essential to investigate the virulence of secondary metabolites.

We performed a differential comparison of proteomes from cotyledons of peanut (*Arachis hypogaea*) infected with atoxigenic and toxigenic *A. flavus* strains, which differed in accumulation of aflatoxins in the infection. Many differential proteins are involved in disease resistance in plants or detoxification in fungus. To confirm that the response is related to aflatoxins but not infection, we analyzed cotyledons imbibed in aqueous solution of aflatoxins for aflatoxin induction of peanut genes. Similarly, we analyzed both plant and fungal genes responding to aflatoxins in cotyledons infected with the atoxigenic *A. flavus* strain with or without exogenous aflatoxins. The expression pattern of all analyzed genes was consistent with the change in protein abundance seen on proteomic analysis. Therefore, both fungal and plant cells responded to aflatoxins.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions

Toxigenic *A. flavus* strain As 3.2890 was obtained from the China General Microbiological Culture Collection Center of the Institute of Microbiology, Chinese Academy of Sciences. It produced 2- to 20-fold increased aflatoxins than other strains.[19] Atoxigenic *A. flavus* strain AF051, with an 89.59-kb fragment deleted from its aflatoxin gene cluster,[20] was kindly provided by Professor Ma Zhonghua (Zhejiang University, China). Mycelia were cultivated on potato dextrose agar medium at 28 °C until they showed numerous sporulation. Spores were collected with use of sterile distilled water containing 0.05% Tween 20 and diluted for density measurements by hemeacytometry and ultraviolet spectrophotometry (Beckman Coulter Du 730, Pasadena, CA).

Infection and Aflatoxin Treatment

Seeds of *A. hypogaea* cv. Luhua 14, which is more susceptible to *A. flavus* than 3 other cultivated peanuts at the late stage of seed maturation[21] were decorticated and de-embryonated to obtain cotyledons, then sterilized with 5% sodium hypochlorite for 10 min, washed with sterile water, and soaked up with sterile filter paper. Cotyledons (4.0 g) were infected with a 1 mL suspension containing 1×10^6 spores and cultivated under aeration at 28 °C. Cotyledons (1.0 g) were imbibed in 1 mL aqueous solution containing 2.5 μg AFB1, B2, G1, and G2 (Sigma-Aldrich) each and incubated at 28 °C for 2 days; cotyledons treated with water were the control. Similarly, cotyledons (1.0 g) were infected with 1 mL atoxigenic *A. flavus* strain AF051 spore suspension containing 1×10^6 spores and 2.5 μg AFB1, B2, G1, and G2 each, and incubated at 28 °C for 2 days; cotyledons infected without aflatoxins were the control. Experiments were performed in triplicate. After treament, cotyledon samples were lyophilized, pulverized, and stored at −80 °C.

To analyze the peanut response to aflatoxin, 100 seeds (*cv.* Silihong) were treated by imbibing with 20 mL water containing 3 μg AFB1; seeds treated with water were the control. Similarly, to analyze the fungal response to aflatoxin, seeds were infected with AF051 or As 3.2890 strains by imbibing with 20 mL spore suspension containing 5×10^4 spores and 3 μg AFB1; seeds infected with *A. flavus* alone were controls. Seeds were incubated in Petri dishes 15 cm in diameter at 28 °C for up to 10 days; 20 mL of water was added to each dish daily. Germination of peanut seeds and growth of mycelium and conidia were analyzed during the incubation.

Aflatoxin Determination by Thin-layer Chromatography (TLC) and High-performance Liquid Chromatography (HPLC)

We transferred 100 mg of peanut cotyledon powder to 1.5 mL of extract buffer (methanol/chloroform/water = 5:2:1, v/v), which was vortexed, sonicated for 30 min, and centrifuged at 12000× *g* for 6 min. We supplemented 1 mL of supernatant with 0.4 mL of chloroform and 0.4 mL of water to re-extract. After centrifugation, 20 μL of chloroform phase was loaded onto TLC plates (Cat. No. 1.05626.0001, Merk KGaA 64271, Darmstadl, Germany). Aflatoxins was separated with use of a chloroform/acetone solvent system (9:1, v/v) and visualized under 365-nm-long ultraviolet wave. Aflatoxins were extracted from 100 mg of sample powder by use of 500 μL of 80% methanol and quantified with use of 10 μL of supernatant on HPLC (Agilent Technologies, 1200 series).

Protein Preparation and 2-D Gel Electrophoresis

Infected cotyledon samples (1.0 g) were ground to a fine powder in liquid nitrogen and further ground with 6 mL ice-cold extraction buffer, which was 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 20 mM NaCl, 5 mM $MgCl_2$, 10 mM DTT, 2 mM phenylmethanesulfonyl fluoride, 10 μg/mL leupeptin, 1 μg/mL pepstatin, and 1 tablet/50 mL protease inhibitor cocktail (Roche Applied Science). The congealed fat and the pellet were removed by centrifugation at 40000× *g* for 20 min at 4 °C. The supernatant was supplemented with chilled acetone to 80% (v/v) and incubated at −20 °C overnight to precipitate proteins. Proteins were pelleted by centrifugation at 18000× *g* for 20 min at 4 °C, air-dried to remove residual acetone, and dissolved in lysis buffer (7 M urea, 2 M thiourea, 2% CHAPs [w/v], 1% DTT [w/v], 0.8% Pharmalyte, pH 3−10). After debris removal by centrifugation at 18000× *g* for 20 min, protein contents were quantified with use of the 2-D Quant kit (GE Healthcare, Piscataway, NJ).

For 2-D gel electrophoresis (2-DE) combined with Coomassie Brilliant Blue (CBB) staining (2-DE-CBB), proteins (800 μg) were diluted with rehydration buffer (6 M urea, 2 M thiourea, 0.5% CHAPs [w/v], 20 mM DTT, 0.5% Pharmalyte, pH 3−10, 0.002% Bromophenol Blue) to 450 μL and loaded onto 24-cm linear gradient immobilized pH gradient (IPG) strips (pH 4−7) (GE Healthcare). Isoelectric focusing involved

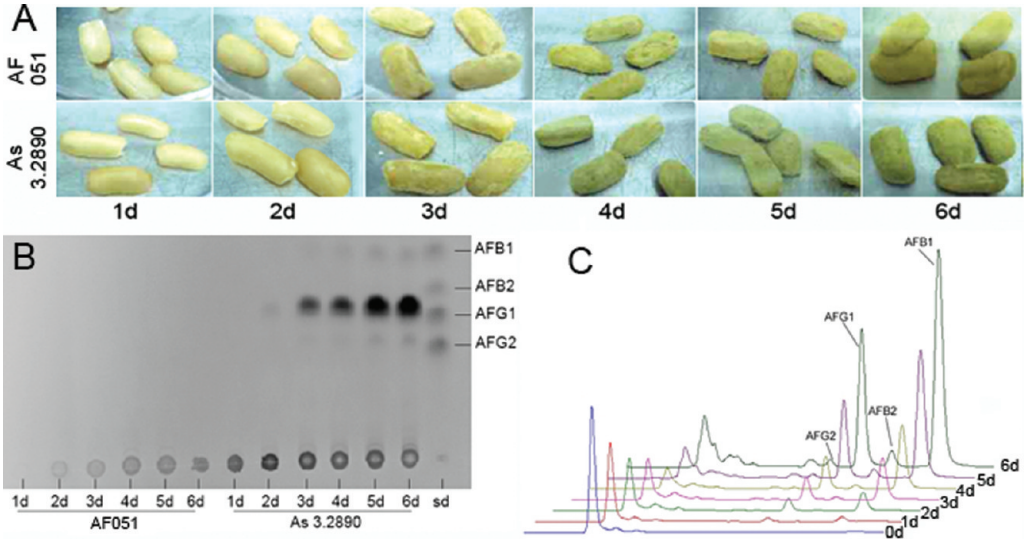


Figure 1. Fungal growth and aflatoxin production after infection of *Arachis hypogaea* cotyledons with toxigenic (As 3.2890) or atoxigenic (AF051) *Aspergillus flavus* strains. (A) Phenotypes of *A. hypogaea* cotyledons and *A. flavus* strains at days 1−6 after the infection; cotyledons were sterilized, infected with *A. flavus* suspension and aerate-cultivated at 28 °C. (B) Aflatoxin production after infection with the 2 *A. flavus* strains by thin-layer chromatography (TLC). (C) High-performance liquid chromatography (HPLC) determination of aflatoxins after infection with toxigenic strain. sd, standard; AFB1-AFG2, aflatoxin B1-G2.

use of the Ettan IPGphor isoelectric focusing system (GE Healthcare). SDS-PAGE involved use of the Ettan DALT Six electrophoresis unit (GE Healthcare), and gels were stained with 0.1% CBB R-250 and decolorized as described.[22] Images were obtained by scanning gels with ImageScanner (GE Healthcare) and analyzed by ImageMaster 2D platinum 5.0 (GE Healthcare).

For 2-D fluorescence-difference gel electrophoresis (2-D DIGE), 50 μg proteins from cotyledons infected with toxigenic or atoxigenic strains were labeled with 200 pmol Cy3 or Cy5 dye, respectively, and the internal standard was prepared by equally mixing all samples and labeled with Cy2 dye by use of the CyDye DIGE Flouors system (GE Healthcare). Cy2-, Cy3-, and Cy5-labeled proteins were pooled and loaded on 24-cm linear gradient strips (pH 4−7, GE Healthcare) for isoelectric focusing and then for SDS-PAGE as described.[23] Images were acquired with use of the Typhoon 9400 scanner (GE Healthcare).

Protein spot detection, spot matching among images or gels, and protein abundance quantification and normalization involved use of Decyder 2D 6.5 software (GE Healthcare). Spot detection performed using pixel data and boundaries of a spot from 3 images of a gel were identical, which were effective in spot matching and quantification, following the software processes. The number of spots expected was set at 2500 and the lowest 10th percentile pixel value on the spot boundary was subtracted on a spot basis as background. Ratios of sample to internal standard were calculated to normalize spot volume and the $\log_{10}$ of these ratio values were used to analyze the difference between toxigenic infection and atoxigenic infection by Student's two-tailed t test when $t < 0.05$ ($p < 0.05$).

Mass Spectrometry (MS) Identification of Protein Spots

Protein spots were manually excised from 2-DE gels. In-gel digestion of protein involved use of trypsin from bovine pancreas (Roche, Penzberg, Germany) as described.[22] The mass spectra were created on an ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonics Inc., Germany). The mass accuracy and mass resolution of mass measurement were set as the default. The time-of-flight spectra were recorded with a mass range from 700 to 4000 m/z following the FlexAnalysis 2.4 and FlexControl 2.4 instructions (Bruker). Peaks were accumulated from 30 shots, and valid peaks were selected when the ratio of signal-to-noise was >5. Peak lists were internally calibrated by a two-point method with typical trypsin autolysis peaks of 805.41630 and 2273.15950 m/z.

Peptide mass fingerprints were searched for protein identification against the National Center for Biotechnology Information nonredundant protein database (NCBInr, version 20081128) (http//www.ncbi.nlm.nih.gov/), containing 7,415,798 sequences and 2,558,340,887 residues. The selections of taxonomy were viridiplantae (527,072 sequence) for identification of proteins from peanut, and fungi (433363 sequences) for identification of proteins from *A. flavus* by use of MASCOT v2.1 (Matrix Science, London, U.K.). Scores >70 were significant ($p < 0.05$) from the viridiplantae database and >69 from the fungi database with the following parameters: allowing 1 missed cleavage; MS tolerance set as ±100 ppm; peptide charge MH+; mass value monoisotopic; fixed modification carbamidomethyl (Cys) and variable modification oxidation (Met) and Gln-pyro-Glu (N-term Gln). In addition, the minimum criteria for identification required >15% coverage of protein by matched peptides, and at least 5 independent peptides matched.

Gene Cloning and RT-PCR

Total RNA was isolated with use of Trizol reagent (Invitrogen, Carlsbad, CA) from infected or aflatoxin-treated cotyledons and their controls. cDNA was generated by the reverse transcriptase method (Invitrogen). Degenerate primers used to clone cDNAs encoding MS-identified proteins (for details see Results section) were designed according to conserved regions of protein homologues revealed by alignment. PCR involved use of Taq DNA polymerase (Biomed, Beijing). Amplified products were cloned into pGEM-T Easy Vector (Promega, Madison, WI) and confirmed by sequencing. The putative polypeptide was aligned with sequences identified by MS. Cloned cDNA fragments were searched for designing

dx.doi.org/10.1021/pr201105d | J. Proteome Res. 2012, 11, 2739−2753

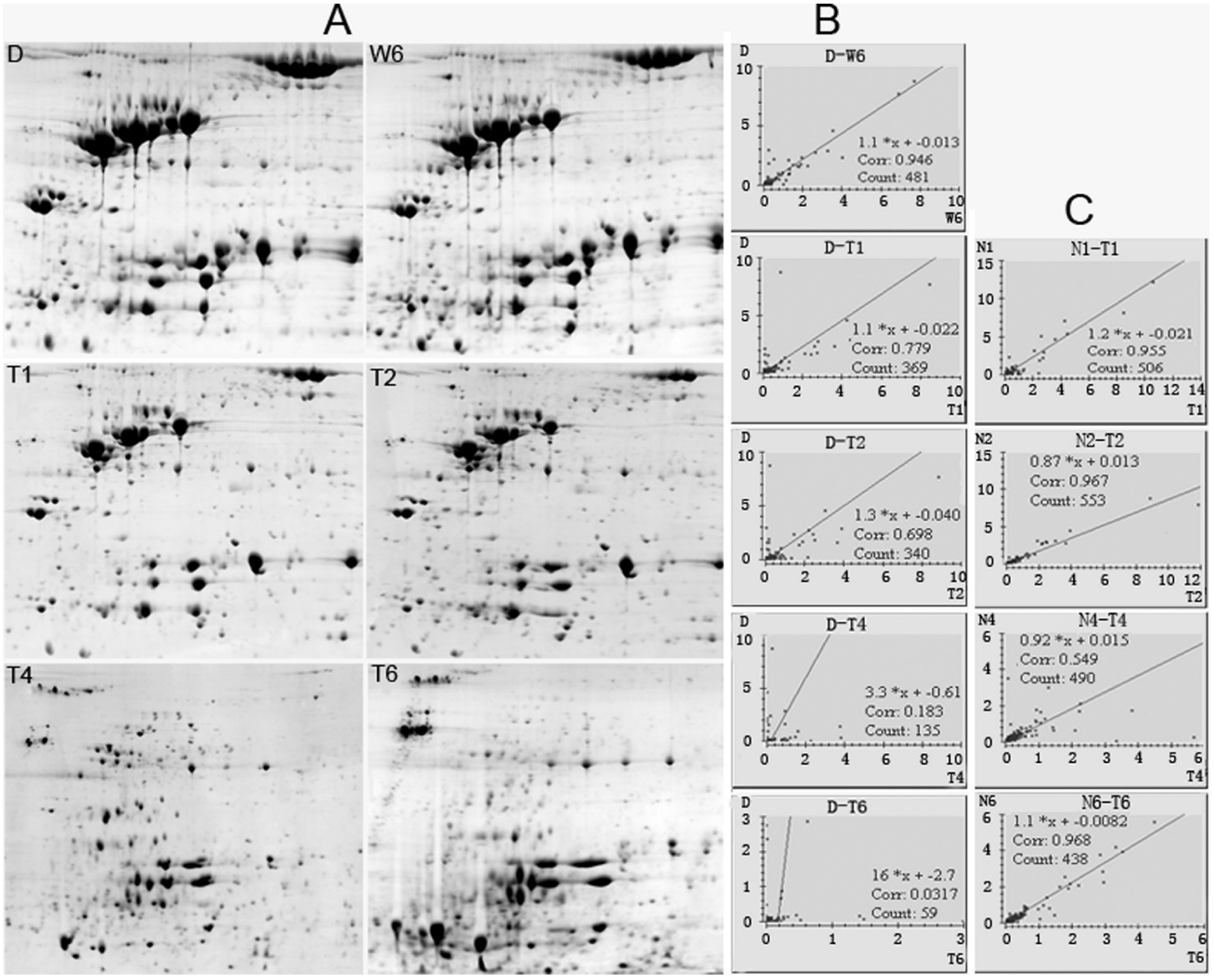


Figure 2. Proteomic changes after peanut cotyledon infection with toxigenic or atoxigenic *A. flavus* strains. (A) 2-D gel electrophoresis (2-DE). (B–C) Scatterplot analysis of protein spots from two 2-DE images. Protein spots were quantified in relative volume (%V), spots from two images were paired, and paired spots were analyzed by scatterplot by use of ImageMaster 2D platinum. (D) Proteins from dry cotyledons; (W6) proteins from cotyledons treated with water for 6 days; (T1–6) proteins from cotyledons infected with toxigenic As 3.2890 strain for 1–6 days; (N1–6) proteins from cotyledons infected with atoxigenic AF051 strain for 1–6 days. Corr., correlation coefficient; $ax + b$, regression line equation; Count, number of protein spot pairs.

primers for RT-PCR. An actin gene of peanut (accession no. DQ873525) was the internal standard. Products of RT-PCR were visualized by ethidium-bromide staining on agarose gel after electrophoresis.

RESULTS

Strain As 3.2890 but Not AF051 of *A. flavus* Produces Aflatoxins

Examination of cultures under a series of temperatures revealed aflatoxins synthesized only at 18–29 °C in *A. flavus*,[24] with 28 °C the optimal condition.[25] Peanut cotyledons were inoculated with spores of two *A. flavus* strains, AF051 and As 3.2890, 1×10^6 spores/mL at 28 °C under aeration. Phenotypes of mycelia and conidiophores of both strains on cotyledons were similar (Figure 1A). Hypha appeared at 2 days after inoculation (DAI) and conidia at DAI 3; thereafter, the conidiophores became thicker and quickly covered the surface of the cotyledon. The cotyledon became softer.

Examination of aflatoxins by TLC detected AFG1 in cotyledons infected with *A. flavus* strain As 3.2890 at DAI 2, which then accumulated quickly; AFB1 and AFG2 were detected at DAI 3 but accumulated slowly (Figure 1B); AFB2 was undetectable. In contrast, no aflatoxins were detected in cotyledons infected with *A. flavus* strain AF051 (Figure 1B). HPLC confirmed that strain AF051 did not produce aflatoxins during the infection (data not shown). However, HPLC revealed that strain As 3.2890 produced AFB1 and AFG1 at DAI 1 and AFB2 and AFG2 at DAI 3, which indicates that HPLC is more sensitive than TLC in detecting aflatoxins. The level of AFB1 was higher than that of AFG1 by HPLC than by TLC, which might be due to the 2 methods having different sensitivity to the 2 aflatoxins. However, both analyses revealed AFB1 and AFG1 at high levels and AFB2 and AFG2 at low levels (Figure 1C), and strain As 3.2890 but not strain AF051 produced aflatoxins.

Interestingly, strain As 3.2890 of *A. flavus* produced not only AFBs but also AFGs on peanut at this temperature. The finding

dx.doi.org/10.1021/pr201105d | *J. Proteome Res.* 2012, 11, 2739–2753

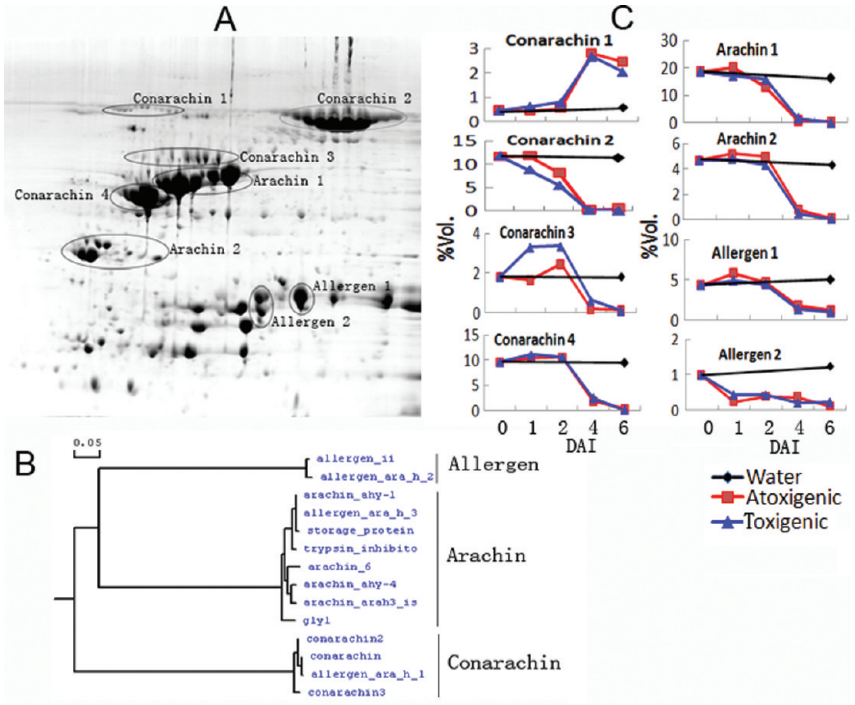


Figure 3. Identification of storage proteins and change in abundance during infection with *A. flavus* strains. Mass spectrometry (MS) identifying protein spots with high abundance and significant change in abundance during infection; the abundance of contiguous or a group of protein spots identified as identical proteins was combined to represent storage proteins (A). These proteins were identified as allergen, arachin, conarachin, trypsin and gly from the database and grouped into 3 protein groups according to sequence similarity (B). Changes in level of these proteins during infection shown by curves (C). W, water treatment; N, infection with atoxigenic AF051 strain; T, infection with toxigenic As 3.2890 strain. DAI, day after infection.

was similar to that for strain NRRL 3145,[26] and strains NRRL 3000, NRRL 2999 and NRRL A1−11,613,[27] which produce AFB1, AFB2, AFG1 and AFG2 in different proportions depending on the strain, balance of nutrients and culture conditions; many other *A. flavus* strains produce only the B group of aflatoxins.

Change in Proteomic Profile during Infection

To understand how infection causes proteomic change in cotyledons, we first analyzed the change in proteome during imbibition as background, then the change during infection, and finally the difference in proteomes with toxigenic and atoxigenic *A. flavus* strains infection on 2-DE-CBB staining. The proteomic profiles of dry and water-treated cotyledons for 6 days were similar (Figures 2A−D and A-W6). On scatterplot analysis, the relative values of 481 pairs of spots from the 2 proteomes were highly correlated (r^2 = 0.946) (Figure 2B-D−W6). Therefore, water treatment for 6 days did not significantly alter the protein pattern in cotyledons.

Infection with strains As 3.2890 or AF051 for 1−6 days greatly changed the proteomic profiles. Some spots with initially large volume in 2-DE gels quickly decreased and disappeared, although most spots with small volume showed little change during the infection (Figures 2A-T1, T2, T4, and T6; Figure S1, Supporting Information). Scatterplots of dry cotyledons and cotyledons infected with strain As 3.2890 at DAI 1, 2, 4, and 6 (Figure 2B) revealed 369, 340, 135, and 59 spot pairs, respectively, for a decrease in number during the infection and great decrease after DAI 4. Similarly, the correlation coefficients of relative abundance between dry cotyledons and cotyledons infected with strain As 3.2890 were 0.779, 0.698, 0.183, and 0.032, respectively, with no correlation after DAI 4 (Figures 2B-D−T1, T2, T4, and T6). Slopes of the regression line increased from 1.1 to 16, which indicated that protein abundance decreased rapidly after infection (Figure 2B). Thus, infection led to a great change in proteomic profile of cotyledons.

The proteomes of cotyledons infected with strains As 3.2890 and AF051 differed little during infection (Figure 2C). The correlation coefficients for spot pairs at DAI 1, 2, 4, and 6 were 0.955, 0.967, 0.549, and 0.968, respectively, with spot pairs of 506, 553, 490, and 438 spots and regression line slopes of 1.2, 0.87, 0.92 and 1.1, respectively (Figures 2C−N1-T1, N2-T2, N4-T4 and N6-T6). The numbers and values of paired spots differed little between the 2 infections, regardless of the large change in protein profiles from DAI 1 to 6. Here, the correlation coefficients were greater than 0.95 ($p < 0.05$), with slopes close to 1.0, except at DAI 4, which indicated a difference in proteomes between the 2 infections then. Therefore, changes in protein profiles in cotyledons infected with toxigenic or atoxigenic strains of *A. flavus* were similar except at DAI 4, when aflatoxins accumulated quickly in aflatoxin-producing infection.

Decrease in Abundance of Storage Proteins after Infection

The greatest change in proteomes during infection was a decrease and disappearance of high-abundance proteins (Figure 2A). These protein spots were collected (Figure 3A) and identified by MS as storage proteins, including arachin and conarachin. However, their denominations were diverse when more storage proteins were identified from differentially expressed proteins (see below and Table S2, Supporting Information). The storage proteins included allergen Arah1 (5 spots), allergen Arah2 (1), allergen Arah3/Arah4 (1), allergen

dx.doi.org/10.1021/pr201105d | *J. Proteome Res.* 2012, 11, 2739−2753

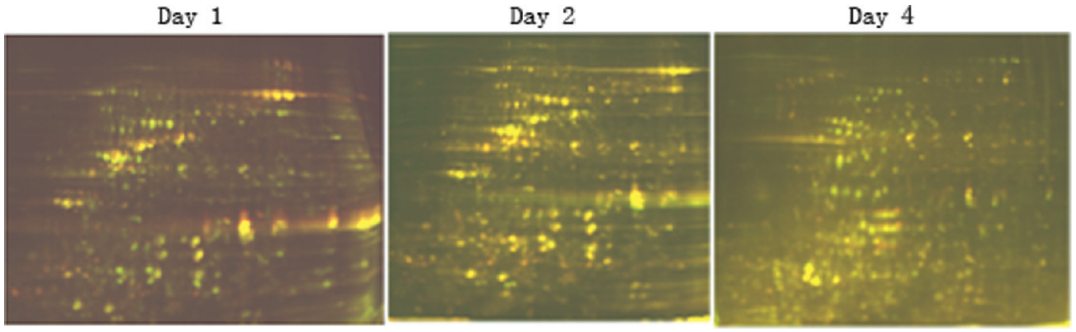


Figure 4. Representative images from 2-D fluorescence-difference gel electrophoresis. Proteins from cotyledons infected with toxigenic or atoxigenic *A. flavus* strains at 1, 2, and 4 DAI were labeled with Cy3 or Cy5 dye, and the internal standard sample was created by equal mixing of both protein samples and labeling with Cy2 dye. Equal samples labeled with Cy2, Cy3, and Cy5 dye were mixed and loaded for 2-DE. Cy2, Cy3, and Cy5 dyes were visualized at excitation 488, 532, and 633 nm, respectively. Signals of Cy3 (green) and Cy5 (red) were merged into yellow if they had the same signal strength. Ratios of sample to internal standard represent the abundance of protein spots. Statistical analysis involved 3 biological replicates. Day 1−4, 1−4 days after infection.

Table 1. Statistical Analysis and Identification of Protein Spots

day after infection	difference (spot)	analysis (spot)	identified (spot)	rate (%)	green plants (identity)	fungi (identity)
1	60	58	25	43.1	21	4
2	141	139	59	42.4	49	10
4	58	51	29	56.8	25	4
Total	259	248	113	45.6	95	18

II (1), arachin 6 (3), arachin Ahy-1 (8), arachin Arah3 (1), conarachin (28), gly1 (3), storage protein (2), and trypsin inhibitor (8). These proteins were incorporated into 14 different sequences. Similarity analysis revealed these sequences grouped into 3 classes: allergen, arachin and conarachin (Figure 3B).

To investigate the change in the abundance of these proteins during infection, we considered a group of spots with neighboring position on gels and their sequences from the same class as one storage protein (Figure 3A). The relative values of these spots were combined to represent the abundance of the protein. These storage proteins included 4 forms of conarachin, 2 of arachin and 2 of allergen (Figure 3A). The amount of these storage proteins could reach 56.3% of protein extracted from cotyledons before infection. However, the amount of these storage proteins dropped to a level close to zero at DAI 4, except for conarachin 1, which remained unchanged in level during infection but increased in level when the level of most storage proteins was decreased and protein loadings were unaltered (Figure 3C). The differences in level of these proteins with the 2 strains appeared only at DAI 1 and 2 (Figure 3C). Therefore, the level of storage proteins decreased quickly after infection with two *A. flavus* strains, but differences occurred mainly at DAI 1 and 2.

Proteins with Differential Abundance after Infection with Toxigenic and Atoxigenic Strains

2-D DIGE running multiple labeled samples on the same 2-D gel can eliminate the integral variability to increase the reproducibility and accuracy of matching and quantification.[28] Therefore, we further analyzed differences in levels of proteins in cotyledons infected with strains As 3.2890 and AF051 at DAI 1, 2, and 4 by 2-D DIGE (Figure 4), with the presence or absence of aflatoxins an important feature. The resolution of separation ranged from 750 to 1000 spots in gels, and 650−800 spots were matched between the 2 infections. Proteins with differential abundance were defined by *t* test ($p < 0.05$) comparison of log-ratio abundance to that of their internal standard between toxigenic and atoxigenic infection (Table S1, Supporting Information). The difference for 259 spots reached statistical significance, including upregulation and downregulation, and 58, 139, and 51 spots were collected from gels of DAI 1, 2, and 4 samples, respectively.

These proteins underwent in-gel digestion with trypsin and then identification by MS. Peak of mass spectra was defined as significant difference from noise and calibrated by trypsin autolysis peaks (Figure S2, Supporting Information). Critical scores of protein identification from the database ($p < 0.05$) were defined as significant following the program and much higher than the score of the next hit (Table S2, Supporting Information) to control false-positive rates. Sequence coverage of matched peptides was greater than 15% (Figure S2; Table S2, Supporting Information). We identified 113 spots with differential abundance, 45.6% of spots analyzed, including 95 from the green plant database and 18 from the fungi database (Table S2; Table 1).

Peanut Proteins with Differential Abundance Are Involved in Systematic Resistance to Pathogens

Among the 95 identified proteins from green plants, 61 were storage proteins, 2 were functionally unknown, and 32 were involved in diverse functional categories, including response and signal transduction (6 proteins), DNA synthesis (3), RNA synthesis (3), defense against disease invasion (12) and metabolism (8) (Table 2). This observation implied an effect of aflatoxins on peanut response pathways because the 2 strains are genetically different mainly in production or not of aflatoxins (Figure 1). Among the 32 proteins, 25 showed aflatoxin-dependent upregulation and 7 downregulation. The downregulated proteins were involved in carbohydrate metabolism (ADP-glucose pyrophosphorylase and glucose-1-phosphate adenylyltransferase), vesicle trafficking (conserved oligomeric Golgi protein), developmental transcription (FUL-

dx.doi.org/10.1021/pr201105d | J. Proteome Res. 2012, 11, 2739−2753

Table 2. Identification of Proteins with Differential Accumulation and Expression in the Presence of Aflatoxins[a]

no.	protein	function	ref.	species	reg	induction In +Af	induction Af	Sim%
		Arachis hypogaea						
		Signal transduction						
637	LRR receptor serine/threonine kinase	PAMPs perception	39	*M. indica*	+	+	+	48.25
49	Serine/threonine protein kinase	Phosphorylation		*O. sativa*	+	+	+	56.15
129	Pto-like receptor kinase resistance protein	Effector-triggered immunity	40	*R. roxburghii*	+	+	+	70.47
617	Protein phosphatase 2A regulatory B subunit	Dephosphorylation		*O. sativa*	+	+	+	
152	Phosphatidylinositol phosphate kinases	Phosphatidylinositol signaling	41	*O. sativa*	+		+	
268	B3 DNA binding protein	Metabolites responsiveness	42	*O. sativa*	+			
		DNA synthesis						
183	RNA-dependent DNA polymerase	Retrotransposition DNA repair	43	*O. sativa*	+		+	
324	ATP binding protein	Chromosome structure	44	*A. thaliana*	+	+	+	
257	Bark storage protein A	Nucleotide biosynthesis	45	*P. deltoides*	+			
		RNA synthesis						
448	FUL-like protein 1	Developmental transcription	46	*N. nucifera*	−			
402	Pentatricopeptide repeat-containing protein	RNA stabilization	47	*O. sativa*	+	+	+	
438	mRNA cleavage factor subunit	mRNA cleavage		*A. thaliana*	−			
		Defense against disease invasion						
1097	S-locus glycoprotein	Induction of defense	48	*B. oleracea*	+			
979	SMZ (SCHLAFMUTZE)	Resistance transcription	49	*A. thaliana*	+			
710	Hypersensitive-induced response protein	Hypersensitive response	50	*C. papaya*	+	+	+	90.27
845	PRL2 protein	Innate immunity	51	*A. thaliana*	+		+	77.78
657	SAM dependent isoflavone 7-*O*-methyltransferase	Biosynthesis of phytoalexins	52	*M. truncatula*	+			
270	Germacrene A synthase	Biosynthesis of phytoalexins	53	*C. sonchifolium*	−			
498	Alcohol dehydrogenase-1F	detoxification	54	*P. acutifolius*	+	+	+	93.51
829	Pectinesterase-1	Cell wall responses to infection	55	*C. sinensis*	+		+	
688	D-ala D-ala ligase	Peptidoglycan assembly	56	*O. sativa*	+	+	+	
821	Cytoskeleton- associated protein	Penetration resistance	57	*O. lucimarinus*	+			
859	myc-like regulatory protein	Condensed tannin synthesis	58	*L. corniculatus*	+			
280	NADH dehydrogenase subunit 7	Cell recovery from stresses	59	*C. atmophyticus*	+			
		Metabolism						
604	Acetyl-CoA carboxylase	Lipid metabolism		*B. napus*	+			
663	Esterase_lipase	Lipid metabolism	60	*C. reinhardtii*	+			
147	ADP-glucose pyrophosphorylase 51kD subunit	Carbohydrate metabolism		*O. sativa*	−			
157	ADP-glucose pyrophosphorylase	Carbohydrate metabolism		*O. sativa*	−			
88	Glucose-1-phosphate adenylyltransferase large chain	Carbohydrate metabolism		*O. sativa*	−			
217	Heat shock protein	Protein folding		*M. domestica*	+			
413	Mitochondrial inner membrane protease subunit 1	Protein translocation	61	*Z. mays*	+			
493	Conserved oligomeric Golgi protein	Vesicle trafficking	62	*P. patens*	−			
		Aspergillus flavus						
		Signal transduction						
141	Sensor histidine kinase	Across membrane signaling	63	*A. fumigatus*	+	+		60.56
		DNA repair						
749	Double-strand breaks repair complex subunit Ku70	DNA repair		*P. marneffei*	+	+		64.47
762	Mismatched base pair and cruciform DNA recognition protein	Mismatched recognition		*A. fumigatus*	+	+		61.76
248	DNA lyase	DNA repair of cytotoxic damage	64	*C. cereale*	+	+		61.65
554	DNA polymerase eta	DNA T-T lesions repair	65	*N. crassa*	−			
428	DNA replication regulator SLD3	Chromosomal DNA replication	66	*P. stipitis*	+	+		
		RNA synthesis						
595	RNA polymerase II transcription mediator	RNA synthesis		*C. glabrata*	+	+		
		Protein translation and modification						
735	Ribosomal protein L30	Protein translation		*N. crassa*	+	+		68.06
718	Ribosomal biogenesis GTPase	Ribosomal biogenesis		*P. chrysogenum*	+	+		80.90
606	HSP90	Posttranslational modification		*P. tritici*	+	+		44.40
701	HNRNP arginine N-methyltransferase	Protein methylation	67	*L. elongisporus*	+	+		
		Structural maintenance of chromosome						
967	Cell cycle control protein Cwf19	Cell cycle		*A. fumigatus*	+			
139	Chromosome segregation protein	Maintenance of chromosome	68	*P. anserina*	+			
268	Chromosome segregation protein	Maintenance of chromosome		*G. zeae*	+			
597	Kinesin, spindle pole protein	Maintenance of chromosome	69	*A. oryzae*	−			

dx.doi.org/10.1021/pr201105d | *J. Proteome Res.* 2012, 11, 2739−2753

Table 2. continued

no.	protein	function	ref.	species	reg	induction In +Af	induction Af	Sim%
		Metabolism and detoxification						
508	Fructose-2,6-bisphosphatase	Glycolysis		*C. neoformans*	+	+		38.26
344	GNAT family acetyltransferase	Arginine biosynthesis	70	*A. fumigatus*	+			
181	Cytochrome P450	Degradation of toxins	30	*A. niger*	+			

[a]Proteomic analysis of proteins in peanut cotyledons infected with toxigenic or atoxigenic *A. flavus* strains. The expression of many of these proteins was confirmed by transcriptional analysis of peanut cotyledons infected with or without atoxigenic *A. flavus* strain but supplemented with or without aflatoxins, respectively (Inf+Afn and Afn). Ref., reference; Reg., protein regulation; In., infection; Af, aflatoxin; Sim, similarity of putative amino acid sequence from cloned fragment and protein sequence from MS identification in other species; +, upregulation; −, downregulation.

like protein), mRNA cleavage and terpene biosynthesis (germacrene A synthase).

In contrast, toxigenic upregulation pathways included stress responses, signal transduction, DNA mismatch repair, structural maintenance of chromosomes, RNA stabilization, defense against disease invasion, lipid metabolism and protein modification and translocation (Table 2). Proteins for signal transduction were involved in PAMP perception, effector-triggered immunity, serine or threonine-specific residue phosphorylation or dephosphorylation of proteins, phosphorylation of inositol, and response to secondary metabolites. Proteins for defense against disease invasion were involved in defense response, disease resistance transcription, hypersensitive response, innate immunity, biosynthesis of phytoalexins, detoxification, cell wall responses to infection, peptidoglycan assembly, penetration resistance, condensed tannin synthesis, and cell recovery from stresses (Table 2).

In general, proteins for development, carbohydrate metabolism and vesicle trafficking were downregulated with aflatoxin, whereas those for DNA repair, chromosome structure maintenance, RNA stabilization, protein modification, lipid metabolism and defense against disease invasion were upregulated. These pathways implied a systematic resistance from upstream to downstream.

A. flavus Proteins with Differential Abundance Are Involved in Facilitating Pathogenic Invasion

We identified 18 fungal proteins in the fungi database (Table S2, Supporting Information), and 16 showed toxigenic-dependent upregulation (Table 2). These proteins are involved in cross-membrane signaling (1 protein), DNA repair (5), protein modification (4), structural maintenance of chromosome (4), and metabolism and detoxification (3). DNA repair proteins included those involved in repair of double-strand-break, mismatched base-pair and cruciform DNA, cytotoxicity-induced-damage DNA, and DNA thymine dimer (T-T) lesions (Table 2). Obviously, DNA repair was over-represented in the analysis.

Another highlighted biological process was structural maintenance of chromosome; the proteins involved were related to cell cycle regulation and chromosome segregation and structural maintenance (Table 2). This finding is consistent with the effect of aflatoxins because activation of these toxins leads to DNA damage and chromosome instability.[4−8] Therefore, DNA repair and chromosome maintenance proteins were likely upregulated to protect *A. flavus* DNA and chromosomes against aflatoxin damage. DNA and chromosomes of both *A. hypogaea* and *A. flavus* cells were damaged with aflatoxin accumulation but were repaired and maintained at a higher level in *A. flavus* than *A. hypogaea*. So, production of aflatoxins in *A. flavus* is a strategy to damage host DNA and chromosomes to facilitate invasion.

Aflatoxin Induces the Expression of the Genes in *A. hypogaea* and *A. flavus*

Most of our MS-identified proteins were homologues of those in other species found in MASCOT databases (Table S2, Supporting Information), and the DNA sequences encoding these proteins are unknown. We designed degenerate primers (Table S3, Supporting Information) and amplified 13 corresponding size bands from the peanut template and 11 from the *A. flavus* template. These amplified products were cloned and sequenced. Similarities of these putative amino acid sequences with respective homologues identified by MS ranged from 38.3% to 93.5%, and each sequence shared conserved motifs with its homologue (Figure 5). These sequences have been submitted to the NCBI database. With these sequences, differential transcription could be analyzed.

Plant infection is a complex system of plant and fungal subsystems. The interaction of both subsystems is the phenotype of the infection. In addition, we cannot completely eliminate differences in the 2 strains of *A. flavus* except for the production of aflatoxins in experimental design (Figures 1−2). Therefore, we treated peanut cotyledons with or without aflatoxins, without infection, to remove the disturbance from infection; also, we incubated cotyledons with atoxigenic *A. flavus* with or without aflatoxins to eliminate differences in strains for analysis of aflatoxin-inducing gene expression.

Figure 6A and C shows the differential expression of 11 peanut genes in cotyledons imbibed with aflatoxins. The expression of genes 402, 498, 829, and 324 was greatly induced and that of 637, 688 and 845, was about two-fold induced, whereas that of 49, 129, and 617 was barely induced by aflatoxin treatment. Figure 6B and D shows the differential expression of 11 peanut genes and 11 *A. flavus* genes in peanut cotyledons infected with the atoxigenic *A. flavus* strain AF051 and synchronously treated with aflatoxins. The expression of peanut genes in response to aflatoxins was similar to that without infection. Genes 402, 498, 637, and 688 were strongly induced, but genes 49, 324, and 617 were induced a little, and the expression of genes 829, 845, and 152 was undetectable. The addition of aflatoxins also induced the expression of genes from *A. flavus*. All genes showed increasing expression but to different degrees. Genes showing aflatoxin-induced expression are in Table 2. The results of gene expression responding to aflatoxins mediated by infection or not were consistent with the change in abundance of proteins in cotyledons infected with toxigenic or atoxigenic *A. flavus* strains (Table 2). Therefore, these genes were induced in the presence of aflatoxins mediated or not by infection.

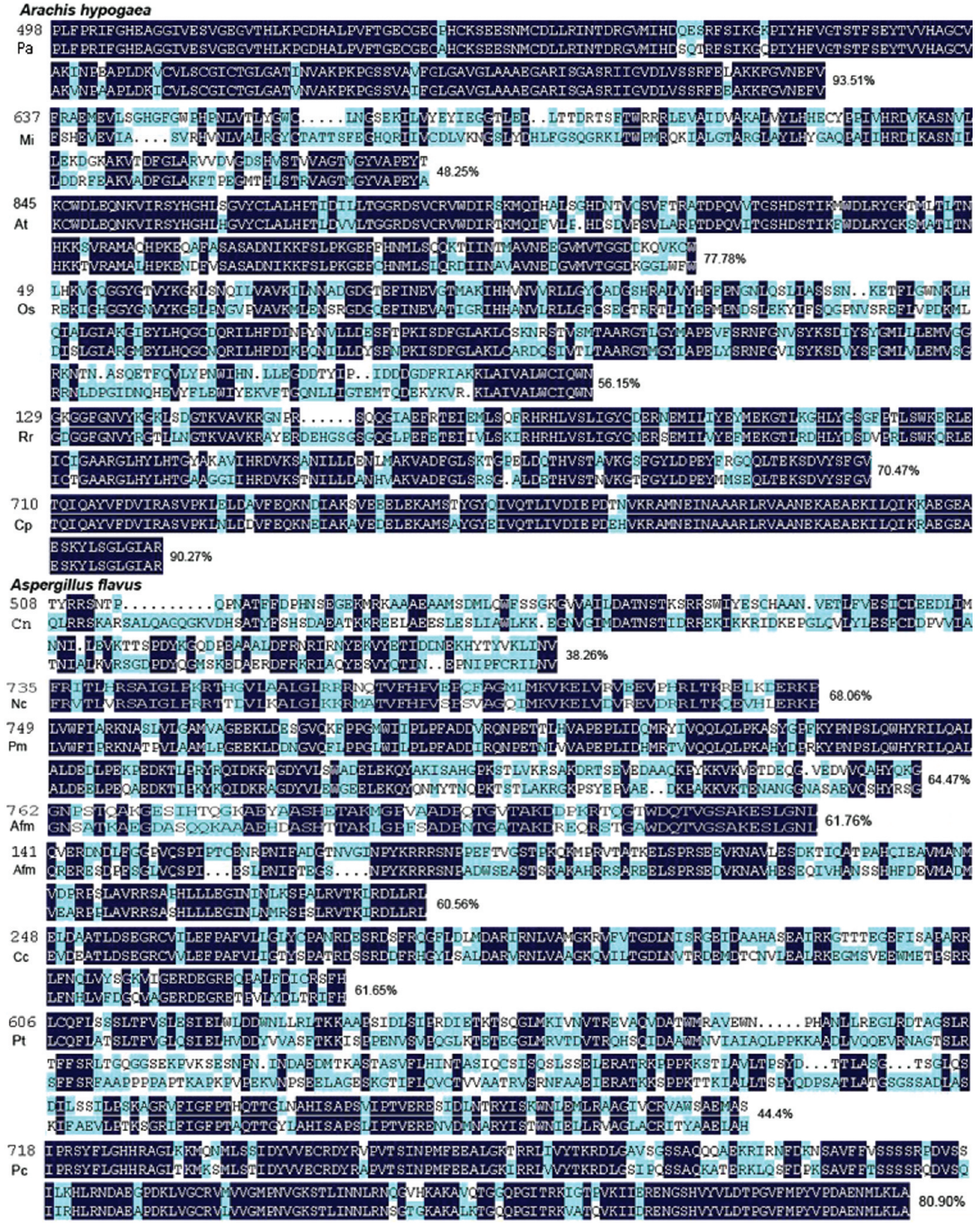


Figure 5. Alignment of amino acid polypeptides of cloned gene fragments with protein sequences identified by MS. The identity in percentage is indicated after each alignment, the upper sequence marked with a number indicates polypeptides of cloned fragments named according to protein spot number, and the lower sequence indicates polypeptides of proteins identified by MS; the name is the abbreviation of the species in protein identification. Pa, *Phaseolus acutifolius*; Mi, *Mangifera indica*; At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Rr, *Rosa roxburghii*; Cp, *Carica papaya*; Cn, *Cryptococcus neoformans*; Nc, *Neurospora crassa*; *Pm*, *Penicillium marneffei*; Afm, *Aspergillus fumigatus*; *Cc*, *Colletotrichum cereale*; Pt, *Pyrenophora triticirepentis*; *Pc*, *Penicillium chrysogenum*. Sequences for 498, 637, 845, 49, 129, 710, 508, 735, 749, 762, 141, 248, 606, and 718 have been submitted to NCBI with the accession numbers JN596992, JN596993, JN596994, JN596995, JN596996, JN596997, JN596998, JN596999, JN597000, JN597001, JN597002, JN597003, JN597004, and JN597005, respectively.

dx.doi.org/10.1021/pr201105d | *J. Proteome Res.* 2012, 11, 2739–2753

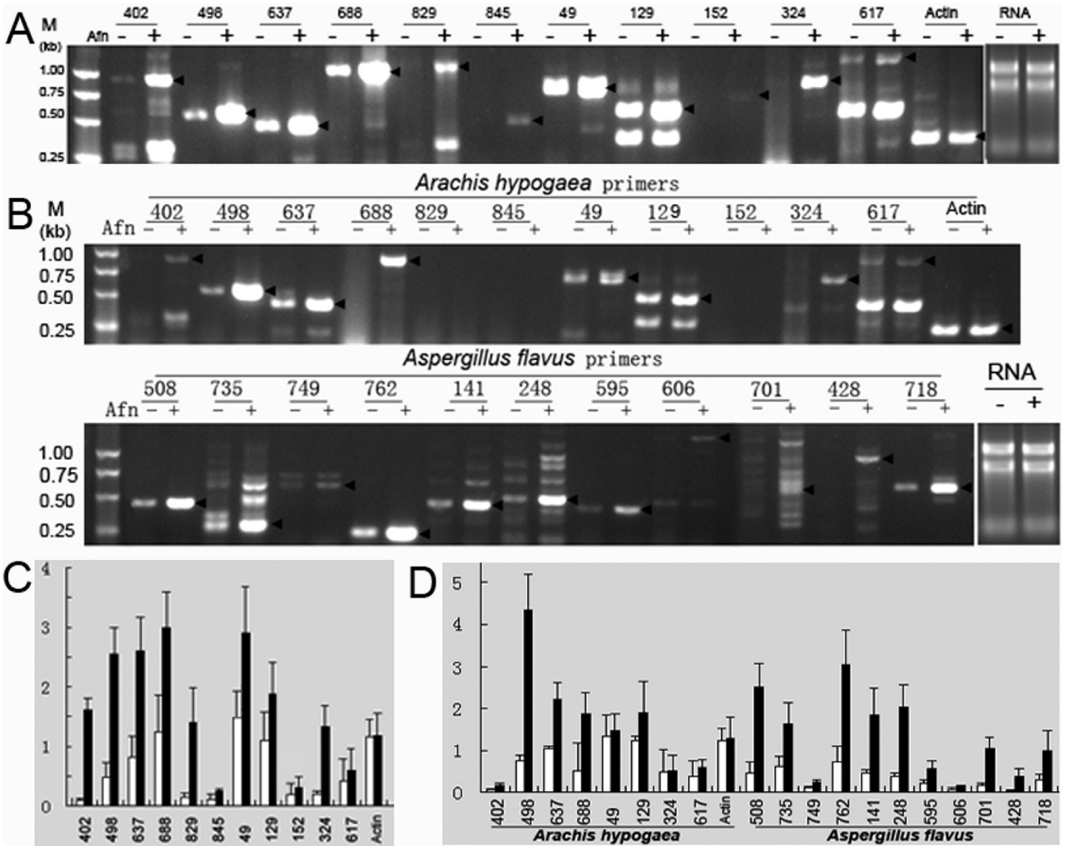


Figure 6. Comparative transcription of genes encoding proteins with differential abundance on aflatoxin treatment. (A and C) RT-PCR analysis of expression of *A. hypogaea* genes with aflatoxin treatment: *A. hypogaea* cotyledons (1.0 g) were treated with 1 mL aflatoxin mixture (Afn, 2.5 μg/mL aflatoxin B1, B2, G1 and G2 each in water; +) or 1 mL water (−) for 2 days. Images are representative of experiments performed in triplicate; corresponding size bands are indicated by black arrowhead (A); amplified DNA bands were quantified by use of ImageMaster 2D platinum 5.0; data are mean ± SD (C). (B and D) RT-PCR analysis of expression of *A. hypogaea* and *A. flavus* genes after aflatoxin treatment during infection of cotyledons with atoxigenic strain AF051. *A. hypogaea* cotyledons (1.0 g) were incubated with 1 mL spore suspension containing 1×10^6 spores and 2.5 μg aflatoxin B1, B2, G1 and G2 each in water (+) or 1 mL water (−) at 28 °C for 2 days. Images are representative of experiments performed in triplicate (B); amplified DNA bands were quantified by use of ImageMaster 2D platinum 5.0. Data are mean ± SD (D). White column, genes expression of control; Black column, expression of genes treated with aflatoxins.

A. hypogaea and *A. flavus* Respond to Aflatoxin

Aflatoxin inducing gene expression for systematic resistance against pathogens in isolated cotyledons suggested that plant cells respond to aflatoxin for disease resistance. We treated intact peanut seeds with AFB1 and found that the elongation of hypocotyls was promoted in the early stage of germination but sprouts were damaged severely after cotyledon opening (Figure 7A–E; Table 3). Hypocotyls of AFB1-treated seeds were much longer than that control seeds (Figure 7A–B), and the number of seeds with hypocotyl elongation with AFB1 treatment was greater than that with control treatment (Table 3). Therefore, germination of peanut seed responded to AFB1 by an unknown mechanism.

However, most AFB1 treated sprouts became hygrophanous, wilted, softened or became brown whereas only a few control sprouts were slightly damaged at day 10 after incubation (Figure 7C–E; Table 3). Therefore, AFB1 promoted but did not damage cell elongation but severely damaged cell division because germination at an early stage chiefly results from cell elongation and cell division thereafter.

In the infection experiment, both strains of *A. flavus* produced similar results of colony phenotype on the peanut seed surface. At DAI 4, some regions of the surface of many seeds were hidden from view because of thick mycelium; these seeds were defined as heavy-mycelium seed. Similarly, at DAI 6, many seeds were defined as heavy-conidia seeds because of thickened conidiophores. Otherwise, seeds were defined as light-mycelium or light-conidia seeds. The number of heavy-mycelium seeds at DAI 4 and heavy-conidia seeds at DAI 6 were lower with AFB1 than control treatment (Figure 7F–I; Table 3). Therefore, AFB1 delayed mycelium growth and conidia production at an early stage of infection.

After DAI 6, almost all seeds in all experiments were heavy-mycelium or heavy−conidia seeds and were difficult to distinguish by the eye. The results of seed germination, hypocotyl elongation and sprout dying with both strains infections were similar to those with AFB1 treatment without infection (Table 3). Therefore, AFB1 promoted hypocotyl elongation and then damaged peanut sprouts but delayed mycelium growth on the surface of the seed.

DISCUSSION

Accumulation of Aflatoxins in *A. flavus* Attack on *A. hypogaea*

From our observation, the toxigenic but not atoxigenic *A. flavus* strain produces and accumulates aflatoxins in peanut cotyledons after infection. As well, an array of *A. flavus* proteins accumulate differentially for signaling, protein

dx.doi.org/10.1021/pr201105d | J. Proteome Res. 2012, 11, 2739–2753

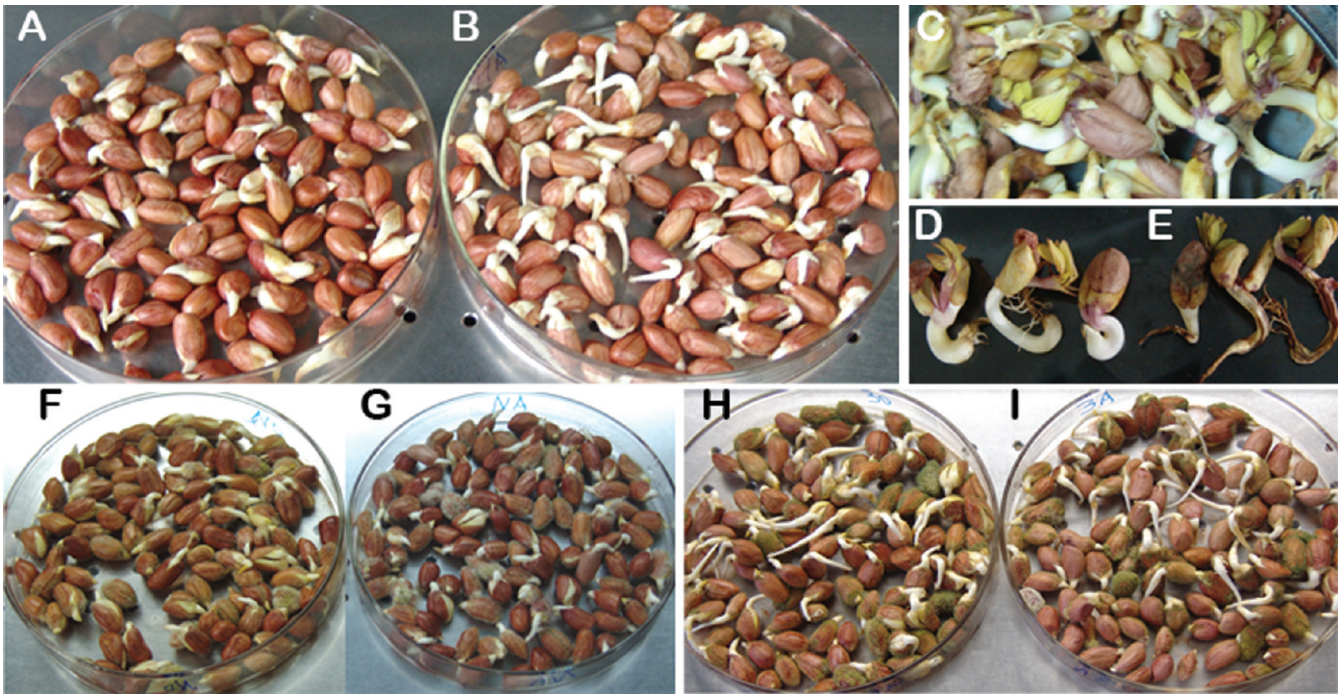


Figure 7. Growth of peanut and *A. flavus* responding to AFB1 during seed germination. (A and B) Seeds were imbibed in sterile water (A) or AFB1 aqueous solution (B) and cultivated at 28 °C for 4 days. (C–E) Phenotypes of peanut sprouts at day 10 after AFB1 treatment (C); (D) living sprouts in water and the (E) dead sprouts with AFB1 treatment. (F and G) Phenotypes of growth of mycelia and appearance of conidiophores in the control (F) or the AFB1 treatment (G) on the surface of seeds infected with atoxigenic AF051 strain at DAI 4. (H and I) Phenotypes of mycelia and conidiophores in the control (H) or the AFB1 treatment (I) on surface of the seeds infected with toxigenic As 3.2890 strain at DAI 6. For details of number of seeds with hypocotyl elongation, germination and nongermination, heavy or light mycelium, heavy or light conidia, and living or dying sprouts, see Table 3.

modification, DNA repair, structural maintenance of chromosome, detoxification and metabolism on infection with the toxigenic rather than the atoxigenic strain. These biochemical processes over-represent detoxification in *A. flavus*, including metabolic detoxification in cytoplasm and DNA repair in the nucleus, which is a fitness mechanism for the toxic circumstance. The pathogen may have evolved toxin production for invasion, a weapon for destroying host cells, but also a mechanism for detoxifying itself.

Table 3. Analysis of Seed Germination, Mycelium Growth and Conidia Phenotype on Seed Surface of Peanut after AFB1 Treatment with or without Infection with *A. flavus* Strains[a]

	Water		AF051		As 3.2890	
AFB1	−	+	−	+	−	+
Hypocotyl elongation (D4)	48	77	35	50	48	61
Germination (D4)	49	14	29	25	29	22
Non germination (D4)	3	9	36	25	23	17
Heavy mycelium seed (D4)	0	0	54	24	41	32
Light mycelium seed (D4)	0	0	46	76	59	68
Heavy conidia seed (D6)	0	0	51	42	56	33
Light conidia seed (D6)	0	0	49	58	44	67
Hypocotyl elongation (D10)	78	82	49	72	70	60
Germination (D10)	22	10	23	21	11	23
Non germination (D10)	0	8	28	7	19	17
Living sprouts (D10)	90	32	64	36	68	31
Dying sprouts (D10)	10	68	36	64	32	69

[a]Each experiment involved 100 peanut seeds; data are seed number. Hypocotyl elongation, hypocotyl longer than 5 mm; heavy mycelium or conidia, mycelium or conidia is thick enough to hide some part of the seed surface; light mycelium or conidia, not thick enough to hide the seed surface; dying sprout, softening and browning of seeds, and hygrophanous and wilted of sprouts. Detailed phenotypes of seed germination and conidia are in Figure 7. D1–10, day 1–10 after AFB1 treatment and infection. −, without AFB1; +, with AFB1.

Aflatoxin is a dose-dependent acute cytotoxic agent leading to cell death.[9,10] To protect fungal cells against self-toxicity, detoxification of aflatoxin is important for *A. flavus*. *A. parasiticus* synthesizes and exports AFB1 in a vesicle transport machinery to compartmentalize the toxin.[29] Cytochrome P450, an extensive system involved in metabolic detoxification,[30] is accumulated in response to aflatoxins. These physical and chemical detoxifications are vital mechanisms for fungal cells. Otherwise, aflatoxins cause DNA damage and chromosome instability by interacting with DNA.[4−8] Aflatoxins damage both plant and fungal DNA. Damage of plant DNA can prevent plant cell resistance by gene expression to facilitate fungal invasion, but fungal cells express a group of proteins for DNA repair to avoid damage of fungal DNA. At both levels of cytotoxic and DNA damage, aflatoxins impair plant cells, but fungal cells are protected by corresponding mechanisms.

The gene expression of these proteins induced by aflatoxins on infection with an atoxigenic strain indicates that the fungal genes can respond to aflatoxin, whether the strain produces aflatoxin or not. That is, the response mainly depends on the presence of aflatoxin but not *A. flavus* strain. These proteins establish a pathway of gene expression from DNA and RNA syntheses to protein translation and modification for DNA repair, structural maintenance of chromosomes and metabolic detoxification in *A. flavus*.

dx.doi.org/10.1021/pr201105d | *J. Proteome Res.* 2012, 11, 2739–2753

Aflatoxin Induces Cotyledon Defense against *A. flavus* Attack

Peanut cotyledons infected with the toxigenic strain accumulated a class of proteins at a higher level than those infected with the atoxigenic strain. The pathways range from signal transduction, DNA repair, RNA stabilization and defense against disease invasion to metabolic detoxification. Similar to the response in *A. flavus*, cotyledon cells also protect DNA and RNA against aflatoxin damage. The corresponding genes respond to aflatoxin as well.

Similarly, the specific response to DNA and RNA damage from aflatoxin is aflatoxin-triggered DNA and RNA repair in plants. In addition, proteins for signal transduction and defense against disease invasion are triggered by aflatoxin in these cotyledons. The signaling includes PAMP perception, effector-triggered immune response and innate immunity. In addition, the defense responds at multiple levels, from downregulation of developmental transcription and carbohydrate metabolism to upregulation of metabolite responsiveness; induction of defense response; increase of cell wall responses; condensed tannin synthesis; peptidoglycan assembly and penetration resistance against invasion; upregulation of disease resistance transcription; biosynthesis of phytoalexins; hypersensitive response and detoxification. These pathways represent most aspects of the systematic resistance in plant.

Similar to the response in *A. flavus*, in plants, differences in protein or transcriptional levels result from the presence of aflatoxins but not from infection. Therefore, peanut cotyledons have developed a pathway from signaling, transcription to translation for defense against aflatoxin attack at both detoxification and DNA repair levels and immune responses from pathogenic stresses. Here, the response to abiotic stress such as aflatoxin shares many features with biotic stresses, which is consistent in that heavy-metal stresses share many responses with pathogenesis.[31]

In the gene-for-gene association, resistance is activated once plant R proteins specifically recognize bacterial, fungal, or viral effectors to initiate defense signaling and host resistance in plant cells.[32] Most R proteins are nucleotide-binding and leucine-rich-repeat (NB-LRR) proteins encoded by *R* genes.[33] However, the plant immunity is first triggered on perception of PAMPs by transmembrane pattern recognition receptors (PRRs).[33] PAMP- and effector-triggered immunities share similar downstream signaling machinery for immune responses.[34] Stomatal closure to restrict bacterial invasion, mediated by signaling in guard cells, is a plant innate immune response in *Arabidopsis*,[35] but the association of immune signaling with host resistance in plants is unknown. The pathways identified here associate many resistance responses at biochemical level with aflatoxin signaling in the peanut cotyledon.

Aflatoxin Triggers an Immune Response in Plant Cells

Acute cytotoxicity of aflatoxin in cells is lethiferous; for example, the relative viability of human hepatocytes treated with aflatoxin is dose dependent and decreases to 20% with 50 μM AFB1 for 24 h.[36] Mice injected with 5 mg/kg AFB1 died within 17 h of massive liver necrosis and some areas of livers showed loss of cell borders and pyknotic nuclei at 3 h after the injection. At the same time, AFB1-N^7-DNA adducts are heavily formed.[37] However, cells of *Aspergillus* evade cytotoxicity by using a vesicle transport system for synthesis and export of aflatoxins[29] to strangle the host. Killing host cells is a strategy for infection and inhabitation because these fungi are saprophytes.[38] Host cells may evolve a mechanism to face with this threat.

In our infection of peanut cotyledons with *A. flavus*, the accumulation of an array of proteins and expression of their encoding genes was strengthened when aflatoxins were increased or supplemented, regardless of infection. Therefore, aflatoxins act as an elicitor or antigen in peanut cells for an immune response, triggering additional resistances.

Upstream signaling of the response includes protein phosphorylation and dephosphorylation, PAMP perception, effector-triggered immunity, phosphatidylinositol signaling and metabolite responsiveness. Output pathways of defense include the induction of defense, innate immunity, hypersensitive response, biosynthesis of phytoalexins, cell wall responses, peptidoglycan assembly, penetration resistance, condensed tannin synthesis, and detoxification.

Along with immunities triggered by PAMPs and effector proteins[11,33] the immunity triggered by aflatoxin is designated the metabolite-triggered immunity in the infection, for a three-grade immune system (Figure 8). At the tip of the *A. flavus* hypha, the growing cells touch the extracellular surface receptors of the peanut cotyledon plasma membrane, which initiates PAMP-triggered immune responses in the host cells; older hypha cells deliver effector proteins, which cross the plasma membrane into peanut cytoplasm mediated likely by haustorium[11] to suppress the PAMP-triggered immune signaling. The foreign proteins initiate effector-triggered immune responses in plant cells. Toxic aflatoxins, produced in mature hypha cells, are confined in a vesicle system to protect hypha against toxicosis[29] but cause cytotoxicity and DNA damage in

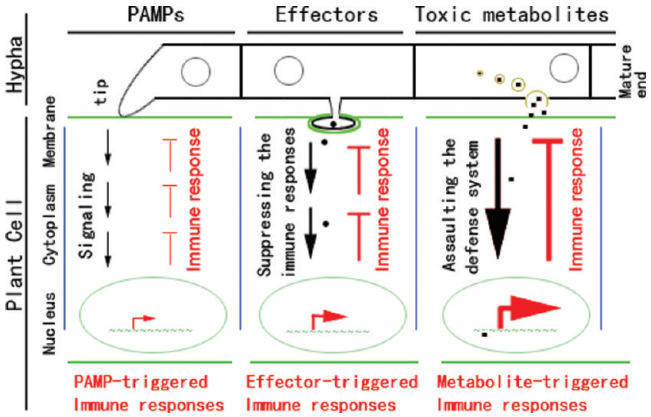


Figure 8. Three grades of immune response in *A. hypogaea* during infection with toxigenic *A. flavus*. At the interface of the hypha tip and cotyledon, host plasma-membrane extracellular surface receptors recognize pathogen-associated molecular patterns (PAMPs) of hypha and initiate PAMP-triggered immunity (PTI, the first immune response) transcribed from nuclear DNA in cotyledon cells (grade 1). To suppress the first immunity for invasion, older hypha cells secrete effectors (round bold spot) via the haustorium into the host cytoplasm to restrain the PAMP-triggered signaling, but host R proteins recognize the activity of these exotic proteins and increase effector-triggered immunity (ETI, the second immunity-grade 2 immune response). As a saprophytic fungus, *A. flavus* developed a pathway to produce toxic aflatoxins through a vesicle system (yellow vesicle) to kill host cells at cytotoxic and DNA damage levels (rectangle bold spot); however, cotyledon cells recognize these toxic metabolites and respond by increasing extensive immune responses (MTI, the third immunity-grade 3 immune response).

dx.doi.org/10.1021/pr201105d | *J. Proteome Res.* 2012, 11, 2739–2753

target cotyledon cells to lethargize host cells for hypha saprophytic inhabitation. Finally, the exotic metabolites produce an additional immune response in the host, including perception of metabolite signaling, response to chemical stress, defense against toxicosis, DNA repair and resistance to aflatoxin production.

Peanut Germination and Fungal Infection Respond to Aflatoxin

Our proteomic and transcriptional analyses revealed that aflatoxin triggers an immune response for disease resistance in peanut cotyledons and a mechanism for detoxification and DNA repair in *A. flavus*. During germination, aflatoxin stimulates hypocotyl elongation at the early stage, which may be a visual cue of resistance because pushing the soil up earlier is an important strategy to avoid stresses for germinating seeds.

More importantly, the germination responds to aflatoxin, a biotoxin, by an unknown mechanism. Aflatoxin does not damage the seed but does promote germination at this dose by accelerating elongation of the hypocotyl. At the same time, damage of sprouts is delayed to about 10 days after AFB1 treatment when hypocotyl elongation finishes and cotyledons open, which suggests that AFB1 damages cell division but not cell elongation. Therefore, DNA damage is more sensitive to AFB1 than cytotoxicity in peanut embryonic cells because cell division requires chromsome remodeling and DNA replication. The process exposes the host DNA to AFB1 interaction. This observation is consistent with the results of our proteomic and transcription analyses, showing over-representation of DNA repair and maintenance of chromosome structure expression.

With infection, AFB1 delays mycelium growth and conidia production on the surface of peanut seeds at the early stage. This observation is consistent with the hypothesis that AFB1 may also damage *A. flavus* before a detoxification machinery is established. In addition, the process costs resources and time to build the machinery, which delays the growth of mycelium. After *A. flavus* is detoxified and peanut DNA is damaged by aflatoxin, infection is easier. Therefore, both peanut and *A. flavus* respond to AFB1, but their responses differ because of differing response pathways and effects.

In addition to the metabolite-triggered immunity, our findings indicate a three-grade coevolution of invasion and defense in plant-pathogen interactions.

■ ASSOCIATED CONTENT

Supporting Information

Table S1. Differential analysis of protein abundances between *Arachis hypogaea* cotyledons infected with toxigenic or atoxigenic *Aspergillus flavus* strains at day 1, day 2, and day 4. Table S2. Mass spectra identification of proteins differentially accumulated in *Arachis hypogaea* cotyledons infected with toxigenic or atoxigenic *Aspergillus flavus* strains at day 1, day 2, and day 4. Table S3. Primers used for PCR amplification of genes and predicted size of gene fragments. Figure S1. Gel images of atoxigenic infection at day 1, 2, 4, and 6 stained by CBB. Figure S2. Annotated mass spectra and amino acid sequence. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tai Wang, Nanxincun 20, Xiangshan, Haidianqu, Beijing 100093, China. Tel: +86-10-62836210. Fax: +86-10-62594170. E-mail: twang@ibcas.ac.cn.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Prof. Ma Zhonghua (Zhejiang Universty, Hangzhou, China) for providing atoxigenic *Aspergillus flavus* strain AF051. The research was supported by grants from the Chinese Academy of Sciences (No. KSCX2-YW-N-033) and the China Ministry of Agriculture (No. 2011ZX08012-002).

■ REFERENCES

(1) Fox, E. M.; Howlett, B. J. Secondary metabolism: regulation and role in fungal biology. *Curr. Opin. Microbiol.* **2008**, *11* (6), 481−7.
(2) Krishnan, S.; Manavathu, E. K.; Chandrasekar, P. H. Aspergillus flavus: an emerging non-fumigatus Aspergillus species of significance. *Mycoses* **2009**, *52* (3), 206−22.
(3) Hof, H. Mycotoxins: pathogenicity factors or virulence factors? *Mycoses* **2008**, *51* (2), 93−4.
(4) Martin, C. N.; Garner, R. C. Aflatoxin B -oxide generated by chemical or enzymic oxidation of aflatoxin B1 causes guanine substitution in nucleic acids. *Nature* **1977**, *267* (5614), 863−5.
(5) Shen, H. M.; Ong, C. N.; Lee, B. L.; Shi, C. Y. Aflatoxin B1-induced 8-hydroxydeoxyguanosine formation in rat hepatic DNA. *Carcinogenesis* **1995**, *16* (2), 419−22.
(6) Bedard, L. L.; Massey, T. E. Aflatoxin B1-induced DNA damage and its repair. *Cancer Lett.* **2006**, *241* (2), 174−83.
(7) Herzog, C. R.; Bodon, N.; Pittman, B.; Maronpot, R. R.; Massey, T. E.; Anderson, M. W.; You, M.; Devereux, T. R. Carcinogen-specific targeting of chromosome 12 for loss of heterozygosity in mouse lung adenocarcinomas: implications for chromosome instability and tumor progression. *Oncogene* **2004**, *23* (17), 3033−9.
(8) Pineau, P.; Marchio, A.; Battiston, C.; Cordina, E.; Russo, A.; Terris, B.; Qin, L. X.; Turlin, B.; Tang, Z. Y.; Mazzaferro, V.; Dejean, A. Chromosome instability in human hepatocellular carcinoma depends on p53 status and aflatoxin exposure. *Mutat. Res.* **2008**, *653* (1−2), 6−13.
(9) Caruso, M.; Mariotti, A.; Zizzadoro, C.; Zaghini, A.; Ormas, P.; Altafini, A.; Belloli, C. A clonal cell line (BME-UV1) as a possible model to study bovine mammary epithelial metabolism: metabolism and cytotoxicity of aflatoxin B1. *Toxicon* **2009**, *53* (4), 400−8.
(10) Golli-Bennour, E. E.; Kouidhi, B.; Bouslimi, A.; Abid-Essefi, S.; Hassen, W.; Bacha, H. Cytotoxicity and genotoxicity induced by aflatoxin B1, ochratoxin A, and their combination in cultured Vero cells. *J. Biochem. Mol. Toxicol.* **2010**, *24* (1), 42−50.
(11) Chisholm, S. T.; Coaker, G.; Day, B.; Staskawicz, B. J. Host-microbe interactions: shaping the evolution of the plant immune response. *Cell* **2006**, *124* (4), 803−14.
(12) Dangl, J. L.; Jones, J. D. Plant pathogens and integrated defence responses to infection. *Nature* **2001**, *411* (6839), 826−33.
(13) Romani, L. Immunity to fungal infections. *Nat. Rev. Immunol.* **2011**, *11* (4), 275−88.
(14) Jia, Y.; McAdams, S. A.; Bryan, G. T.; Hershey, H. P.; Valent, B. Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. *Embo J* **2000**, *19* (15), 4004−14.
(15) Daub, M. E.; Herrero, S.; Chung, K. R. Photoactivated perylenequinone toxins in fungal pathogenesis of plants. *FEMS Microbiol. Lett.* **2005**, *252* (2), 197−206.
(16) Steinkamp, M. P.; Martin, S. S.; Hoefert, L. L.; Ruppel, E. G. Ultrastructure of lesions produced in leaves of *Beta vulgaris* by cercosporin, a toxin from *Cercospora beticola*. *Phytopathology* **1981**, *71*, 1272−81.

(17) Daub, M. E. Peroxidation of tobacco membrane lipids by the photosensitizing toxin, cercosporin. *Plant Physiol.* **1982**, *69* (6), 1361–4.
(18) Choquer, M.; Dekkers, K. L.; Chen, H. Q.; Cao, L.; Ueng, P. P.; Daub, M. E.; Chung, K. R. The CTB1 gene encoding a fungal polyketide synthase is required for cercosporin biosynthesis and fungal virulence of Cercospora nicotianae. *Mol. Plant-Microbe Interact.* **2005**, *18* (5), 468–76.
(19) Li, S. Research progress of peanut resistance to Aspergillus flavus in Guangdong. *Guangdong Agric. Sci.* **2006**, *10*, 17–20 (in Chinese).
(20) Jiang, J.; Yan, L.; Ma, Z. Molecular characterization of an atoxigenic Aspergillus flavus strain AF051. *Appl. Microbiol. Biotechnol.* **2009**, *83* (3), 501–5.
(21) Yang., W.; Ma, G. Population and dynamic analysis of Aspergillus flavus and other fungi in groundnut. *J. Hebei Vocation-Tech. Teachers College* **2003**, *17*, 19–22.
(22) Wang, Z.; Wang, T. Dynamic proteomic analysis reveals diurnal homeostasis of key pathways in rice leaves. *Proteomics* **2011**, *11* (2), 225–38.
(23) Xu, S. B.; Yu, H. T.; Yan, L. F.; Wang, T. Integrated proteomic and cytological study of rice endosperms at the storage phase. *J. Proteome Res.* **2010**, *9* (10), 4906–18.
(24) Schindler, A. F.; Palmer, J. G.; Elsenberg, W. V. Aflatoxin production by Aspergillus flavus as related to various temperatures. *Appl. Microbiol.* **1967**, *15*, 1006–9.
(25) Georgianna, D. R.; Hawkridge, A. M.; Muddiman, D. C.; Payne, G. A. Temperature-dependent regulation of proteins in Aspergillus flavus: whole organism stable isotope labeling by amino acids. *J. Proteome Res.* **2008**, *7* (7), 2973–9.
(26) Applegate, K.; Chipley, J. Daily variations in the production of aflatoxins by Aspergillus flavus NRRL-3145 following exposure to ^{60}Co irradiation. *J. Appl. Microbiol.* **1974**, *37* (3), 359–72.
(27) Hesseltine, C.; Shotwell, O. L.; Ellis, J.; Stubblefield, R. Aflatoxin formation by Aspergillus flavus. *Bacteriol. Rev.* **1966**, *30* (4), 795–805.
(28) Marouga, R.; David, S.; Hawkins, E. The development of the DIGE system: 2D fluorescence difference gel analysis technology. *Anal. Bioanal. Chem.* **2005**, *382* (3), 669–78.
(29) Chanda, A.; Roze, L. V.; Kang, S.; Artymovich, K. A.; Hicks, G. R.; Raikhel, N. V.; Calvo, A. M.; Linz, J. E. A key role for vesicles in fungal secondary metabolism. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106* (46), 19533–8.
(30) Scott, J. G.; Liu, N.; Wen, Z. Insect cytochromes P450: diversity, insecticide resistance and tolerance to plant toxins. *Comp. Biochem. Physiol. C: Pharmacol. Toxicol. Endocrinol.* **1998**, *121* (1–3), 147–55.
(31) Ahsan, N.; Renaut, J.; Komatsu, S. Recent developments in the application of proteomics to the analysis of plant responses to heavy metals. *Proteomics* **2009**, *9* (10), 2602–21.
(32) Flor, H. H. Current status of the gene-for-gene concept. *Annu. Rev. Phytopathol.* **1971**, *9*, 275–96.
(33) Jones, J. D.; Dangl, J. L. The plant immune system. *Nature* **2006**, *444* (7117), 323–9.
(34) Tsuda, K.; Katagiri, F. Comparing signaling mechanisms engaged in pattern-triggered and effector-triggered immunity. *Curr. Opin. Plant Biol.* **2010**, *13* (4), 459–65.
(35) Melotto, M.; Underwood, W.; Koczan, J.; Nomura, K.; He, S. Y. Plant stomata function in innate immunity against bacterial invasion. *Cell* **2006**, *126* (5), 969–80.
(36) Li, A. P. Metabolism comparative cytotoxicity assay (MCCA) and cytotoxic metabolic pathway identification assay (CMPIA) with cryopreserved human hepatocytes for the evaluation of metabolism-based cytotoxicity in vitro: proof-of-concept study with aflatoxin B1. *Chem. Biol. Interact.* **2009**, *179* (1), 4–8.
(37) Ilic, Z.; Crawford, D.; Vakharia, D.; Egner, P. A.; Sell, S. Glutathione-S-transferase A3 knockout mice are sensitive to acute cytotoxic and genotoxic effects of aflatoxin B1. *Toxicol. Appl. Pharmacol.* **2010**, *242* (3), 241–6.
(38) Tekaia, F.; Latge, J. P. Aspergillus fumigatus: saprophyte or pathogen? *Curr. Opin. Microbiol.* **2005**, *8* (4), 385–92.
(39) Gomez-Gomez, L.; Boller, T. FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. *Mol. Cell* **2000**, *5* (6), 1003–11.
(40) Song, W. Y.; Wang, G. L.; Chen, L. L.; Kim, H. S.; Pi, L. Y.; Holsten, T.; Gardner, J.; Wang, B.; Zhai, W. X.; Zhu, L. H.; Fauquet, C.; Ronald, P. A receptor kinase-like protein encoded by the rice disease resistance gene, Xa21. *Science* **1995**, *270* (5243), 1804–6.
(41) Anderson, R. A.; Boronenkov, I. V.; Doughman, S. D.; Kunz, J.; Loijens, J. C. Phosphatidylinositol phosphate kinases, a multifaceted family of signaling enzymes. *J. Biol. Chem.* **1999**, *274* (15), 9907–10.
(42) Yamasaki, K.; Kigawa, T.; Inoue, M.; Tateno, M.; Yamasaki, T.; Yabuki, T.; Aoki, M.; Seki, E.; Matsuda, T.; Tomo, Y.; Hayami, N.; Terada, T.; Shirouzu, M.; Osanai, T.; Tanaka, A.; Seki, M.; Shinozaki, K.; Yokoyama, S. Solution structure of the B3 DNA binding domain of the Arabidopsis cold-responsive transcription factor RAV1. *Plant Cell* **2004**, *16* (12), 3448–59.
(43) Farkash, E. A.; Prak, E. T. DNA damage and l1 retrotransposition. *J. Biomed. Biotechnol.* **2006**, *2006* (1), 37285.
(44) Hirano, M.; Hirano, T. Hinge-mediated dimerization of SMC protein is essential for its dynamic interaction with DNA. *Embo J.* **2002**, *21* (21), 5733–44.
(45) Bzowska, A.; Kulikowska, E.; Shugar, D. Purine nucleoside phosphorylases: properties, functions, and clinical aspects. *Pharmacol. Ther.* **2000**, *88* (3), 349–425.
(46) Leseberg, C. H.; Eissler, C. L.; Wang, X.; Johns, M. A.; Duvall, M. R.; Mao, L. Interaction study of MADS-domain proteins in tomato. *J. Exp. Bot.* **2008**, *59* (8), 2253–65.
(47) Johnson, X.; Wostrikoff, K.; Finazzi, G.; Kuras, R.; Schwarz, C.; Bujaldon, S.; Nickelsen, J.; Stern, D. B.; Wollman, F. A.; Vallon, O. MRL1, a conserved Pentatricopeptide repeat protein, is required for stabilization of rbcL mRNA in Chlamydomonas and Arabidopsis. *Plant Cell* **2010**, *22* (1), 234–48.
(48) Maimbo, M.; Ohnishi, K.; Hikichi, Y.; Yoshioka, H.; Kiba, A. S glycoprotein-like protein regulates defense responses in Nicotiana plants against Ralstonia solanacearum. *Plant Physiol.* **2010**, *152* (4), 2023–35.
(49) McGrath, K. C.; Dombrecht, B.; Manners, J. M.; Schenk, P. M.; Edgar, C. I.; Maclean, D. J.; Scheible, W. R.; Udvardi, M. K.; Kazan, K. Repressor- and activator-type ethylene response factors functioning in jasmonate signaling and disease resistance identified via a genome-wide screen of Arabidopsis transcription factor gene expression. *Plant Physiol.* **2005**, *139* (2), 949–59.
(50) Jung, H. W.; Lim, C. W.; Lee, S. C.; Choi, H. W.; Hwang, C. H.; Hwang, B. K. Distinct roles of the pepper hypersensitive induced reaction protein gene CaHIR1 in disease and osmotic stress, as determined by comparative transcriptome and proteome analyses. *Planta* **2008**, *227* (2), 409–25.
(51) Palma, K.; Zhao, Q.; Cheng, Y. T.; Bi, D.; Monaghan, J.; Cheng, W.; Zhang, Y.; Li, X. Regulation of plant innate immunity by three proteins in a complex conserved across the plant and animal kingdoms. *Genes Dev.* **2007**, *21* (12), 1484–93.
(52) He, X. Z.; Dixon, R. A. Genetic manipulation of isoflavone 7-O-methyltransferase enhances biosynthesis of 4′-O-methylated isoflavonoid phytoalexins and disease resistance in alfalfa. *Plant Cell* **2000**, *12* (9), 1689–702.
(53) Bouwmeester, H. J.; Kodde, J.; Verstappen, F. W.; Altug, I. G.; de Kraker, J. W.; Wallaart, T. E. Isolation and characterization of two germacrene A synthase cDNA clones from chicory. *Plant Physiol.* **2002**, *129* (1), 134–44.
(54) Grey, M.; Schmidt, M.; Brendel, M. Overexpression of ADH1 confers hyper-resistance to formaldehyde in Saccharomyces cerevisiae. *Curr. Genet.* **1996**, *29* (5), 437–40.
(55) Albrecht, U.; Bowman, K. D. Gene expression in Citrus sinensis (L.) Osbeck following infection with the bacterial pathogen Candidatus Liberibacter asiaticus causing Huanglongbing in Florida. *Plant Sci.* **2008**, *175* (3), 291–306.
(56) Kitamura, Y.; Ebihara, A.; Agari, Y.; Shinkai, A.; Hirotsu, K.; Kuramitsu, S. Structure of D-alanine-D-alanine ligase from Thermus thermophilus HB8: cumulative conformational change and enzyme-

ligand interactions. *Acta Crystallogr., D: Biol. Crystallogr.* **2009**, *65* (Pt 10), 1098−106.
(57) Hardham, A. R.; Jones, D. A.; Takemoto, D. Cytoskeleton and cell wall function in penetration resistance. *Curr. Opin. Plant Biol.* **2007**, *10* (4), 342−8.
(58) Robbins, M. P.; Paolocci, F.; Hughes, J. W.; Turchetti, V.; Allison, G.; Arcioni, S.; Morris, P.; Damiani, F. Sn, a maize bHLH gene, modulates anthocyanin and condensed tannin pathways in Lotus corniculatus. *J. Exp. Bot* **2003**, *54* (381), 239−48.
(59) Ghosh, R.; Girigoswami, K. NADH dehydrogenase subunits are overexpressed in cells exposed repeatedly to H_2O_2. *Mutat. Res.* **2008**, *638* (1−2), 210−5.
(60) Fojan, P.; Jonson, P. H.; Petersen, M. T.; Petersen, S. B. What distinguishes an esterase from a lipase: a novel structural approach. *Biochimie* **2000**, *82* (11), 1033−41.
(61) Schneider, A.; Behrens, M.; Scherer, P.; Pratje, E.; Michaelis, G.; Schatz, G. Inner membrane protease I, an enzyme mediating intramitochondrial protein sorting in yeast. *Embo J.* **1991**, *10* (2), 247−54.
(62) Smith, R. D.; Lupashin, V. V. Role of the conserved oligomeric Golgi (COG) complex in protein glycosylation. *Carbohydr. Res.* **2008**, *343* (12), 2024−31.
(63) Ueguchi, C.; Koizumi, H.; Suzuki, T.; Mizuno, T. Novel family of sensor histidine kinase genes in Arabidopsis thaliana. *Plant Cell Physiol.* **2001**, *42* (2), 231−5.
(64) Sobol, R. W.; Prasad, R.; Evenski, A.; Baker, A.; Yang, X. P.; Horton, J. K.; Wilson, S. H. The lyase activity of the DNA repair protein beta-polymerase protects from DNA-damage-induced cytotoxicity. *Nature* **2000**, *405* (6788), 807−10.
(65) Madril, A. C.; Johnson, R. E.; Washington, M. T.; Prakash, L.; Prakash, S. Fidelity and damage bypass ability of Schizosaccharomyces pombe Eso1 protein, comprised of DNA polymerase eta and sister chromatid cohesion protein Ctf7. *J. Biol. Chem.* **2001**, *276* (46), 42857−62.
(66) Kamimura, Y.; Tak, Y. S.; Sugino, A.; Araki, H. Sld3, which interacts with Cdc45 (Sld4), functions for chromosomal DNA replication in Saccharomyces cerevisiae. *Embo J.* **2001**, *20* (8), 2097−107.
(67) Wada, K.; Inoue, K.; Hagiwara, M. Identification of methylated proteins by protein arginine N-methyltransferase 1, PRMT1, with a new expression cloning strategy. *Biochim. Biophys. Acta* **2002**, *1591* (1−3), 1−10.
(68) Sun, Y.; Kucej, M.; Fan, H. Y.; Yu, H.; Sun, Q. Y.; Zou, H. Separase is recruited to mitotic chromosomes to dissolve sister chromatid cohesion in a DNA-dependent manner. *Cell* **2009**, *137* (1), 123−32.
(69) Sindelar, C. V.; Downing, K. H. An atomic-level mechanism for activation of the kinesin molecular motors. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107* (9), 4111−6.
(70) Xu, Y.; Labedan, B.; Glansdorff, N. Surprising arginine biosynthesis: a reappraisal of the enzymology and evolution of the pathway in microorganisms. *Microbiol. Mol. Biol. Rev.* **2007**, *71* (1), 36−47.