Insight into Genes Regulating Postharvest Aflatoxin Contamination of Tetraploid Peanut from Transcriptional Profiling

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ABSTRACT Postharvest aflatoxin contamination is a challenging issue that affects peanut quality. Aflatoxin is produced by fungi belonging to the Aspergilli group, and is known as an acutely toxic, carcinogenic, and immune-suppressing class of mycotoxins. Evidence for several host genetic factors that may impact aflatoxin contamination has been reported, *e.g.*, genes for lipoxygenase (PnLOX1 and PnLOX2/PnLOX3 that showed either positive or negative regulation with *Aspergillus* infection), reactive oxygen species, and WRKY (highly associated with or differentially expressed upon infection of maize with *Aspergillus flavus*); however, their roles remain unclear. Therefore, we conducted an RNA-sequencing experiment to differentiate gene response to the infection by *A. flavus* between resistant (ICG 1471) and susceptible (Florida-07) cultivated peanut genotypes. The gene expression profiling analysis was designed to reveal differentially expressed genes in response to the infection (infected *vs.* mock-treated seeds). In addition, the differential expression of the fungal genes was profiled. The study revealed the complexity of the interaction between the fungus and peanut seeds as the expression of a large number of genes was altered, including some in the process of plant defense to aflatoxin accumulation. Analysis of the experimental data with "keggseq," a novel designed tool for Kyoto Encyclopedia of Genes and Genomes enrichment analysis, showed the importance of α-linolenic acid metabolism, protein processing in the endoplasmic reticulum, spliceosome, and carbon fixation and metabolism pathways in conditioning resistance to aflatoxin accumulation. In addition, coexpression network analysis was carried out to reveal the correlation of gene expression among peanut and fungal genes. The results showed the importance of WRKY, toll/Interleukin1 receptor–nucleotide binding site leucine-rich repeat (TIR-NBS-LRR), ethylene, and heat shock proteins in the resistance mechanism.

KEYWORDS peanut; aflatoxin; *Aspergilli*; *Aspergillus flavus*; keggseq; KEGG enrichment analysis

Peanut (*Arachis hypogaea* L.), an oilseed crop, is a suitable substrate for fungal growth and mycotoxin production, and is the most susceptible species for aflatoxin production as compared with other oilseed crops such as soybean (Bean *et al.* 1972). Different mycotoxins are formed on peanuts, *e.g.*, cyclopiazonic acid, zearalenone, trichothecene-toxins, and aflatoxin (Chang *et al.* 2013). The latter is the most common and destructive mycotoxin produced on peanut and other crops such as corn, cottonseed, rice, wheat, oat, and barley (Stubblefield *et al.* 1967; Cotty and Jaime-Garcia 2007; Mateo *et al.* 2011; Suárez-Bonnet *et al.* 2013; Dunham *et al.* 2017). Aflatoxin has received widespread attention since the discovery that it was the causative agent of "Turkey X disease," a disease that killed 100,000 young turkeys on English poultry farms in 1960 (Spensley 1963). Aflatoxins are in an acutely toxic, carcinogenic, and immunosuppressive class of mycotoxins affecting animals including humans (Scheidegger and Payne 2003). In addition, aflatoxins are considered mutagenic agents as they cause oxidative damage to DNA (Verma 2004). Aflatoxins are classified in four major classes: B1, B2, G1, and G2 (Ehrlich *et al.* 2004); however, aflatoxin B1 is the most potent and carcinogenic naturally occurring substance known (Squire 1981).

Aflatoxin is produced in agricultural products mainly by contaminating *Aspergillus flavus* and *A. parasiticus*. Not only are the fungal products harmful, the fungus *A. flavus* is an

Copyright © 2018 by the Genetics Society of America
doi: https://doi.org/10.1534/genetics.118.300478
Manuscript received November 5, 2017; accepted for publication March 7, 2018; published Early Online March 15, 2018.
Supplemental material available at Figshare: https://doi.org/10.25386/genetics.5984860.
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ascomycetous fungus that can infect humans, plants, animals, and insects (Klich 2007). In humans, it is the second leading cause of invasive aspergillosis disease after *A. fumigatus* (Hedayati *et al.* 2007). *A. flavus* may infect peanut and lead to aflatoxin accumulation in the field (preharvest aflatoxin contamination) or during storage (postharvest aflatoxin contamination).

Abiotic stress is an important factor contributing to preharvest aflatoxin accumulation. Drought-tolerant genotypes, sufficient irrigation, and best management practices may reduce preharvest aflatoxin contamination since drought conditions and heat stress exacerbate aflatoxin contamination (Kisyombe *et al.* 1985; Holbrook *et al.* 2000a; Craufurd *et al.* 2006; Nigam *et al.* 2009). However, better understanding of the resistance mechanisms and the development of resistant genotypes for postharvest aflatoxin contamination is needed.

Different genetic factors that may affect *Aspergillus spp*. infection and/or aflatoxin accumulation have been proposed; however, the exact role of such factors remains unclear. Lipoxygenase (LOX) is a gene super family that encodes dioxygenases. It was found to have a critical role in many disease-response mechanisms of plants such as those against nematodes (Gao *et al.* 2008; Ozalvo *et al.* 2014), rust (Choi *et al.* 2008), downy mildew (Babitha *et al.* 2004, 2006), and insects (Wang *et al.* 2008; Tang *et al.* 2009; Yan *et al.* 2013). However, in peanut it has received most attention for its potential role in resistance to *A. flavus*. Burow *et al.* (2000) isolated the first peanut LOX, PnLOX1, from a seed cDNA library and found expression to be enhanced after infection by *A. parasiticus*. An opposite result was obtained by Tsitsigiannis *et al.* (2005) while studying two more LOXs, PnLOX2 and PnLOX3, where they observed reduced expression upon infection by *A. flavus*. Two additional LOXs were discovered later, which showed various responses to *A. flavus* inoculation (Müller *et al.* 2014). In addition, LOX expression differences have been observed upon interaction of *Aspergillus spp*. with plants other than peanut, *e.g.*, soybean (Bean *et al.* 1972; Doehlert *et al.* 1993; Boué *et al.* 2005), maize (Gao *et al.* 2009; Huang *et al.* 2013), cottonseeds (Zeringue 1996), and almond (Mita *et al.* 2007).

Additionally, β-1,3-glucanases, chitinases, pathogenesis-related proteins 10 and 10.1, ribosome-inactivating proteins, and zeamatin may be related to *A. flavus* resistance (Fountain *et al.* 2014), along with WRKY transcription factors (Fountain *et al.* 2015b). Furthermore, the drought stress-responding compounds such as reactive oxygen species (ROS) are highly associated with aflatoxin production (Jayashree and Subramanyam 2000; Reverberi *et al.* 2012; Fountain *et al.* 2015a) and antioxidant enzymes are highly coexpressed with fungal growth under infection conditions (Fountain *et al.* 2016a).

Cultivated peanut, *Ar. hypogaea*, is an allotetraploid ($2n = 4\times = 40$) that was formed by spontaneous doubling of a cross between two diploid progenitors, *A. duranensis* and *A. ipaensis* (Seijo *et al.* 2004). The whole-genome sequence of tetraploid peanut is not yet available. However, high-quality, well-annotated genomes of *A. duranensis* and *A. ipaensis* have been released (Bertioli *et al.* 2016; https://peautbase.org/). The two subgenomes together have a size of ~2.7 Gb with 88,876 annotated proteins. The whole-genome sequence of *A. flavus* also has been released (https://www.aspergillusflavus.org/genomics/). The genome is 40 Mbp, containing 13,478 predicted genes on eight chromosomes. Aflatoxin biosynthesis is encoded by a 70-kbp gene cluster and has been extensively studied for *A. flavus* and *A. parasiticus* (Yu *et al.* 2004; Ehrlich *et al.* 2005; Georgianna and Payne 2009). Although only these two fungi are responsible for aflatoxin production in food products, the cluster region is conserved across other species such as *A. bombycis* and *Emericella astellata* (Amaike and Keller 2011). The aflatoxin biosynthetic pathway is responsive to environmental conditions such as temperature, stress, lipids, and salts (Bhatnagar *et al.* 2003), which makes breeding for resistance to aflatoxin production challenging.

In this study, we utilized the published peanut and *A. flavus* genomes to study the genes that respond to *A. flavus* infection and are differentially expressed during fungal interaction with resistant *vs.* susceptible peanut genotypes. Extended analysis comprising self-organizing maps, gene ontology (GO) term enrichment, Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment, and coexpression network analysis was conducted.

Materials and Methods

Plant material and infection

ICG 1471 and Florida-07 were planted on the Tifton Campus of the University of Georgia in June and harvested in October 2015. Thirty seeds from each genotype were inoculated with fungal spores, alongside 10 mock-treated seeds according to Korani *et al.* (2017). Briefly, seeds were surface sterilized for 15 min under UV light (LABCONCO purified class II biosafety cabinet, Kansas City, MO). The AF-70-GFP strain (Rajasekaran *et al.* 2008) was used for infection at a concentration of 1000 conidia/ml. The fungus was grown on potato dextrose agar (PDA) medium in petri dishes for 2 weeks at 30°, and conidia were suspended in 0.01% Tween-20 solution. The seeds were harvested at 16, 32, and 64 HAI (hours after inoculation) time points. The experiments were conducted using a randomized complete block design (10 seeds/block). Every individual seed was ground in liquid nitrogen and divided into three aliquots. The first portion was used for GFP quantification, the second for aflatoxin analysis, and the third for RNA-seq (RNA- sequencing) analysis. Sterilization, all infection procedures, and GFP and aflatoxin analyses were carried out according to the methods described previously (Korani *et al.* 2017). A Student's *t*-test was used to test the differences in GFP expression and aflatoxin contamination between the two genotypes under infection conditions for every time point (R v3.2.2) (R Core Team 2014).

RNA extraction

For every time point and genotype, the third pulverized portion of six mock-treated seeds and six infected seeds was used for RNA extraction with QIAGEN RNeasy Plant Mini kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. The quality of RNA was checked with an Agilent 2100 Bioanalyzer (Georgia Genomics Facility, University of Georgia, Athens, GA).

RNA sequencing

DNA was eliminated from the extracted RNA using DNase I, amplification grade (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Seventy-two RNA libraries were constructed using a KAPA stranded RNA-seq library preparation kit (KR0934-v1.13; Kapa Biosystems, Wilmington, MA) and the Illumina set B indexes (Illumina, San Diego, CA) according to the manufacturer's instructions. The integrity analysis and quantification of the libraries were carried out using an Agilent 2100 Bioanalyzer and Qubit 2.0 Fluorometer (Georgia Genomics Facility, University of Georgia, Athens, GA). Sequencing was done on an Illumina HiSeq2500 in six lanes, with 12 samples pooled per lane (HudsonAlpha Institute for Biotechnology, Huntsville, AL).

Differential expression analysis

The sequence quality for all libraries was determined using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/, v. 0.11.4 2015). Trimmomatic v0.36 (Bolger *et al.* 2014) was used to trim the low-quality bases and filter out low-quality sequences. The cleaned paired-end reads were aligned to a Bowtie-indexed (v1.1.0, Langmead *et al.* 2009) peanut synthetic tetraploid reference genome, containing the genomes of *A. duranensis* and *A. ipaensis* (Bertioli *et al.* 2016, https://peautbase.org/), using Tophat v2.0.14 (Trapnell *et al.* 2009). Only the cleaned paired-end reads of infected libraries were aligned to a Bowtie-indexed *A. flavus* NRRL3357 reference genome [National Center for Biotechnology Information (NCBI), txid5059] using Tophat v2.0.14. The raw counts were calculated using HTSeq v0.6.1p1 (Anders *et al.* 2015).

Differential expression analysis of counts was carried out using edgeR (Robinson *et al.* 2010). DESeq2 (Love *et al.* 2014) and Next MaSigPro (Nueda *et al.* 2014) were used to repeat the analysis for *in silico* validation. Supplemental Material, Table S1 shows different models used in the analysis. Two models were applied to test the differences between the genotypes (resistant and susceptible) due to the infection *vs.* control treatments; the second model was applied to test the differences between the responses of the fungal genes during fungal growth on the two genotypes for only high aflatoxin-contaminated treatments, since control treatments had no fungal growth and the low aflatoxin-contaminated treatment had limited fungal growth.

Cufflinks v2.2.1 (Trapnell *et al.* 2010) was used to calculate the fragments per kilobase of transcript per million mapped reads (FPKM), then the Z-score was calculated using R v3.2.2 (R Core Team 2014). The expression profile of differentially expressed genes was clustered using self-organization maps (SOMs) of the kohonen package (R Core Team 2014).

GO enrichment analysis

Libraries GenomicFeatures (Lawrence *et al.* 2013) and biomaRt (Durinck *et al.* 2005) were used to extract gene lengths and GO terms from annotation files, respectively. GO enrichment analysis of differentially expressed genes was implemented using GOseq v2.12 (Young *et al.* 2010) with a correction for gene-length bias.

KEGG enrichment analysis

KEGG enrichment analysis was carried out using the "keggseq" package for the three models described above. The KEGG enrichment analysis for a synthetic tetraploid genome requires merging the two subgenomes in one analysis. However, the available tools for KEGG analysis do not support combining two species. Therefore, we designed R packages to carry out this type of analysis designated keggseq. The *P*-value was calculated according to Yang *et al.* (2015) within the keggseq package, which is freely available to the public under Massachusetts Institute of Technology (MIT) license and can be downloaded from https://github.com/w-korani/keggseq.

The keggseq package provides some other advantages over the available tools. (1) It allows application of KEGG enrichment analysis for diploids or polyploids with any level of genome duplication; (2) it generates ready-to-publish plots and produces graphs of interested pathways that have differentially expressed enzymes marked; (3) it generates . csv files containing detailed information of enzymes included in pathways of interest; (4) it allows editing of gene identifiers (IDs) if the user wants to use an annotation different from KEGG annotation; (5) It is a run-time package since the data are downloaded directly from the KEGGs database, so it does not require an internal database for specific species; and (6) it is step-by-step and easily implemented.

De novo assembly of transcripts

The unmapped reads of ICG 1471 controls, remaining after alignment with *A. duranensis*, *A. ipaensis*, and *A. flavus* genomes, were converted back to paired-end fastq files using bamtools v2.25.0 (Barnett *et al.* 2011) and concatenated. Trinity v. 2.0.6 (Haas *et al.* 2013) was used to assemble the concatenated reads with normalization to maximum coverage of 50×. The transcripts were given IDs starting with "RC."

The process was repeated for ICG 1471 treatments, Florida-07 controls, and treatments with given IDs, starting with RT, SC, and ST, respectively. The four assemblies were combined and the redundant transcripts were filtered out using EvidentialGene pipeline (http://arthropods.eugenes.org/). Since the assembly contained sequence from peanut and sequences from *A. flavus*, BLAST+ (basic local alignment search tool; Camacho *et al.* 2009) was used to cluster the

assembly into peanut and fungal transcripts by applying BLASTn for the transcripts against the NCBI nucleotide database. Transcripts that matched plant sequences were identified as peanut transcripts and those that had fungal matches were defined as *A. flavus* transcripts. The peanut filtered assembly was merged with the peanut tetraploid assembly produced by Clevenger *et al.* (2016a). Blast2GO was used to annotate GO terms of the new transcripts (https://www.blast2go.com/). Differential expression analysis, GO, and KEGG enrichment analyses were carried out as described above in the first model (differences between resistant and susceptible genotypes due to the infection *vs.* control) (Table S1).

Coexpression network analysis

Differentially expressed gene analysis of the fungal response to the infection was carried out separately for each genotype using edgeR (Robinson *et al.* 2010). Since a fungal control treatment was lacking, the 16-hr treatment was used as control. In addition, differential expression analysis was carried out for both genotypes to test the treatment effect (controls *vs.* treatments) for time points 32 and 64 HAI for each genotype separately. The Z-scores for fungal and peanut genes were combined in one matrix/genotype with rows for gene ID and columns for time points. Pearson correlation analysis was done using the R v3.2.2 package (R Core Team 2014) as described by Musungu *et al.* (2016). Only pairs that showed correlation > 0.99 were loaded into cytoscape network v3.4.0 (Shannon *et al.* 2003). As the data set containing the correlated paired genes of the susceptible genotype was huge, the network was clustered only for the resistant genotype using the MCODE app (Bader and Hogue 2003), and then the genes that matched those of the susceptible genotype matrix were excluded from the clusters of the resistant genotype.

Data availability

The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and supplemental figures and tables. Table S1: statistical models for differential expression analysis; Figure S1: sequence read integrity and mapping results; Figure S2: SOM clusters of differentially expressed genes between genotypes; Figure S3: KEGG pathway of α-linolenic acid metabolism; Figure S4: KEGG pathway of protein processing in the endoplasmic reticulum; Figure S5: KEGG pathway of the spliceosome; Figure S6: KEGG pathway of carbon fixation; Figure S7: KEGG pathway of carbon metabolism; Figure S8: expression profile of the novel transcripts; Figure S9: GO/KEGG enrichment analysis of differently expressed genes between peanut genotypes due to the infection *vs.* control of the newly assembled transcripts; Figure S10: SOM cluster groups of fungal differentially expressed genes; File S1.xlsx: Z-scores of differentially expressed genes; File S2.fasta: 2026 novel peanut transcripts; and File S3.txt: ICG 1471 coexpression network clusters. All raw data fastq sequences are deposited at the NCBI (http://www.ncbi.nlm.nih.gov/) under BioProject PRJNA417591. All raw sequences are deposited as BioSamples SAMN08000482: SAMN08000553. The keggseq package is freely available to the public under MIT license and can be downloaded from https://github.com/w-korani/keggseq. Supplemental material available at Figshare: https://doi.org/10.25386/genetics.5984860.

Results and Discussion

Fungal growth and aflatoxin accumulation

It was shown previously that the peanut genotype ICG 1471 is a strong candidate for resistance to aflatoxin accumulation upon *in vitro* inoculation of mature peanut seeds with *A. flavus* (Korani *et al.* 2017). In addition, ICG 1471 has been reported as a resource for resistance to pre- (Waliyar *et al.* 2003; Nigam *et al.* 2009) and postharvest (Waliyar *et al.* 2008) aflatoxin contamination. Therefore, it was used in this study along with Florida-07 (Gorbet and Tillman 2009), which was a susceptible genotype for both pre- and postharvest aflatoxin accumulation (Clevenger *et al.* 2016b; Korani *et al.* 2017).

To estimate the dynamic change in gene expression, infected seeds and their controls were harvested at three different time points: 16, 32, and 64 HAI. Figure 1 shows the interaction between aflatoxin B produced by *A. flavus* and the progression of fungal growth estimated indirectly by relative fluorescence units of GFP protein signal. The data were consistent with our previous findings (Korani *et al.* 2017) as ICG 1471 showed less aflatoxin production as compared with Florida-07 (Table S1). A Student's *t*-test revealed no significant differences between the two genotypes for GFP relative fluorescence for the three time points, yet aflatoxin levels between genotypes were significantly different for all three time points.

The interaction plots revealed that not only do peanut genotypes interact differently with the fungus, but also every individual seed produced different amounts of aflatoxin within the same genotype/treatment/time point. This supports previous reports that aflatoxin accumulation is responsive to environmental influence (Blankenship *et al.* 1984; Kisyombe *et al.* 1985; Bhatnagar *et al.* 2003; Craufurd *et al.* 2006). The samples that were chosen for RNA-seq analysis were circled in the figure (Figure 1). Picking such samples with a diverse range of aflatoxin accumulation gave a realistic representation of the biological replication; however, it increases SD. Therefore, six biological replicates were used to study the differentially expressed genes due to genotypic effect.

Peanut genotypic differential expression analysis

The cleaned paired-end reads that were mapped to the synthetic tetraploid peanut and the *A. flavus* genomes are presented in Figure S1. Except for the highly fungal contaminated libraries (treatments of 64 HAI of Florida-07), 3.3–9.9 million paired-end reads were mapped to the peanut genome for every

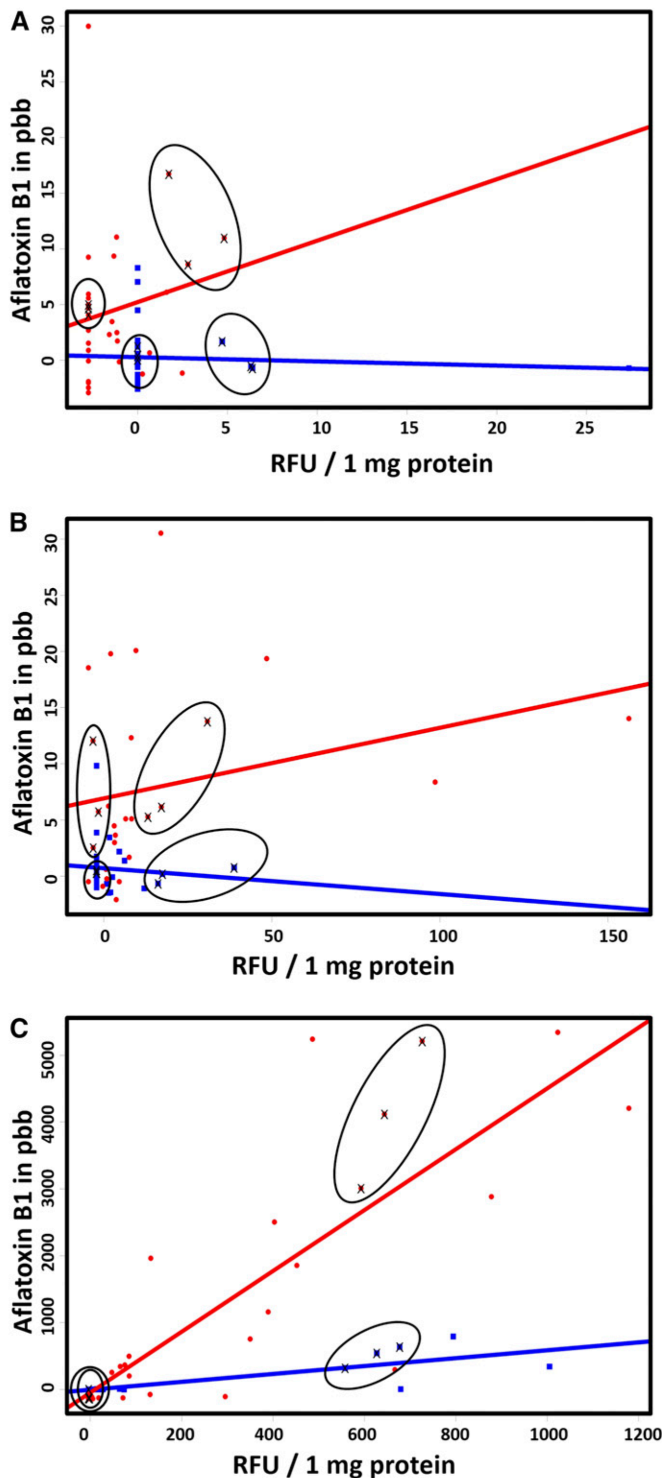

Figure 1 Interaction between GFP signals and aflatoxin levels for 16 (A), 32 (B), and 64 (C) hours after inoculation. The red line and points represent Florida-07 data; the blue line and points represent ICG 1471 data; the cross marks show the samples that were chosen for RNA-sequencing analysis; and the ovals reveal the diverse range of aflatoxin contamination. RFU stands for GFP relative fluorescence unit.

sample. This gave an average of 6 million paired-end reads/library and a total average of 5.6 million paired-end reads/library, including the highly contaminated libraries (which had 2.4–4.4 million mapped fragments/library). These results showed a reasonable coverage of the 2.7 Gb peanut genome (Bertioli *et al.* 2016).

In total, 4272 genes were differentially expressed between the two genotypes (resistant *vs.* susceptible) due to the infection by *A. flavus* (treatment *vs.* control). The Z-scores are provided in the supplemental materials (File S1.xlsx). The general pattern groups represented by SOM clusters are shown in Figure S2. Since the expression profile included the dynamic change across the three time points, some SOM clusters of these genes may have similar general trends and only differ slightly in the dynamic change from one time point to another. The clustering showed that some genes were downregulated due to the infection in the susceptible genotype and upregulated (Figure S2A) or unaffected (Figure S2B) in the resistant genotype. Conversely, some genes were upregulated due to the infection in the susceptible genotype and downregulated (Figure S2C) or unaffected (Figure S2) in the resistant genotype. A fifth group was upregulated in both genotypes but it was more highly expressed in the resistant genotype (Figure S2E).

To mitigate postharvest aflatoxin contamination of peanut, the resistance mechanisms have to be understood. Therefore, genes that are responsive to the infection (infection *vs.* mock-treatment) were studied. The large number of significant differentially expressed genes and GO terms generated by the analysis revealed the complexity of the interaction between *A. flavus* and peanut in terms of aflatoxin production and the significant effect of individual seed physiology on the process. In addition, it can be misleading, even after clustering/grouping, to assign a resistance function to a particular gene or small group of genes of interest; however, KEGG enrichment analysis gives clues to pathways that are associated with the resistance response.

GO enrichment analysis of the differentially expressed genes generated 146 significant GO terms out of 1672 GO terms found in the annotation of the two subgenomes of peanut. The 20 most significant GO terms (Figure 2A) included several for protein processing, protein polymerization, protein complex, unfolded protein binding, protein folding, protein heterodimerization activity, and protein binding. The latter GO term represented 529 differentially expressed genes. On the other hand, KEGG enrichment analysis (Figure 2B) only generated five significant pathways: α-linolenic acid metabolism, protein processing in the endoplasmic reticulum, spliceosome, carbon fixation, and carbon metabolism (Figure S3, Figure S4, Figure S5, Figure S6, and Figure S7, respectively).

As only five KEGG pathways were identified as significant for resistance (excluding individual seed effects), it can be assumed that they are the main keys controlling the defense mechanism in ICG 1471. The most interesting significant pathway is α-linolenic metabolism, which contains different components that have been reported as related to or responsive for biotic and abiotic stresses of plants. α-linolenic acid accounts for 0.37–1.11% of peanut total oil content (Ozcan

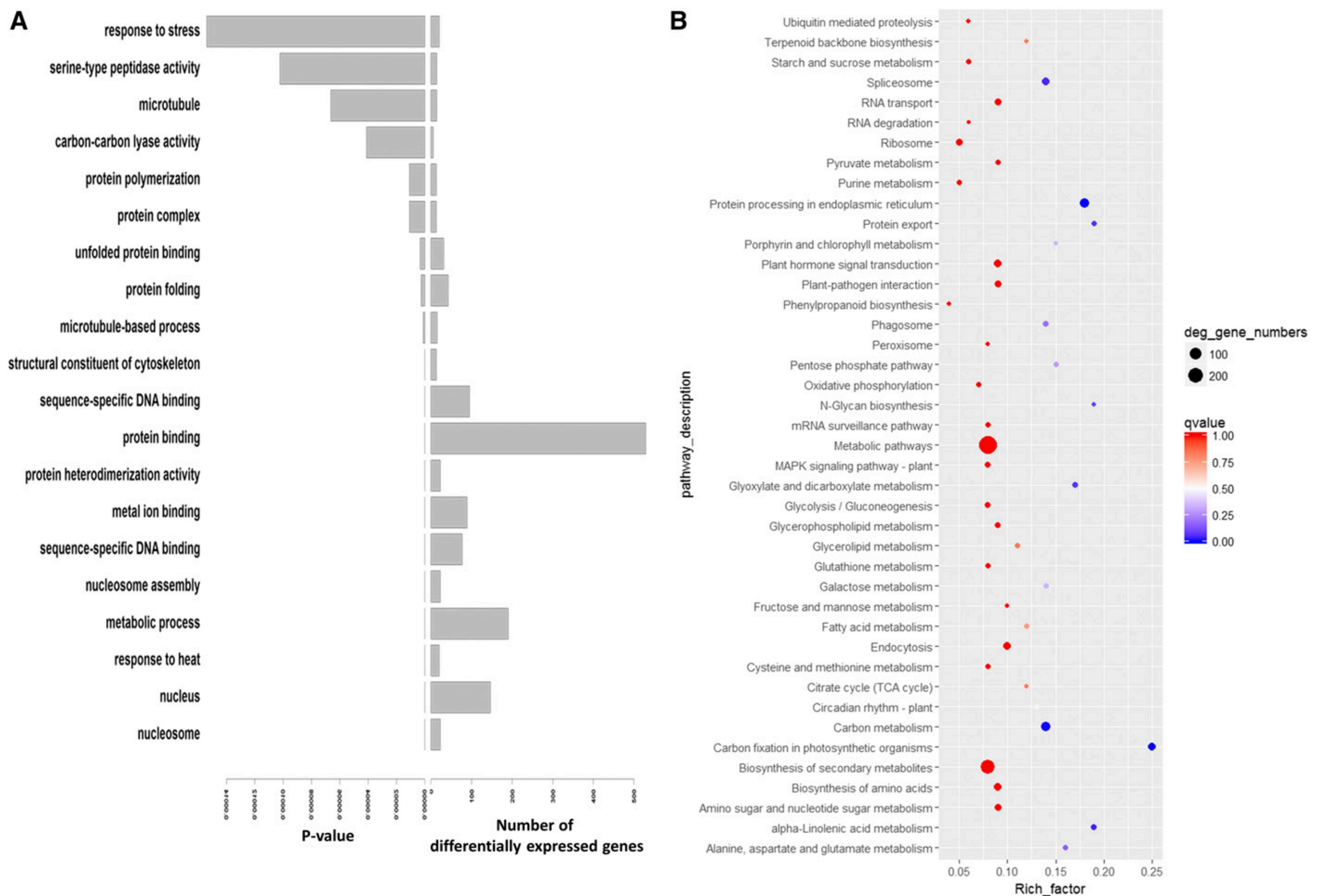

Figure 2 Gene ontology (GO)/Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of peanut differently expressed genes between genotypes due to infection *vs.* control. (A) The 20 most significant GO terms extracted by GO enrichment analysis and (B) KEGG enrichment analysis carried out by the keggseq package. Rich_factor: the ratio of differentially expressed genes to the all genes that were annotated in the pathway.

2010). Although it is a minor component of peanut oil, the pathway catabolizes α-linolenic acid into jasmonate and methyl-jasmonate and was significantly regulated in the resistant genotype ICG 1471. Jasmonates are synthesized though this pathway in two main cellular compartments: the chloroplast where α-linolenic acid is converted to 12-oxo-phytodienoic acid (OPDA) in a process initiated by chloroplast 13S-LOX (Bell *et al.* 1995), and the peroxisome where 12-OPDA is localized and converted to jasmonates (Stintzi 2000).

LOXs were documented to play a role in *Aspergillus spp*. infection and the subsequent aflatoxin contamination of different crops including peanut (Burow *et al.* 2000; Tsitsigiannis *et al.* 2005; Kumari *et al.* 2012; Müller *et al.* 2014), soybean (Bean *et al.* 1972; Doehlert *et al.* 1993; Boué *et al.* 2005), maize (Gao *et al.* 2009; Huang *et al.* 2013), cottonseeds (Zeringue 1996), and almond (Mita *et al.* 2007). Figure 3 shows nine LOXs that were found among the differentially expressed genes. BLASTp search against the NCBI nonredundant protein database was used to estimate their function; eight were predicted to generate 13-hydroxyperoxides and six had features of plastidial enzymes.

Additionally, both 12-OPDA and jasmonates were documented to play independent roles in the wound response of *Arabidopsis*, and each influences the expression of an overlapping set of genes as well as different sets of responsive genes (Taki *et al.* 2005; Sham *et al.* 2015). In addition, numerous reports showed the importance of jasmonates in plant responses to biotic and abiotic stresses, *e.g.*, insects (Thaler *et al.* 1996; McConn *et al.* 1997; Kessler *et al.* 2004), fungi (Vijayan *et al.* 1998; Thomma *et al.* 2000; Mei *et al.* 2006; Zeneli *et al.* 2006), and wounding, (Baldwin *et al.* 1997) and during development (Creelman and Mullet 1997). In particular, methyl-jasmonate was found to delay spore germination, and inhibit mycelial pigment formation and aflatoxin production of *A. flavus* (Goodrich-Tanrikulu *et al.* 1995). Interestingly, it was found to enhance aflatoxin production by *A. parasiticus* (Vergopoulou *et al.* 2001). However, Meimaroglou *et al.* (2009) showed that methyl-jasmonate might enhance or reduce aflatoxin production by *A. parasiticus* depending on its concentration. Moreover, fungal pathogens can manipulate, enhance, or suppress jasmonate signaling in plant hosts (Zhang *et al.* 2017).

Metabolic products such as 10-OPDA, which has a high phytotoxicity, are produced by the α-linolenic acid metabolism

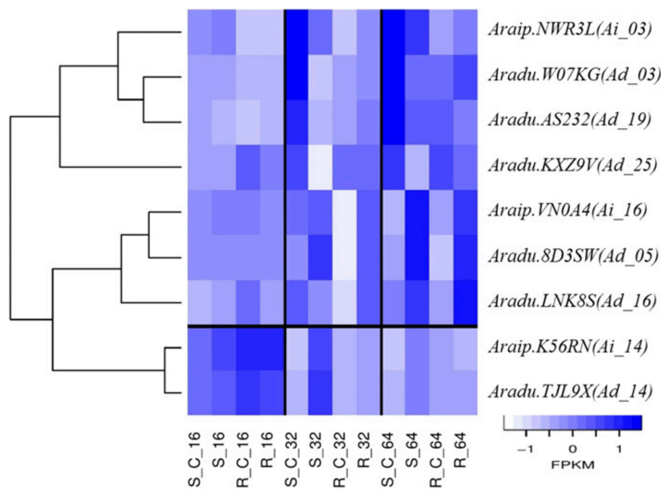

Figure 3 Differentially expressed lipoxygenases. Left, middle, and right panels are 16, 32, and 64 hours after inoculation, respectively. The upper panel is plastidial genes and the lower panel is extraplastidial genes. All genes except Ad_25 are predicted to generate13-S-hydroxyperoxides. Ad_25 was not classified. FPKM: Z-scores of fragments per kilobase of transcript per million mapped reads.

pathway enzyme 9S-LOX (Sherif *et al.* 2016). Another route through the α-linolenic acid metabolism pathway produces 8,11,14-heptadecatrienoic acid using α-dioxygenase 1 (DOX1) without LOX activity. Both the enzyme and its product were documented to increase in tobacco during interaction with *Pseudomonas syringae* pv *syringae* (Hamberg *et al.* 2003). In addition, DOX1 was upregulated in *Arabidopsis* after 12-OPDA treatment (Sham *et al.* 2015), which functioned to protect the plant from oxidative stress (De León *et al.* 2002).

Therefore, evidence suggests that, regardless of the direction of the effect, jasmonates and 12-OPDA produced by the α-linolenic pathway, in addition to other pathway components, have an important role in aflatoxin biosynthesis of *Aspergillus spp*. ICG 1471, as a resistant genotype, may regulate the synthesis of jasmonates to reduce aflatoxin production. On the other hand, Florida-07, as a susceptible genotype and a high-oleic variety, may not respond to infection with the same level of jasmonate production, possibly because of altered substrate amounts or membrane properties in a high-oleic fatty acid background, thereby resulting in elevated aflatoxin accumulation. Jasmonate levels will be tested in the future. Previously, high-oleic lines were shown to have double the aflatoxin contamination compared to normal oleic lines with similar genetic background under *in vitro* inoculation conditions (Xue *et al.* 2003). In the present study, the magnitude of aflatoxin contamination in Florida-07 is 15 times that of ICG 1471, which is a normal oleic variety. Therefore, the postharvest aflatoxin resistance conferred by ICG 1471 surpasses the effect of just having the normal oleic acid content.

Protein processing in the endoplasmic reticulum also was a significant pathway that may contribute to the resistance mechanism. However, its role may be integrated with the α-linolenic pathway since the endoplasmic reticulum contributes to the formation of peroxisomes (Hoepfner *et al.* 2005). Fountain *et al.* (2016b) showed that alternative carbon sources have different effects on aflatoxin and kojic acid production by the fungus; kojic acid has an important role in remediating damage resulting from ROS. These results reveal the importance of carbon fixation and metabolism pathways for aflatoxin production by *Aspergillus spp*. and protection of the fungus against oxidative damage.

As a synthetic reference genome of tetraploid peanut was used in our differential expression analysis, some genes/transcripts having roles in resistance to aflatoxin accumulation may not be represented within the two subgenomes. Therefore, *de novo* assemblies were constructed to capture such novel transcripts. Four assemblies were created for ICG 1471 control and treatments, and Florida-07 controls and treatments, which generated 61,176, 67,813, 90,543, and 109,068 total transcripts, respectively, and among them 413, 457, 551, and 505 were new transcripts, respectively.

To validate the genes and pathways involved in resistance, analysis of differential expression between peanut genotypes was repeated using a combined reference transcriptome (88,626 transcripts) that included the 2026 novel transcripts (supplemental materials: File S2.fasta) and the previously published tetraploid peanut transcriptome (86,600 transcripts) (Clevenger *et al.* 2016a). The differential expression analysis generated 3879 significant genes for which Z-scores are given in the supplemental materials (File S1.xlsx). The expression profile of the novel transcripts also is given (Figure S8); out of the 2026 novel genes, 66 were differentially expressed. GO enrichment analysis identified 406 out of 8530 significant GO terms (Figure S9A). Most significant GO terms resulting from genomic analysis (using predicted transcripts) also were significant in transcriptomic analysis. However, interestingly, KEGG enrichment analysis generated four of the same significant pathways as with genomic analysis (Figure S9B), except for α-linolenic acid metabolism, which was near the significance threshold with a q-value of 0.06. These outputs confirmed the key roles of these five pathways and their respective genes in the resistance of peanut to aflatoxin produced by Aspergilli.

Differential expression of fungal genes and coexpression network analysis

The interaction between peanut seeds and Aspergilli encompasses responsive pathways inside the plant and those inside the fungi, and genes regulating the signaling between organisms. Furthermore, some fungal genes may be affected differentially by growth of the fungus on different peanut genotypes. To investigate host–pathogen interaction, differential expression analysis was carried out for fungal genes, which generated 1197 significant genes (Z-scores in supplemental materials, File S1.xlsx). SOM clusters of the expression patterns of these genes (Figure

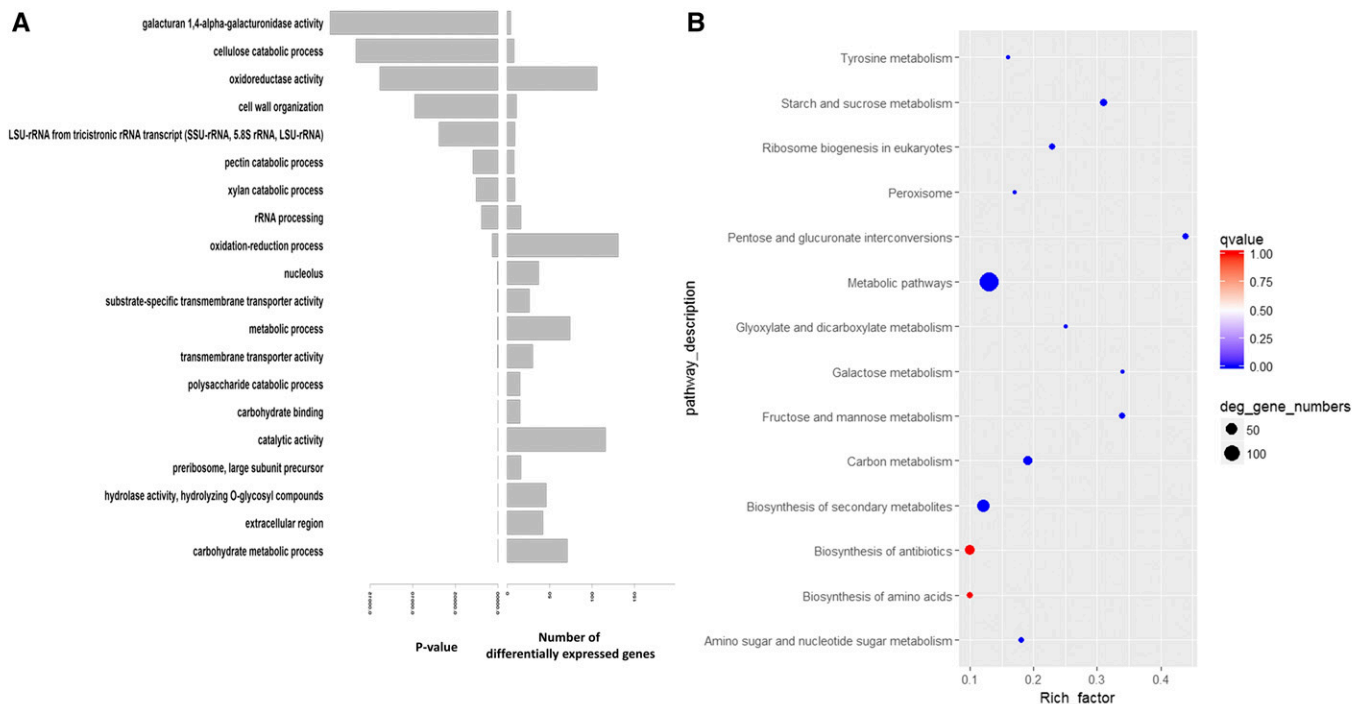

Figure 4 Gene ontology (GO)/Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of differentially expressed *A. flavus* genes due to growth of the fungus on resistant *vs.* susceptible genotypes. (A) The 20 most significant GO terms extracted by GO enrichment analysis and (B) KEGG enrichment analysis carried out by the keggseq package. Rich_factor: the ratio of the differentially expressed genes to all genes that were annotated in the pathway.

S10) and GO term enrichment analysis showed 97 significant GO terms, out of 4918 in total (Figure 4A). KEGG enrichment analysis identified eight significant pathways (Figure 4B): one interconversion pathway (pentose and glucuronate), one degradation pathway (valine, leucine, and isoleucine), and six metabolic pathways (fructose/mannose, galactose, starch/sucrose, glycerolipid, carbon, and metabolic pathways). Interestingly, seven of these pathways include carbohydrate processing. These results are in agreement with previous studies that showed changes in aflatoxin production by *A. flavus* or *A. parasiticus* using different sugar sources (Davis and Diener 1968; Abdollahi and Buchanan 1981). Growth of *A. flavus* on ICG 1471 may result in the production of different sugars than growth on Florida-07, leading to lower aflatoxin production by the fungus. A further consequence may be reduced kojic acid production and a subsequent increase in the sensitivity of the fungus to ROS. These two hypotheses need to be tested in future work.

To further investigate the differential response of fungal genes due to host genotype, coexpression network analysis based on Pearson correlation was conducted (Figure 5). In total, 1265 and 1111 differentially expressed peanut and fungal genes, respectively, were found in *A. flavus*/ICG 1471 interaction (for the time points of 64 and 32 HAI for the comparison of treatments *vs.* controls), which formed a matrix of 0.5 million correlated pairs (edges). More (6795 peanut and 1265 fungal genes) were differentially expressed in the *A. flavus*/Florida-07 interaction, which created a huge matrix of 14 million correlated pairs (edges). Figure 5 shows the interspecies peanut/*A. flavus* coexpression network for ICG 1471 (Figure 5A) and Florida-07 (Figure 5D). The MCODE cluster analysis of the ICG 1471 coexpression network generated 45 clusters (supplemental materials, File S3.txt). The most interesting clusters (subnetworks) were 1 and 15; subnetwork 1 had 1037 peanut genes and eight *A. flavus* genes (Figure 5B), including *gene10037* (AflNA), and subnetwork 15 had 28 peanut genes and only one *A. flavus* gene, *gene10043* (AflH) (Figure 5C). AflNA [averantin hydroxylase (EC 1.14.13.174)] and AflH [versiconal hemiacetal acetate reductase (EC 1.1.1.353)] encode two upstream enzymes regulating the aflatoxin biosynthetic pathway.

Out of the 1037 and 28 ICG 1471 peanut genes whose expression were highly correlated with *gene10037* and *gene10043* of *A. flavus*, respectively, 640 and 24 genes were not found in the Florida-07/*A. flavus* matrix. Among these genes, eight WRKY family transcription factors, nine toll/Interleukin1 receptor–nucleotide binding site leucine-rich repeat (TIRNBS-LRRs), six ethylene signaling proteins, and one heat shock protein were upregulated, and expression was correlated with *gene10037*. One heat shock and an ethylene signaling gene were upregulated, and expression was correlated with *gene10043*. Figure 6 represents the expression profile of these genes. Although gene expression was upregulated in both genotypes for all genes,

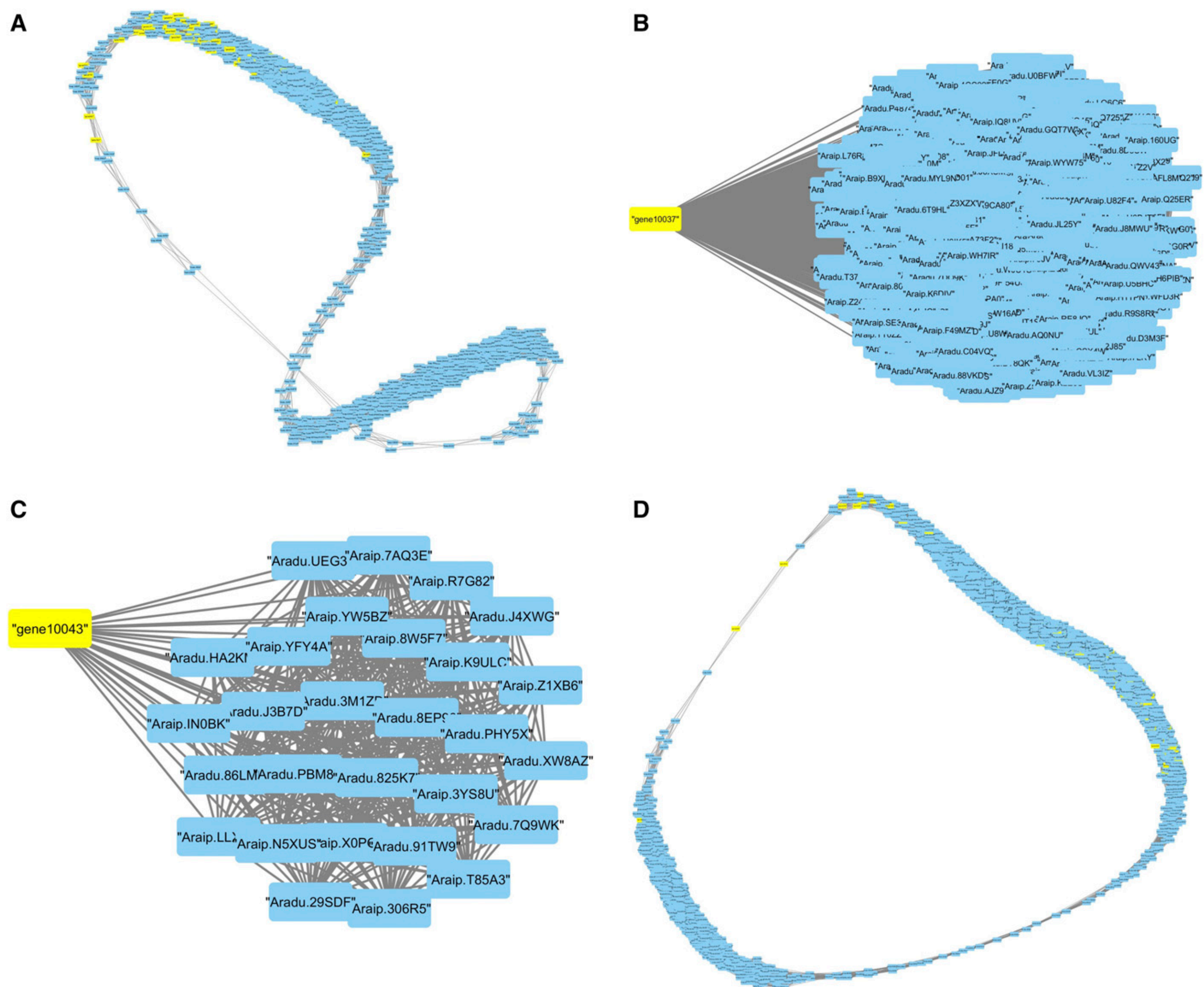

Figure 5 Coexpression network analysis of peanut/*A. flavus* genes. (A) ICG 1471/*A. flavus* network. (B) subnetwork 1 of ICG 1471/*A. flavus* network. (C) subnetwork 15 of ICG 1471/*A. flavus* network. (D) Florida-07/*A. flavus* network; lines represent edges, blue rectangles are peanut nodes, and yellow rectangles are *A. flavus* nodes.

ICG 1471 genes were coexpressed with *gene10037* or *gene10043* of *A. flavus*.

Many plant disease-resistance genes encode NBS-LRR proteins (McHale *et al.* 2006; Sekhwal *et al.* 2015). Ethylene signaling genes were significantly upregulated in response to *A. flavus* infection of maize (Musungu *et al.* 2016). Heat shock proteins may play a role in plant defense by affecting R protein stability and their regulation (Lee *et al.* 2012). WRKY transcription factors were differentially expressed in the response of resistant and susceptible genotypes of maize to infection by *A. flavus* (Fountain *et al.* 2015b). In addition, they were found to have an effect on pathways for ethylene-jasmonate-mediated defense (Birkenbihl *et al.* 2012), plant response to heat stress (Li *et al.* 2010), and defense triggered by jasmonates, either negatively (Gao *et al.* 2011) or positively (Journot-Catalino *et al.* 2006). These eight WRKY genes may be important in controlling jasmonate defense mechanisms. In addition, the high correlations between expression of these genes in ICG 1471 and *gene10037* of *A. flavus* reveals their importance in the defense mechanism and suggests that they may be involved in regulation of the α-linolenic acid metabolism pathway in ICG 1471.

In silico validation of differential expression analysis

In this study, different complex factors were involved in the RNA-seq experiment, *e.g.*, genotypic effect, *A. flavus* infection, and time-course dynamic change. Therefore, three analytical models were compared (Figure 7). Across all analyses, DESeq2 showed similar results to edgeR for identifying differentially expressed genes. On the other hand, Next maSigPro identified many genes that were not discovered

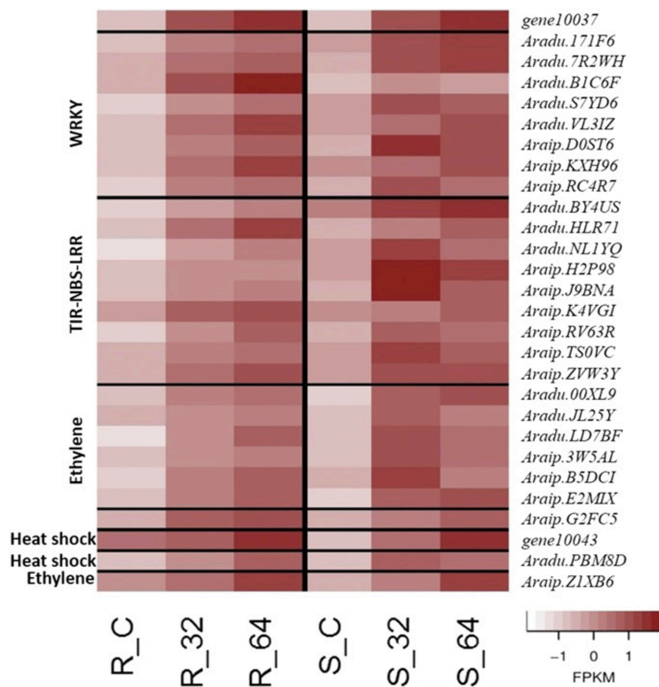

Figure 6 Peanut coexpressed genes with *gene10037* and *gene10043* of *A. flavus*. FPKM: Z-scores of fragments per kilobase of transcript per million mapped reads.

by the other two methods and failed to extract many other genes that were determined to be differently expressed by the other two methods.

EdgeR is one of the most common methods used for differential expression analysis of RNA-seq data. However, it is not a standard method to handle the time course experiments as it uses a negative binomial model, which deals with time points as independent factors (Robinson *et al.* 2010). Methods have been designed to account for time course experiments that used different models such as Next maSigPro (polynomial regression model) (Nueda *et al.* 2014), DyNB (nonparametric Gaussian processes regression negative binomial likelihood model) (Äijö *et al.* 2014), TRAP (β-negative binomial model) (Jo *et al.* 2014), SMARTS (input–output hidden Markov model) (Wise and Bar-Joseph 2015), EBSeq-HMM (empirical Bayes mixture model) (Leng *et al.* 2015), FunPat (different distribution models) (Sanavia *et al.* 2015), and timeSeq (negative binomial mixed-effect model) (Sun *et al.* 2016). All these methods had limitations and none was standardized to this type of analysis. Next, maSigPro was initially designed to analyze microarray data using polynomial regression and later was updated to handle RNA-seq data (Nueda *et al.* 2014). This method relies on R^2 factor to extract the significant differentially expressed genes, which is considered a drawback since the threshold is user-defined (Spies and Ciaudo 2015). Although both edgeR and DESeq2 use a negative binomial model, DESeq2 has different implementation, tests, and normalization (Love *et al.* 2014). Both gave a reasonable level of analysis validation.

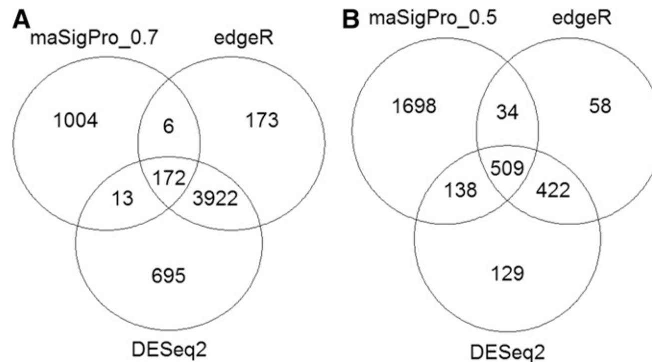

Figure 7 Differential expression analysis with multiple programs for *in silico* validation of peanut genotypic differences due to *A. flavus* infection (A) and fungal/peanut-genotype effects (B).

Conclusions

The objective of this study was to identify genetic factors and biochemical pathways that function to limit aflatoxin production in resistant peanut genotypes. Differential expression analysis revealed five important biochemical pathways regulating resistance. In addition, results captured the fungal pathways that are differentially affected by fungal infection and aflatoxin production on resistant *vs.* susceptible peanut genotypes. The study highlighted the critical role of the α-linolenic acid metabolism pathway and certain WRKY genes likely regulating the jasmonate-based defense pathways to mitigate aflatoxin production. To further estimate the effects of these components on aflatoxin production and/or identify effective QTL, we have created a population between ICG 1471 and Florida-07 that is being advanced to recombinant inbred lines.

Acknowledgments

We thank Jeff Cary, Unites States Department of Agriculture (USDA)-Agricultural Research Service-Southern Regional Research Center, New Orleans, for providing AF-70-GFP, the GFP-expressing *A. flavus*. This work was supported by the Peanut Foundation, the Agriculture and Food Research Initiative competitive grant 2012-85117-19435 of the USDA National Institute of Food and Agriculture, and the Feed the Future Innovation Lab for Collaborative Research on Peanut Productivity and Mycotoxin Control (Peanut and Mycotoxin Innovation Lab), supported by funding from the United States Agency for International Development. The authors declare no conflict of interest.

Author contributions: W.K. performed the experiments, designed keggseq, applied the data analysis, and wrote the manuscript. Y.C. assisted with experimental design and helped in laboratory work training. C.C.H. provided the peanut genotypes, field data, and assistance with aflatoxin analysis. P.O.-A. conceived and supervised the project, secured funding, and revised and submitted the manuscript.

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Communicating editor: J. Birchler