Comprehensive insights and their role in pre-harvest aflatoxin accumulation, comparative proteomics provide insights on the basis of resistance to *Aspergillus flavus* infection and aflatoxin production in peanut (*Arachis hypogea* L.)

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**ABSTRACT**

*Aspergillus flavus* is an opportunistic fungal pathogen that produces carcinogenic aflatoxin, a serious constraint for food safety and human health. In this study, to better understand the molecular mechanism/s of peanut resistance to *A. flavus* growth and aflatoxin accumulation, comparative proteomic analysis was performed in two contrasting peanut genotypes, variety JL 24 (susceptible) and its near-isogenic resistant transgenic derivative expressing an alfalfa defensin gene. Several resistance proteins associated with secondary metabolic pathways were strongly induced in the resistant genotypes including phenylalanine ammonia lyase, cinnamic acid-4-hydroxylase, chalcone synthase, resveratrol synthase, flavanone-3-hydroxylase, lipoxygenase, diacylglycerol-glycerol-3-phosphate-3-phosphatidyltransferase, β-ketoacyl-ACP-reductase, monoacylglycerol acyltransferase, and diacylglycerol acyltransferase, indicating their roles in resistance. Besides, several putative susceptibility-associated proteins were revealed providing knowledge on potential candidate target genes for precise breeding interventions for aflatoxin mitigation. This is the first study to demonstrate comparative proteomics analysis using contrasting near-isogenic lines to elucidate the underlying molecular mechanisms of resistance.

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1. Introduction

Peanut (*Arachis hypogaea* L.) is an important grain legume and nutritious food ingredient, being the third most important source of vegetable protein and oil that plays a significant economic role in small-scale farming systems (Variath and Janila 2017). However, predominantly grown as a rainfed crop, it is often exposed to end-of-season dry spells during harvest, thereby aggravating *Aspergillus flavus* infection resulting in pre- and post-harvest aflatoxin contamination, diminishing the crop quality (Klich 2007). Aflatoxins not only pose a threat as a carcinogen and hepatotoxic to the health of humans and livestock (Bryden et al. 2012; Sharma et al. 2018), but also lead to considerable economic losses due to international trade restrictions (Bandyopadhyay and Cotty 2013). Besides, due to the lack of sustainable resistance sources in the available peanut germplasm, there is an urgent need to deploy genetic solutions for durable resistance to the fungal infection and aflatoxin contamination in elite peanut cultivars. However, this can only be accomplished following an in-depth understanding of the mechanisms of resistance.

Qualitative or quantitative resistance to *A. flavus* infection may be governed by either a single or several genes (Chen et al. 2010; Nigam et al. 2009; Pandey et al. 2014) and numerous efforts have been made to develop aflatoxin-free peanut lines through breeding (Pandey et al. 2019). The identification of quantitative trait loci (QTL) reported for aflatoxin resistance in peanut showed 8–21% phenotypic variance (Pandey et al. 2014; Yu et al. 2019). Nevertheless, no remarkable success in developing durable resistance has been achieved so far through breeding due to the non-availability of highly resistant germplasm (Nigam et al. 2009). Limited success has been achieved through host–plant resistance strategies using transgenics that overexpressed anti-fungal peptides/proteins such as chitinases, β-1,3-glucanase, protease inhibitors, thionins, lectins and polygalacturonase inhibitor proteins (Bhatnagar-Mathur et al. 2015; Cary et al. 2011). Our previous research demonstrated that overexpression of defensins and host-induced gene silencing (HIGS) of *Aspergillus* biosynthetic pathway genes has a potential to provide heritable resistance, thereby offering opportunities for achieving durable quantitative resistance to fungal infection and reduction in aflatoxin contamination using a two-pronged strategic interventions (Arias et al. 2015; Sharma et al. 2018).

Technological advances in ‘omics’ have been explored to identify and characterize resistance-associated proteins and genes for aflatoxin contamination (Wang et al. 2013; Zhao et al. 2019). While transcriptomic studies related to *A. parasiticus* infection under drought conditions have revealed a number of genes potentially associated with drought tolerance and *A. parasiticus* interactions, these do not offer comprehensive insights and their role in pre-harvest infection of peanut is still not well understood (Luo et al. 2005; Nayak et al. 2017). Hence, it is pivotal to understand the *Aspergillus*–peanut pathosystem and underlying molecular mechanisms for better insights on the genes and pathways that play a critical role in host–pathogen interaction as well as the resulting aflatoxin production.

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Proteomics is a powerful tool to gain insights on the dynamics of proteins expressed by genomes under different environmental stresses and biological processes (Graves and Haystead 2002; Jamshidi Goharrizi et al. 2019, 2020). Linking proteome analyses with genomic information is essential to define the function of the genes associated with any trait. Proteomics approaches have been previously used to study several molecular events underlying the development of peanut seed (Kang et al. 2007), gynophore (Zhao et al. 2015), leaf (Katam et al. 2010), allergens (Porterfield et al. 2009) and drought tolerance (Kottapalli et al. 2013). Efforts have also been made to investigate the seed proteins associated with aflatoxin contamination in a few resistant peanut genotypes (Wang et al. 2010, 2012; Zhao et al. 2019), thereby revealing some differentially expressed proteins (DEPs) associated with peanut pre-harvest aflatoxin contamination. Non-target proteomics, based on 2D gel electrophoresis combined with LC-MS/MS, has been earlier used to explain the resistance mechanism against A. flavus (Ouakhssase et al. 2019). Moreover, diverse mechanisms of resistance such as oxidative stress response, induction of PR proteins and activation of secondary metabolic pathways have been revealed using proteomic approaches (Balof et al. 2021; Elmore et al. 2021). However, most of these comparative host–pathogen proteome responses were carried out using genotypically diverse backgrounds posing bottlenecks with respect to pinpointing the role of candidate genes underlying resistance (Chen et al. 2009).

Our previous studies have demonstrated a successful host–plant resistance strategy to create peanut germplasm with improved genetic resistance to A. flavus infection and aflatoxin contamination (Sharma et al. 2018). Considering that the transgenic events overexpressing MtDef genes were nearly isogenic to the wild-type parent JL 24, except for a single gene insert offered opportunities to deep dive into the molecular basis of resistance to fungal infection and aflatoxin production in Aspergillus–peanut pathosystem (Sharma et al. 2018). Considering that the transgenic events overexpressing MtDef genes were nearly isogenic to the wild-type parent JL 24, except for a single gene insert offered opportunities to deep dive into the molecular basis of resistance to fungal infection and aflatoxin production in Aspergillus–peanut pathosystem without any background noise (Chen et al. 2009). To better understand the molecular mechanisms underlying resistance/susceptibility to A. flavus infection and resulting aflatoxin contamination, we used non-target proteomics based on liquid chromatography hybrid mass spectrometry (LC-MS/MS) of two contrasting peanut lines (resistant transgenic and susceptible wild-type) under aeration at 30 °C in dark. Inoculated and un-inoculated samples were collected at 24, 48, 56, and 72 h post-inoculation (hpi) for all the experiments. All samples were immediately frozen in liquid nitrogen and stored at −80 °C until use.

For aflatoxins quantitation, the tissue samples collected at 72 hpi were subjected to ELISA assays. For this, 100 mg of cotyledon sample was extracted overnight in 0.5 mL methanol (70% methanol + 0.5% KCI). Sample extracts were diluted 1:10 in PBST-BSA for quantitative ELISA using the standard protocol (Kumar et al. 2017).

2.2. Aspergillus flavus growth conditions
Aspergillus flavus strain AF 11–4 obtained from the groundnut pathology collections at ICRISAT, India (Mehan et al. 1995), was used for fungal bioassays. Fungal mycelia were cultivated on potato dextrose agar (PDA) medium at 28°C until they showed sporulation. Virulent spores were collected in sterile distilled water containing 0.05% Tween 20 and diluted to a concentration of 5 × 10^6 spores/mL using a Neubauer haemocytometer, and the colony-forming units (CFUs) determined by standard 10-fold dilutions to obtain ~40,000 cfu/mL on A. flavus parasiticus agar (AFPA) medium.

2.3. Fungal bioassays and aflatoxin analysis
Peanut seeds were screened for infection and aflatoxin resistance and susceptible as previously described (Arias et al. 2015) with minor modifications. Matured seeds from the resistant OE-Def line (Def4Ec 97-1-8) and susceptible WT parent (JL 24) were surface sterilized with 0.1% (w/v) mercurochloride for 3 min, followed by two to three times washes with sterile distilled water and soaked in sterile distilled water for 2 h. Subsequently, the seed coats were removed to eliminate the potential barrier to A. flavus growth and cotyledons were cut into two vertical halves. Embryonated half was used for generation advancement and the other cotyledon was further halved and placed in Petri dishes containing sterile agar (1.7% agar/water; 12 vertically cut cotyledons (¼) per plate), with cut surface exposed. Each piece was infected with 2 µl of A. flavus spore suspension containing 5 × 10^7 spores/mL and incubated under aeration at 30 °C in dark. Inoculated and un-inoculated samples were collected at 24, 40, 56, and 72 h post-inoculation (hpi) for all the experiments. All samples were immediately frozen in liquid nitrogen and stored at −80 °C until use.

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2.4. Fungal load detection
Fungal genomic DNA was isolated using 100 mg of mycelium from A. flavus cultures using PureLink Plant Total DNA Purification kit (Invitrogen, Carlsbad, CA, USA). The purified DNA was evaluated in 0.8% (w/v) agarose gel followed by quantitative and qualitative determination using Qubit Fluorometer 2.0 and spectrophotometer (GE Healthcare, NJ, USA), respectively, and stored at −20°C until use.

Aspergillus flavus biomass in the infected samples of WT control and OE-Def lines was quantified based on quantitative PCR (qPCR). Fungal gene, FLAV-specific primers
(Sardiñas et al. 2011) was used to amplify A. flavus ITS2 region using qPCR, according to the manufacturer’s instructions. Aspergillus flavus DNA ranging from 10 ng to 0.01 pg was used to prepare the standard curve. The logarithm of starting quantity of template for each dilution was plotted against the cycle threshold values (Ct) to obtain the standard curve. Amplification efficiencies were calculated from the slopes of the standard curves.

### 2.5. Gene expression studies

Total RNA was extracted from the pulverized frozen cotyledons collected at 0 and 40 hpi using the RNeasy Plant Mini kit (Qiagen, Tokyo, Japan), and quantified with Nanodrop ND 1000 Spectrophotometer (Nanodrop, Wilmington, DE, USA) followed by DNase treatment using the DNase Kit (Fermentas, Hanover, MD, USA) as per the manufacturer’s instructions. A 2.0 μg aliquot of purified RNA was used for cDNA synthesis following the recommended protocol (Thermoscript RT–PCR system, Invitrogen, Carlsbad, CA, USA). Quantitative PCR was carried out with gene-specific primers (Table S1) in a Realplex Real Time PCR system (Eppendorf, USA). The peanut (EG529529) and G6PD (EG030635) genes were used as housekeeping genes for normalization of cycle threshold (Ct) values (Reddy et al. 2013). qRT–PCR reaction conditions were set in triplicates as: 2 min at 95°C, 40 cycles of 10 s at 95°C, 20 s at 60°C followed by fluorescence measurement at each 0.5°C variation from 60°C to 95°C in 20 min was included to obtain the melting curve. Relative fold expression was calculated by $2^{-ΔΔCt}$ method (Livak and Schmittgen 2001).

### 2.6. Protein extraction and digestion

Total proteins were extracted following an earlier reported protocol (Kottapalli et al. 2013). Briefly, three replicates of 0.2 g of frozen cotyledons were homogenized and suspended in 1 mL of extraction buffer containing 0.9 M sucrose, 0.1 M Tris (pH 8.8), 10 mM EDTA, 50 mM dithiothreitol (DTT), 1 mM phenyl methyl sulfonyl fluoride (PMSF), and 25 μL of protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA). An equal volume of Tris-saturated phenol was added and mixed thoroughly by shaking for 30 min at 4°C followed by centrifugation (5000 g, 4°C) for 20 min. The upper phenolic phase was collected and re-extracted twice as described above. The proteins in the phenolic phase were precipitated by centrifugation (5000 g for 30 min at 4°C) by adding 5 volumes of 0.1 M ammonium acetate containing 10 mM DTT. The protein pellet was washed twice with methanol containing 10 mM DTT followed by another wash with acetone containing 10 mM DTT. The pellet was either dissolved in rehydration buffer (8 M Urea, 4% CHAPS (w/v), 40 mM Tris-base) or stored at −80°C until further use. The protein yield was quantified and normalized for equal concentrations by polyacrylamide gel electrophoresis (PAGE).

### 2.7. In-gel digestion

In-gel digestion was carried out and peptides were extracted based previously reported protocol (Kilambi et al. 2016). Briefly, 100 μg of proteins dissolved in rehydration buffer (8 M Urea, 4% CHAPS (w/v), 40 mM Tris-base) and then separated on 12% SDS–PAGE as per standard protocol (He 2011). After completing de-staining, each gel lane was cut into 36 slices, and 12 slices were pooled in a single fraction (total 3 fractions were obtained). The proteins in each slice were prepared for mass spectrometry by reduction and alkylation using 10 mM DTT for 30 min and 50 mM iodoacetamide for 45 min, respectively, followed by overnight trypsin digestion using sequencing grade pore size trypsin (Promega, USA) in 1:25 of enzyme: protein ratio at 37°C (Parankusam et al. 2017). Peptides from each fraction were separately extracted by addition of 60% (v/v) acetonitrile (ACN) containing 0.1% (v/v) formic acid and sonicated in ice for 30 min. This step was repeated thrice, and peptides obtained from each extraction step were pooled. The pooled peptides for each fraction were then concentrated using a speed vacuum concentrator (Thermo Scientific, USA), and desalted using C18 spin columns (Thermo Scientific, USA). These samples were either subjected to proteomics analysis immediately or stored at −80°C.

### 2.8. Mass spectrometry and data acquisition

The trypsin digests were reconstituted in 50 μl of 0.1% formic acid followed by separation on the Acquity BEH C18 UPLC column (75 μm × 150 cm × 1.7μm; Waters, UK) connected to UPLC system for 90 min using LC-MS grade water in 0.1% formic acid (v/v; mobile phase A) and acetonitrile in 0.1% formic acid (mobile phase B). The mobile phase B was linearly ramped from 53 to 85% over 90 min. The initial flow rate was 5 μL/min of 97% mobile phase A for 3 min, while the flow rate was maintained at 200 μL/min for the remaining period. The column temperature was kept at 60°C. The UPLC separated peptides were analyzed for MS and MS/MS fragmentation on Xevo-G2-XS (Waters, Milford, MA, USA) with ESI source on a positive mode. Data were acquired in resolution mode using the following settings (Collision Energy: 1. Low Energy: Trap: 4 V, Transfer: 4 V; 2. High Energy: Trap Collision Energy Ramp: 15–40 V; Transfer Collision Energy Ramp: OFF; Cone Voltage: 40 V) (Parankusam et al. 2017). The scan time was set to 0.5 s in continuum mode and the set mass range was 50–2000 Da in TOF. The Leucine encephalin (Sigma-Aldrich, USA) at 200 pg/μL was used as an external calibrant and lock mass acquisition was performed every 30 s. Mass spectra of the samples were acquired by MassLynx v4.0 software (Waters, Milford, MA, USA) and further submitted for protein identification and expression.

### 2.9. Data analysis and bioinformatics

Proteins were identified and quantified from raw spectra by Progenesis QI for Proteomics Software V.4.0 (Nonlinear Dynamics, Newcastle, UK). Progenesis aligned the imported MS scans based on retention time and processed them to generate peak lists containing m/z and abundance for each replicate. To increase sensitivity for peak detection, parameters were set to 5 and captured proteins with charge higher than 5, fall in detection range 100–1600 m/z and between 5 and 80 min. Triplicate MS scans were grouped according to the treatment followed by normalization and comparison of abundance data before statistical evaluation. However, conflicting peptides assigned to more than one protein were excluded from quantification. Protein
identifications were performed using an inbuilt ion accounting algorithm of Progenesis against the peanut proteome downloaded from UniProt (http://www.uniprot.org/). The following parameters were set for searches against target database: tryptic cleavage with a maximum of two missed cleavages; maximum mass errors of 20 ppm; minimum fragment ion per peptide equal to two, minimum fragment ion per protein equal to five, minimum peptide per protein equal to two; a minimum of two unique peptides matching the protein; carbamidomethylation of Cys was considered as fixed modifications and oxidation of Met and Gln-PyroGlu (N-term Gln) as variable modifications. The false discovery rate (FDR) for the statistically significant proteins was found to be 5% (Benjamini and Hochberg 1995). Quantitative analysis was performed based on the ratio of protein ion counts among contrasting samples or treatments. The resulting dataset was further filtered for proteins quantified based on at least two unique peptides and considered up- or downregulated only if the fold change was 1.5 with significant P-value ANOVA (P ≤ 0.05). Protein subcellular localization was predicted for significant proteins using Plant-mPLoc (Chou and Shen 2010). The mass spectrometry proteomics data have been deposited to the ProteomeXchange consortium via jPOSTrepo (Okuda et al. 2017) with the database identifier PXD027315.

2.10. Functional annotation and pathway mapping

The unique and DEPs were classified into Gene Ontology (GO) categories using MapMan application (http://mapman.gabipd.org) (Lohse et al. 2014). GO assignments are helpful to classify the functions of the identified proteins. To understand the potential involvement of these predicted proteins in biological pathways, the proteins were mapped to the reference canonical pathways using Arachis genus as reference in KEGG automated annotation server KAAS (http://www.genome.jp/kaas-bin/kaas_main). To identify the similar expression patterns of A. flavus-responsive proteins, a hierarchical clustering of 50 common A. flavus-responsive proteins that had at least 1.5-fold variation were clustered using MeV software (Version 4.8, USA) based on the Euclidean distance method.

2.11. Statistical analysis

The data were analyzed using Sigmaplot (ver.11) based on the mean and standard error (SE) values in all assays including in vitro seed colonization experiments. Statistically significant differences between treatments were determined by one-way ANOVA using Student’s t-test method. The statistical analysis was carried out with at least three biological replicates. By analyzing protein abundance values, only statistically significant results were considered (P ≤ 0.05), and DEPs with a ratio of at least 1.5 in absolute value observed in at least two biological replicates were used for data analyses.

3. Results

3.1. Confirmation of transgenic resistance to A. flavus infection and aflatoxin

The cotyledons of T4 progenies of peanut transgenic OE-Def lines were reconfirmed for transgene heritability and evaluated for fungal resistance using in vitro seed colonization assays using A. flavus strain AF 11–4 (Figure 1(A,B)). Based on visual scoring, the transgenic lines showed less than 20% mycelial coverage of kernels after 72 hpi, compared to WT controls that showed over 85% coverage. Furthermore, fungal biomass was measured as fungal load from the inoculated cotyledons of OE-Def and WT controls using qPCR analyses. On an average, 7.22 ng/µL of A. flavus-specific DNA was detected from OE-Def events, compared to 88.02 ng/µL in the WT control lines at 72 hpi, respectively (Figure 1C). These results revealed that the infected host tissues of the WT control lines showed significantly higher fungal development than OE-Def lines. No fungal growth was detected in the un-inoculated cotyledon samples. ELISA revealed significantly lower aflatoxin levels in the inoculated OE-Def cotyledons (P ≤ 0.05) than the WT controls (Figure 1D). Transgenic OE-Def lines accumulated <5 ppb aflatoxin compared to >4000 ppb detected in the WT controls following 72 h of fungal infection, indicating very high levels of resistance to aflatoxin contamination.

3.2. Impacts of A. flavus infection on the proteomes

To understand the proteome changes in OE-Def and WT control lines, a label-free quantitative proteomics analysis was performed at 0, 24, 40, 56, and 72 h after A. flavus inoculation using UPLC-ESI-MS/MS. A time point comparison within a genotype revealed expression of 2975, 1989, 2085, 1993, and 2014 proteins in WT control cotyledons when compared with 3508, 2108, 2118, 2045, and 2040 proteins in OE-Def lines. However, comparison between WT control and OE-Def lines revealed the expression of 3020, 2983, 2446, 2446, 2410, and 2880 proteins over a 72 h period (Tables S2 and S3).

Comparative quantitative protein expression analysis was performed to identify the A. flavus-responsive proteins using the proteins having at least two unique peptides with 1.5-fold change at P ≤ 0.05 between the uninfected and infected groups in both control and transgenic lines. Expression analysis indicated differential regulation of 2327 proteins across various time points, of which 1269 and 1058 proteins were down- and up-regulated in WT controls, respectively (Figure 2A). Similarly, in the transgenic lines, 2612 proteins were differentially regulated upon infection, of which 1422 proteins were down-regulated, and 1190 proteins were up-regulated (Figure 2B). Further, we identified 569/592, 579/589, 664/698, and 515/733 DEPs in control/transgenic cotyledons and over 879, 871, 907, and 900 DEPs between the WT control and transgenic samples (Figure 2C) under A. flavus infection at 24, 40, 56, and 72 hpi, respectively, indicative of the proteome changes in response to pathogen at different time points (Figure 2D). In addition, 125 proteins in control and 201 proteins in the transgenic lines, and 290/310, 279/292, 209/243, and 167/385 unique proteins were revealed between WT control and transgenic lines consistent in their presence across the tested time points.

3.3. Functional classification of identified proteins based on GO annotation

GO annotation was performed to functionally classify the identified proteins providing details of the hierarchical relationships of cellular components, biological processes,
and molecular functions (Figure 3). The detailed information, including protein IDs, KEGG, and subcellular localizations of all identified proteins, is listed in Tables S2 and S3. Analysis of putative functions revealed 21 GO terms related to biological processes, eight GO terms for molecular functions and six GO terms for cellular components. In terms of the cellular components, the most represented categories were cell, cell parts, and organelles. In the molecular function category, proteins with binding and catalytic activity were highly represented. Regarding biological process, most proteins were involved in cellular processes, response to stimulus, and metabolic processes. Broad functional distribution analysis showed that the DEPs in the contrasting lines shared the same categories in biological, molecular, and cellular process with the differences in the proportional distribution of the proteins (Figure 3).

3.4. Enrichment analysis of DEPs in OE-Def and WT lines under A. flavus infection

The DEPs from OE-Def and WT control lines were mapped individually, and in comparison, to each other and to different functional categories in the MapMan tool to reveal the A. flavus-affect ed pathways. All DEPs were associated with 35 pathways (Figure 4A–C), significantly enriched are ones associated with stress (8–19%), signaling (5–10%), protein (9–11%), lipid metabolism (5–10%), and photosynthesis (5%). Functional protein association network built using STRING indicated 633 mapped nodes in WT controls, while 656 nodes were mapped in OE-Def samples. According to the interaction network, 28 KEGG pathways significantly enriched in WT controls while 34 pathways enriched in OE-Def lines under A. flavus infection. K-means clustering indicated five protein co-interacting clusters in control lines while six clusters were prominent in transgenic lines which mainly belongs to fatty acid metabolism, nitrogen and carbohydrate metabolism, flavonoid synthesis, receptor-like protein kinase, and disease resistance NBS-LRR proteins (Figure 4D,E). A few A. flavus-responsive proteins that could not be identified by the UniProt database were matched with their corresponding homologous proteins using UniProt blast.

Nine protein clusters were identified while comparing the proteins from WT control and transgenic samples (Figure 4F). In Group 1, the proteins were mainly involved in energy production and conversion, lipid transport and metabolism, nucleotide transport and metabolism, posttranslational modification, protein turnover, and chaperones. Group 2 included ribosomal proteins with different functions involved in translation, ribosomal structure, and biogenesis. Group 3 included proteins mainly involved in carbohydrate transport, energy production, and conversion. Group 4 had proteins belonging to energy production and metabolism, Groups 5 and 6 contain linked proteins belongs to fatty acid biosynthesis and metabolism. While the proteins in Group 7 represent photosystems, Groups 8 and 9 have proteins involved in flavonoid synthesis and cytoskeletal rearrangements, respectively. The STRING analysis suggested a strong interaction between the proteins involved in photosynthesis-related proteins, ribosomal family proteins, energy metabolism, lipid metabolism, cytoskeletal proteins, and proteins involved in secondary metabolite
synthesis. These results provided clear indications that proteins belong to vital metabolic pathways and the induced metabolic pathways had strong interactions in OE-Def lines under *A. flavus* infection.

### 3.5. Comparative proteomic changes between OE-Def and WT controls

Changes in proteomes between the OE-Def transgenic and WT control samples were captured across different time points viz. 24, 40, 56, and 72 hpi, with 290/310, 279/292, 209/243, and 167/385 unique proteins were revealed following *A. flavus* infection (Tables S2 and S3). OE-Def samples consistently exhibited a higher number of unique proteins at all time points compared to WT controls. The difference in the number of proteins observed between lines at early stages of infection provided information about the proteins that are potentially involved in early compatible and incompatible responses to pathogen infection. Proteins involved in signaling, phosphorylation, and early resistance mechanisms, such as citrate synthase, glutamine synthase, phytol kinase, and acyl carrier protein in resistant OE-Def plants and putative CBL-interacting protein kinase, serine threonine protein kinase, resistant proteins PRG and PNTR were observed in WT control plants at 24 hpi (Tables S2 and S3). Likewise, a comparison between these two contrasting lines was made at 72 hpi to examine protein changes at late *A. flavus* infection stage. Furthermore, transcription factors (NAC, putative R2R3 MYB 8, MYB22, auxin signaling F-box3) and secondary metabolic pathway genes such as diacylglycerol acyltransferase, fatty acid hydroxylase, Ω-6 fatty acid desaturase, and fatty acid hydroxylase were also observed in resistant OE-Def lines at 72 hpi (Tables S2 and S3). Interestingly, as the *A. flavus* infection progressed, a clear reduction in the expression of unique proteins in the WT control lines was observed in contrast to their incrementally higher number in OE-Def transgenic samples. Moreover, distinct differences were observed in the protein expression between the WT control and transgenic samples at late stages of infection, positively contributing to the establishment of the disease resistance phenotype in the OE-Def transgenics. While the pathway analysis of DEPs in contrasting lines at 72 hpi supported this assumption, proteins representing 65 metabolic pathways were altered in WT control, whereas 83 metabolic pathways responded to infection in transgenic at 72 hpi.

### 3.6. Validation of protein expression

The expression patterns of 14 selected genes previously reported to have roles in either disease resistance or susceptibility were validated experimentally using Quantitative RT-PCR to investigate whether changes captured in the expression of proteins were regulated at the transcriptional
level (Figure 5). These include genes involved in biotic stress responses, proteolysis, flavonoid and fatty acid biosynthesis, and signal transduction pathways categorized into different subcategories, which were either directly or indirectly linked to plant resistance or susceptibility. The results revealed substantial agreement at the two levels, for all 14 genes differentially expressed upon pathogen infection in resistant transgenic lines and exhibited specific differential expression profile between the contrasting genotypes (Figure 5). The expression of lipoxygenase (3.00 FC), CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyl transferase (3.90 FC), diacylglycerol acyltransferase (3.44 FC), sphingolipid delta (4)-desaturase (3.62 FC), fatty alcohol oxidase-1 (35.56 FC), triacylglycerol lipase 3 (71.48 FC), cysteine proteinase inhibitor (55.83 FC), ubiquitin-conjugating enzyme-1 (3.85 FC) and chitinase (23.38 FC) were significantly \( (P \leq 0.05) \) increased in OE-Def lines compared to WT control lines following A. flavus infection. However, expression of annexin 5 (4.22 FC), syntaxin 52 (3.25 FC), and MLO-like protein 1 (4.70 FC) were significantly \( (P \leq 0.05) \) higher in WT controls. Whereas expression of kunitz trypsin inhibitor 4 (1.15 FC) and glycosyltransferase (1.39 FC) was higher in WT control compared to OE-Def lines inoculated with pathogen but was not significant.

3.7. Identification of resistance-associated proteins in A. flavus–peanut pathosystem

To identify the resistance-associated proteins, contrasting proteomes were compared individually and between during A. flavus infection progression (Tables S2 and S3). At least 872 A. flavus-responsive proteins common in both the transgenic and control samples were identified following differential expression patterns. The A. flavus-responsive DEPs were found to be mainly involved in processes such as stress response, cell wall biosynthesis, hypersensitive response, synthesis of secondary metabolic pathways including phenylpropanoids and flavonoids, lipid metabolism, and plant–pathogen interactions (Figure 6, Tables 1 and 2).

Proteins related to the upregulation of cell wall biosynthesis were identified in transgenic samples at different time points (Table 1). Cell wall enzymes such as chitinase (108.30-fold change (FC)) and pectinesterase (10.51-FC) were higher in OE-Def lines, while \( \beta \)-1,3-glucanase (14.37 FC) was present in abundance in WT controls. Further, disease resistance genes (R genes), such as NBS-LRR proteins (2.0–89.00 FC), TIR-NBS-LRR (2.5-20.00 FC), PR protein 4A (7.26 FC), and NPR1 protein (75.00 FC) were significantly higher in OE-Def transgenic lines than the WT control lines.

Proteins encoding secondary metabolite biosynthesis were detected in both OE-Def and WT control samples (Table 2). These proteins belonged to different chemical groups where some of the important and high-fold change proteins included phenylpropanoids viz., phenylalanine ammonia-lyase 2-like (EC:4.3.1.24) (60.57 FC), caffeic acid methyl transferase (7.83 FC), cinnamic acid 4-hydroxylase (9.80 FC); flavonoids: chalcone synthase (47.64 FC), chalcone-flavanone isomerase (10.13 FC), resveratrol synthase (100.00 FC), flavanone-3-hydroxylase (12.83 FC), 2-methyl-6-phytylbenzoquinone methyltransferase (32.75 FC), and resveratrol 3’-dimethyl allyl transferase (368.87 FC) that were up-regulated in OE-Def transgenic lines. Similarly, the fatty acid pathway proteins such as acyl carrier protein (397.56 FC), lipoxygenase (131.89 FC), diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase (153.72 FC), \( \beta \)-ketoacyl-ACP-reductase (14.19 FC), \( \beta \)-ketoacyl-ACP synthase-II (158.71 FC), 1-acyl-sn-glycerol-3-phosphate acyltransferase (128.79 FC), monoacylglycerol acyltransferase (33.47 FC), diacylglycerol acyltransferase (14.80 FC), triacylglycerol lipase (31.17 FC), long-chain fatty alcohol oxidase
1 (14.14 FC), acyl-acyl-carrier-protein hydrolase (112.00 FC), and lipase (37.27 FC) were higher in OE-Def lines.

3.8. Identification of susceptibility-associated proteins

The study allowed the identification of abundantly expressing putative susceptibility-associated proteins (SAPs) in WT control samples compared to OE-Def lines as the infection progressed (Table S3). A majority of SAP were present in the early stage of infection (24 hpi) during compatible pea–A. flavus interactions (Table 3). Higher expression of several proteins involved in plant–pathogen interactions included annexin (4.20 FC), syntaxin (9.72 FC), kunitz trypsin inhibitor 4 (64.72 FC), mildew resistance locus O (MLO)-like protein (7.68 FC), heat shock protein HSP70 (4.67 FC),

Figure 4. Overview of the A. flavus-responsive proteins in OE-Def transgenic and control lines. (A–C) Functional classification of differentially expressed proteins in (A) control, (B) transgenics, and (C) comparison of transgenic and control samples based on map man bin codes. (D–F) Protein interaction network of the significant proteins that were differentially regulated by A. flavus infection in (D) control samples, (E) transgenic lines. (F) Comparison of transgenic and controls. The protein integration map was predicted using STRING with high confidence (0.7) based on the proteins mapped to Medicago truncatula proteome. Red dotted circles show clusters of interest.

Figure 5. Relative transcript expression of differentially expressed proteins from OE-Def transgenic and control lines, following A. flavus inoculation at 40 hpi based on qRT–PCR in comparison to reference gene ADH3 and Gpdh. (A) Lipoxigenase (Q9M5D3), (B) Glycosyltransferase (A0A0K0KBH5), (C) CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyl transferase (H9L792), (D) Diacylglycerol acyltransferase (Q2KP14), (E) Sphingolipid delta(4)-desaturase (A0A384R632), (F) Fatty alcohol oxidase-1 (A0A345G), (G) Triacylglycerol lipase 3 (F1AM72), (H) Cysteine proteinase inhibitor (Q647G6), (I) Ubiquitin-conjugating enzyme-1 (Q45W77), (J) Chitinase (D3U314), (K) annexin 5 (A0A0F7GF8K), (L) Syntaxin 52 (Q06H39), (M) Kunitz trypsin inhibitor 4 (B4UW82), and (N) MLO-like protein 1 (A0A290G010). Different letters on the error bars indicate significant differences using Student’s t-test.
cationic peroxidase 1 (149.91 FC), R genes, NBS-LRR proteins (89.14 FC), TIR-NBS-LRR (8.95 FC), flavonoid pathway proteins such as, stilbene synthase 2 (99.78 FC), dihydroflavonol-4-reductase (28.20 FC), resveratrol 3'-dimethylallyltransferase (28.82 FC), and anthocyanidin synthase (5.22 FC), fatty acid pathway proteins, β-ketoacyl-ACP-reductase 1–2 (13.42), β-ketoacyl-ACP synthase III-1 (3.00 FC), and ω-6 fatty acid desaturase (7.37 FC), and MYB transcription factors, MYB4 (5.09 FC) and MYB9 (15 FC) (Table 3).

**4. Discussion**

Plants have developed several defense mechanisms to restrict the growth of pathogens. Changes in proteome composition and protein activity are major key elements of plant–pathogen interactions (Elmore et al. 2021; Jamshidi Goharrizi et al. 2019, 2020). Proteome profiling during pathogen infection can identify specific proteins and associated biological pathways that contribute to disease resistance and susceptibilities. Developing elite cultivars by understanding the molecular mechanisms of *Aspergillus*–peanut interaction is the most
Table 1. Cell wall and hypersensitivity-related proteins showing >2-fold increase in OE-Def transgenic peanut samples compared to WT control during Aspergillus flavus infection.

| Accession | Mass (kDa) | Description | Fold change over WT control |
|-----------|------------|-------------|-----------------------------|
| D3U314    | 4.83       | Chitinase (Fragment) | –  –  6.10  – |
| B4UW30    | 16.00      | Chitinase-like protein (Fragment) | 8.03  –  6.53  – |
| B4UWE0    | 19.11      | Pectinesterase (Fragment) | –  –  10.51  – |
| Q42515    | 29.44      | Chitinase (Class II) | 10.79  –  –  – |
| Q43322; D8V552 | 30.97     | Chitinase (Class II) | 108.31 22.82  –  – |
| D8V52    | 36.19      | Class II chitinase | 15.95  –  5.44  2.58 |

Table 2. List of up-regulated proteins (>2-FC) involved in secondary metabolic pathways in OE-Def transgenic peanut samples compared to WT controls during Aspergillus flavus infection.

| Accession | Mass (kDa) | Description | Fold change over WT control |
|-----------|------------|-------------|-----------------------------|
| A. Phenylpropanoids and flavonoid pathway |
| D2JVK3    | 13.67      | Phenylalanine ammonia-lyase (Fragment) | 60.57 4.55 41.54  – |
| Q850G5    | 13.88      | Putative caffeic acid methyl transferase | –  7.83  –  – |
| G3LT02    | 23.47      | Chalcone-flavonone isomerase family protein | 41.11  –  10.13  3.16 |
| E3NYG3    | 25.54      | Cinnamic acid 4-hydroxylase (Fragment) | 9.80  –  1.86  – |
| JQ9G3M    | 27.68      | Chalcone-flavonone isomerase family protein | –  –  11.54  13.87 |
| G3F842;G3F838;G3F840 | 40.16 | 2-methyl-6-phytylbenzoquinone methyltransferase | 30.42  –  –  – |
| B. Fatty acid biosynthesis |
| D8KXY3;D8KXY4 | 14.25 | Acyl carrier protein | 253.91 7.83  –  – |
| Q430KKCGL2 | 41.49 | Flavanone-3-hydroxylase | 12.83 12.10 2.74 1.95 |
| Q4LSL0    | 43.26      | Resveratrol synthase 3 | 57.99 100.37 2.82 6.47 |
| Q5VSX6    | 43.28      | Chalcone synthase | 2.27  –  47.64 5.21 |
| A0A4HGADD0 | 44.70 | Resveratrol 3-dimethylallyltransferase | 368.87 7.01 15.07 4.35 |
| B. Fatty acid biosynthesis |
| D2JVK3    | 13.67      | Phenylalanine ammonia-lyase (Fragment) | 60.57 4.55 41.54  – |
| Q850G5    | 13.88      | Putative caffeic acid methyl transferase | –  7.83  –  – |
| G3LT02    | 23.47      | Chalcone-flavonone isomerase family protein | 41.11  –  10.13  3.16 |
| E3NYG3    | 25.54      | Cinnamic acid 4-hydroxylase (Fragment) | 9.80  –  1.86  – |
| JQ9G3M    | 27.68      | Chalcone-flavonone isomerase family protein | –  –  11.54  13.87 |
| G3F842;G3F838;G3F840 | 40.16 | 2-methyl-6-phytylbenzoquinone methyltransferase | 30.42  –  –  – |
| A0A4HGADD0 | 44.70 | Resveratrol 3-dimethylallyltransferase | 368.87 7.01 15.07 4.35 |
| B. Fatty acid biosynthesis |
| D2JVK3    | 13.67      | Phenylalanine ammonia-lyase (Fragment) | 60.57 4.55 41.54  – |
| Q850G5    | 13.88      | Putative caffeic acid methyl transferase | –  7.83  –  – |
| G3LT02    | 23.47      | Chalcone-flavonone isomerase family protein | 41.11  –  10.13  3.16 |
| E3NYG3    | 25.54      | Cinnamic acid 4-hydroxylase (Fragment) | 9.80  –  1.86  – |
| JQ9G3M    | 27.68      | Chalcone-flavonone isomerase family protein | –  –  11.54  13.87 |
| G3F842;G3F838;G3F840 | 40.16 | 2-methyl-6-phytylbenzoquinone methyltransferase | 30.42  –  –  – |
| A0A4HGADD0 | 44.70 | Resveratrol 3-dimethylallyltransferase | 368.87 7.01 15.07 4.35 |
effective strategy to mitigate aflatoxin contamination in peanuts. However, poor genetic background and lack of sustainable resistance to *A. flavus* infection and aflatoxin production in the available germplasm has been a major bottleneck for breeding of elite cultivars in peanut (Fountain et al. 2015).

In this study, OE-Def transgenic lines developed in our previous study (Sharma et al. 2018) were reconfirmed for their significantly enhanced capacity to resist *A. flavus* infection and significantly reduced aflatoxin accumulation compared to their wild-type counterparts. Comparative proteomic analysis in the resistant and susceptible lines revealed 872 differentially expressed proteins (DEPs) involved in diverse metabolic processes during progressive *A. flavus* infection. The highest number of DEPs in OE-Def lines was observed to be induced at 56 and 72 hpi clearly indicating differential regulation of pathogenicity in OE-Def transgenic lines during mid–late stages of infection. While there have been no studies on how defense genes regulate these downstream resistance genes, our study revealed DEPs involved in hypersensitive responses, which possibly is an inducible defense reaction mediated by the *Def*-transgene and further activates the downstream regulatory genes and resistance-associated proteins that contribute to the observed quantitative durable resistance (Kaur et al. 2011; Kushalappa et al. 2016). This study revealed over 106 pathways to be in regulation of pathogenicity in OE-Def transgenic lines during *A. flavus* infection (Chen et al. 2012; Gupte et al. 2013). The observed results concur with earlier reports where stronger induction of PR10 was observed in resistant maize compared to the susceptible genotype against *A. flavus* infection (Chen et al. 2010, 2006, Lanubile et al. 2017).

4.1. Differentially expressed proteins related to hypersensitive responses to *A. flavus*

Plants recognize the pathogen-produced elicitors through their receptors localized in cell membrane or in cytosol (Jones and Dangl 2006). We observed the upregulation of several families of receptor-like kinases such as receptor-like protein kinase 1, receptor protein kinases, and serine–threonine protein kinases in resistant OE-Def samples. An earlier report in maize also identified serine–threonine protein kinases as resistance-associated proteins during *A. flavus* infection and have been shown to play a role in both broad-spectrum, elicitor-initiated defense responses and as dominant resistance (R) genes in race-specific pathogen defense (Jones and Dangl 2006). As the first level of defense, plants produce pathogenesis-related (PR) proteins such as β-1,3-glucanase and chitinase to breakdown the cell wall components of pathogen and act as a signaling molecules to trigger resistance (Funnell et al. 2004). In the present study, higher production of chitinases, pectinesterases, β-1,3-glucanases and PRA4 was detected upon *A. flavus* infection indicating their role in one of the crucial processes in plant defense. These enzymes occur frequently in many isoforms and are involved in synthesis of defense substances or a direct antimicrobial activity (Balasubramanian et al. 2012; Gupta et al. 2013). The observed results concur with earlier reports where stronger induction of PR10 was observed in resistant maize compared to the susceptible genotype against *A. flavus* infection (Chen et al. 2010, 2006, Lanubile et al. 2017).

Table 3. Putative SAPs identified in WT controls during *Aspergillus flavus* infection.

| Accession | Mass (kDa) | Description | 24 h | 40 h | 56 h | 72 h |
|-----------|------------|-------------|------|------|------|------|
| K0H635    | 8.63       | Heat shock protein Hsp70 | 4.67 | –    | 31.61 | –    |
| B4UWC9    | 14.34      | MCT-1 protein-like protein | –    | –    | 31.61 | –    |
| Q06H39    | 17.10      | Syntaxin | 9.72 | 2.75 | –    | –    |
| G0Y6V2    | 19.03      | TIR disease resistance protein | 8.95 | –    | –    | –    |
| B4UWB2    | 22.09      | Kunitz trypsin inhibitor 4 | 64.72 | –    | 28.20 | –    |
| Q06H37    | 23.85      | Syringolide-induced protein 19-1-5 | –    | 2.57 | –    | –    |
| M4T2P8    | 25.06      | F-box family protein 6 | 5.15 | –    | –    | –    |
| V5T684    | 25.58      | Putative R2R3 MYB protein 9 | 15.00 | –    | –    | –    |
| COL2U0    | 27.57      | Putative cyclase family protein | 10.12 | –    | –    | –    |
| A0A0U3DHM7| 30.13      | Inositol polyphosphate multikinase | –    | 2.61 | –    | –    |
| H9L7Q9    | 33.48      | CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase | –    | 2.09 | –    | –    |
| A0M023UN1 | 33.82      | Asbetic acid 8-hydroxylase 3 | –    | 2.59 | –    | –    |
| P22195    | 34.03      | Cationic peroxidase 1 | 149.91 | 21.15 | –    | –    |
| D8KXZ3/D8KXZ4 | 34.14 | β-ketoacyl-ACP-reductase 1-2 | –    | 13.42 | –    | –    |
| P20077    | 34.25      | Putative stilbene synthase 2 | 99.78 | –    | –    | –    |
| V5T679    | 34.74      | Putative R2R3 MYB protein 4 | 5.09 | –    | –    | –    |
| A0A0F7GFK8| 36.11      | Annexin | 2.11 | 4.20 | –    | –    |
| A0A0K0K8G3| 40.17      | Anthocyanidin synthase | 5.22 | –    | 2.22 | –    |
| A0A0K0K8G1| 40.24      | Dihydroflavonol-4-reductase | –    | 28.20 | –    | –    |
| D8KXZ1    | 43.06      | β-ketoacyl-ACP synthase III-1 | –    | 3.00 | –    | –    |
| A0A0H4GDD0| 44.70      | Resveratrol 3′-dimethylallyltransferase | 28.82 | –    | –    | –    |
| D0MQ49    | 45.76      | Mitogen-activated protein kinase | 2.45 | –    | –    | –    |
| A0A290G010| 56.63      | MLO-like protein | –    | 7.68 | –    | –    |
| A1DZF0    | 60.72      | Arachin 6 | 12.28 | 2.86 | –    | –    |
| V5T6P4    | 61.29      | Putative MYB-related protein 12 | –    | 2.46 | –    | –    |
An upregulation of NBS-LRR and TIR-NBS-LRR proteins were observed in the resistant OE-Def lines. Specialized pathogens suppress elicitor-initiated defense responses, penetrate into the cells, and produce race-specific effectors encoded by avirulence (AVR) genes (Oliver and Solomon 2010) that are recognized by receptors with NBS-LRR domains to activate effector-triggered immunity (ETI). While these ETI result in hypersensitive response to contain the pathogen, there have been very few reports on the involvement of peanut NBS-LRR proteins against A. flavus infection (Li et al. 2013; Nayak et al. 2017; Song et al. 2017). However, it is still a possibility that these receptors trigger downstream regulatory, and resistance-associated proteins to activate secondary metabolic pathway genes.

4.2. Differentially expressed proteins related to signal transduction

Following the perception of pathogen-produced elicitors, plants synthesize a complex blend of phytohormones which activate downstream genes that regulate distinct sets of resistance genes (Pieterse et al. 2012). In the present study, the resistant OE-Def lines showed an overproduction of several plant hormone-induced proteins including auxin-induced protein, auxin-induced putative aldo/keto reductase, auxin-induced putative tetratricopeptide-containing protein, ethylene-responsive proteins such as, ethylene-responsive element binding factor-2, aminocyclopropane-carboxylate oxidase, S-adenosylmethionine synthase, and ABA-responsive proteins such as abscisic acid 8-hydroxylase 1 and ABA response element binding protein-1. Ethephon-generated ethylene has been earlier shown to inhibit aflatoxin biosynthesis in vitro in A. flavus, that was partially due to its effects on ROS production in peanuts (Huang et al. 2009) and ethylene-responsive factor (ZmERF1)-induced defensin proteins to resist A. flavus in maize (Fountain et al. 2015). Further, treatment of ethylene in combination with CO₂ was shown to prevent aflatoxin production in peanut against A. parasiticus (Roze et al. 2007). Likewise, homolog of abscisic acid-insensitive gene, ABR1, has been identified as a repressor of the ABA signaling pathway which confers resistance against pre-harvest aflatoxin contamination (Clevenger et al. 2016). Studies on direct involvement of auxins and ABA in plant defense response are very limited and restricted only to model plants. However, these phytohormones either activate a variety of transcription factors that regulate plant defense responses involving up- or down-regulation of downstream resistance genes (Alves et al. 2014) or cross-talk between them to regulate defense response in plants (Asghar et al. 2019).

4.3. Differentially expressed regulatory proteins and TFs

Transcription factors (TFs) are excellent candidates in stress resistance breeding by directly regulating downstream resistance genes (Alves et al. 2014; Kou and Wang 2012; Lanubile et al. 2017; Yoon et al. 2020; Yuan et al. 2019). A total of 20 high-fold change TF-related proteins were detected in resistant OE-Def transgenic and WT control lines, including genes encoding MYB binding proteins in OE-Def transgenic lines. While the ones up-regulated in OE-Def lines included MYB1, MYB3, MYB12, MYB13, MYB20, MYB21, MYB23, MYB24, and MYB30. These findings are not unusual as increased levels of MYB and NAC proteins are known to regulate secondary defense response genes following infection by several microorganisms (Yoon et al. 2020; Yuan et al. 2019). Among these, MYB12 has been reported to be involved in specific regulation of flavonoid biosynthesis by regulating chalcone synthase (CHS) and flavanol synthase (FLS) accounting for flavanol glycoside accumulation in Arabidopsis (Mehrtens et al. 2005; Stracke et al. 2010). In addition, proteins encoding for ethylene-responsive TF, AP2-like ethylene-responsive transcription factor ANT-like protein, MADS box TF, MADS box protein M8, and NAC TF were significantly induced in OE-Def transgenic lines. While TFs, NAC, and ethylene-responsive factors are known to play a vital role in complex signaling networks during plant stress responses, their specific roles in biotic stress resistance remain largely unexplored. Previous studies demonstrate that NAC proteins are activated via an ethylene signaling pathway (Kim et al. 2014; Yogendra et al. 2017) and individually or together with TF may possibly act as transcriptional activators or repressors to regulate downstream resistance genes.

4.4. Differentially expressed proteins involved in secondary metabolite production

In the current study, 67 proteins belonging to the secondary metabolism were differentially induced in OE-Def-resistant transgenic line compared to the susceptible WT control. These resistance proteins are known to produce secondary metabolites such as phytoalexins and phytoalexins which may be antimicrobial and suppress the progression of pathogen infection (Kushalappa et al. 2016; Nawrot et al. 2014). Most of the proteins identified in the resistant transgenic lines belonged to the phenylpropanoid pathway, flavonoids, and fatty acids, suggesting their major role in aflatoxin resistance.

4.4.1. Phenylpropanoid biosynthesis

Secondary metabolic pathways including those for phenylpropanoids and flavonoids, well-known antioxidants with role in plant defense were observed to be differentially induced during the A. flavus infection. An increased expression of proteins viz. phenylalanine ammonia-lyase 2-like, caffeic acid methyl transferase, cinnamic acid 4-hydroxylase, chalcone synthase, chalcone-flavonone isomerase, resveratrol synthase, flavanone-3-hydroxylase, and 2-methyl-6-phytylbenzoquinone methyltransferase were captured in OE-Def transgenic samples under A. flavus infection. Phenylalanine ammonia lyase (PAL), a precursor of lignin and phytoalexins, has also been previously shown to increase in a resistant peanut genotype upon A. flavus infection (Xuanqiang et al. 2001). Similarly, Resveratrol, another key protein up-regulated in OE-Def transgenic lines in our study, is a well-known phytoalexin that enhance the host-plant resistance against biotic and abiotic stresses (Chang et al. 2011). The inhibitory effect of resveratrol on aflatoxin synthesis and mycelial growth has previously been reported in peanut (Wang et al. 2013, 2015). Furthermore, our results are consistent with the previous transcriptomic data, where the upregulation of resveratrol and flavonoids in resistant peanut lines were reported to affect the fungal growth and aflatoxin production (Nayak et al. 2017; Wang et al. 2015).
4.4.2. Fatty acid biosynthesis

In the present study, over 46 proteins involved in fatty acid biosynthesis, elongation, and degradation were found to be responsive to A. flavus infection in OE-Def transgenic lines. We observed higher abundance of acyl carrier protein, lipoxygenase, diacylglycerol-glycerol-3-phosphate-3-phosphatidyltransferase, β-ketoacyl-ACP-reductase, β-ketoacyl-ACP synthase-II, monoacylglycerol acyltransferase, diacylglycerol acyltransferase, triacylglycerol lipase, long-chain fatty alcohol oxidase-1 and acyl-[acyl-carrier-protein] hydrolase in transgenic samples during A. flavus infection. These results are in line with the previous reports where lipoxygenase mediated hydroperoxy fatty acids production acted as the substrate for oxylipin synthesis (Dave and Graham 2012), antimicrobial for a wide range of pathogens including A. flavus (Jayashree and Subramanyam 2000). Lipoygenase-3 (LOX3) and few other 9-oxylipins have been reported to be crucial for resistance to A. flavus and suppression of aflatoxin biosynthesis in maize and soybean (Gao and Kolomiets 2009). Fatty acids mainly involve in the synthesis of signaling molecules, such as jasmonate, again an antimicrobial compound which plays a key role in resistance to Aspergillus infection and act as the physical barrier for pathogen entry in peanut (Van der Ent et al. 2009).

4.5. Susceptibility-associated proteins involved in A. flavus infection and aflatoxin contamination

While the knowledge of genes regulating plant disease resistance has dramatically increased in recent years, the characterization of disease susceptibility genes has been limited. A gain or loss of resistance is defined as host susceptibility (Chisholm et al. 2006). A gap between the nature of resistance and that of susceptibility is likely due to differences in their genetic tractability (Lorang et al. 2007). Specialized pathogens like A. flavus often possess multiple virulence factors called effectors which incrementally contribute to the disease phenotype. The early stage of compatible interaction of peanut–A. flavus are likely to involve protein phosphorylation, ion fluxes, reactive oxygen species (ROS), and other signaling transduction (Shen et al. 2017). However, as the disease progresses, the pathogen keep the infection going by producing more mycelia and virulent spores, resulting in a susceptible phenotype. In our study, we observed high expression of several proteins such as syntaxin-52, annexin-5, kunitz trypsin inhibitor-4, MLO-like protein-1, and R genes in the WT controls at early stages of infection. Syntaxins, family of membrane integrated Q-SNARE proteins have been reported to be key susceptibility factors for a number of plant diseases. Silencing of Syntaxin SYP121 and SYP122 have shown to improve disease resistance in Arabidopsis thaliana against the virulent powdery mildew fungus Erysiphe cichoracearum and bacterial pathogen Pseudomonas DC3000 in a salicylic acid (SA)-independent manner (Zhang et al. 2007). In potato, RNAi silencing of SISYR1 and SiSNAP33 genes increased the constitutive accumulation of SA and PR1 transcripts, enhancing resistance against Phytophthora infestans (Eschen-Lippold et al. 2012). Likewise, silencing of MdSYP121 gene increased tolerance to Botryosphaeria dothidea infection in apple (He et al. 2018). Similarly, RNAi silencing of AtANN8 encoding for Ca²⁺ regulated phospholipid-binding and membrane-binding annexins proteins in A. thaliana led to enhanced expression of Resistance to Powdery Mildew 8.1 (RPW8.1) and provided defense against powdery mildew (Zhao et al. 2021). RNAi silencing of Kunitz trypsin inhibitor (AtKTI11) in A. thaliana led to enhanced resistance to the virulent pathogen Erwinia carotovora subsp. carotovora, while overexpression of AtKTI11 resulted in higher susceptibility towards this pathogen (Li et al. 2008).

MLO, another susceptibility gene that contains seven transmembrane helices, was observed to be abundant in the susceptible WT line. The loss-of-function mutations of MLO genes in barley (Jørgensen 1992), tomato (Bai et al. 2008), wheat (Wang et al. 2014), rose (Fang et al. 2021), and grapes (Pessina et al. 2016) have shown to confer resistance to powdery mildew pathogen. It is intriguing to note higher expression of NBS-LRR genes during infection in the WT control lines, considering that this family of genes have been reported as resistance-associated genes across several crops. A previous report in Arabidopsis revealed an NBS-LRR gene underlying the locus LOV1 induced susceptibility to Cochliobolus victoriae infection (Lorang et al. 2007). In addition, induction of several MYB transcription factors viz. MYB4, MYB9, and MYB12 were also up-regulated in WT control lines. An earlier report in tobacco and switchgrass pointed to MYB4 as a transcriptional repressor that reduced the activities of phenylalanine ammonia lyase (PAL) and caffeoyl CoA 3-O-methyltransferase (CCoAOMT) in lignin biosynthetic pathway (Shen et al. 2012). We hypothesize that a higher fold increase in MYB4 in the WT control might have affected the phenylpropanoid pathway genes, increasing its susceptibility to A. flavus infection.

Besides these, the role of several other fatty acid and flavonoid pathway proteins/genes observed to be induced during early infection in WT controls is not very clear in the literature and needs further investigation. Nevertheless, this knowledge could potentially provide opportunities for transcriptional inactivation of these candidate susceptibility-associated proteins (SAPs) to aid the development of durable disease resistance using targeted breeding interventions such as genome editing.

5. Conclusions

The comparative proteomics approaches employed in Aspergillus–peanut pathosystem provides insights into the molecular events underlying an enhanced resistance to A. flavus in transgenic line compared to its near-isogenic susceptible WT parent. We identified hierarchies of genes including PR proteins, phytohormones, and transcription factors which regulate the downstream secondary metabolic pathway genes known to directly suppress pathogen development in its host. The identified proteins can be used as biomarkers in plant breeding for the selection of suitable aflatoxin-free crops. In addition, these data sets reveal underlying genetic mechanisms and discovery of several defense and susceptibility-associated proteins, that provide novel targets for precision breeding in peanuts for resistance to Aspergillus and the resulting aflatoxin contamination.

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