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Identifying cotton (*Gossypium hirsutum* L.) genes induced in response to *Aspergillus flavus* infection

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ABSTRACT

Development of aflatoxin-resistant cotton against *Aspergillus flavus* is handicapped by the lack of resistance source in available germplasm. Genetic engineering warrants for identification of resistance-associated genes in cotton. As a first step toward this, we isolated 44 differentially expressed genes (DEGs) in response to *A. flavus* infection, using annealing control primer system. Different functional classification of the DEGs suggested a complex and multi-factorial plant–fungus interaction. Eight DEGs, including transcription factors, kinase, and downstream stress responsive genes, showed a tissue- and time-dependent differences in their expression. The upregulated genes can be used as transgenes and/or functional markers for breeding aflatoxin-resistant cottonseed.

1. Introduction

Aflatoxins are dangerous and carcinogenic mycotoxins produced as secondary metabolites of *Aspergillus flavus* and *Aspergillus parasiticus*. These polyketides contaminate a variety of important agricultural products, such as corn, cottonseed, peanut, and tree nuts, both in the field and after harvest, and are extremely carcinogenic when ingested in small amount by animals and humans [1]. The wide occurrence of aflatoxin contamination in food and feed and the subsequent serious health and economic impact are well recognized internationally. So far, over 50 countries have established or proposed regulations on the permissible level of aflatoxins in food and feed because low dose consumption of aflatoxin contaminated food stuff causes chronic aflatoxicosis resulting in cancer, suppression of immunological responses, and other "slow" pathological conditions in both animals and humans [2]. To minimize exposure to these potent toxins, the US Food and Drug administration imposed 20 ng/g (ppb) limits of aflatoxin content allowed in foodstuffs for human consumption, while the European Community has imposed more stringent level of 4 ng/g [3]. In addition to the adverse effects that aflatoxin has on human and animal health worldwide there are also significant economic costs incurred trying to mitigate aflatoxin contamination of crops. *A. flavus* and *A. parasiticus* cause worldwide annual losses of approximately $270 M [4]. In the U.S. the southeast peanut, southern cotton belt, and mid-south corn farming regions are considered to be endemic to severe outbreak of aflatoxin contamination.

A. flavus is the primary causal agent of aflatoxin contamination of cottonseed. The concern of aflatoxin contamination of cottonseed in the United States is due to the feed-mediated transfer of contaminated seeds to milk of dairy cows [5]. Aflatoxin contamination of cottonseed is frequent in pre-mature seeds associated with insect damage to the boll, particularly by pink boll worm [6]. The contamination also occurs after seed maturity and boll opening, and involves direct infection of seed by *A. flavus* [6,7]. Biotic and abiotic factors, either nutritional or environmental including temperature, pH, and drought stress, are also known to trigger aflatoxin production in toxigenic *Aspergillus* species, although the molecular mechanisms for these effects are still unclear [8–11].

The morphological process of invasion [12,13] and molecular mechanism of *A. flavus* involved in cottonseed invasion process have been extensively studied [14,15]. Fungal invasion of cottonseeds is associated with the production of a specific pectinase

Abbreviations: ACP, annealing control primer; DEG, differentially expressed genes; L1, locule; Adj, adjacent locules; qRT-PCR, quantitative reverse transcription PCR.

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isozyme, lipases, and hydrolytic enzymes [14–17]. Moreover, the availability of *A. flavus* whole genome microarray makes easier to identify genes expressed in the fungus during its invasion of crops.

Current strategies employ both pre-harvest and post-harvest measures to reduce the risk of aflatoxin contamination in food and feed. Biological control with non-aflatoxigenic strains that outcompete toxigenic strains in nature has shown promise as a strategy to reduce pre-harvest aflatoxin contamination of cotton, peanut, and corn. Enhancing host resistance is the most widely explored strategy for eliminating pre-harvest aflatoxin contamination by *A. flavus*. Success in conventional breeding for resistance to mycotoxin-producing or phytopathogenic fungi is reliant upon the availability of resistance gene(s) in the germplasm and there is no genetic resistance available in cotton genotypes. This warrants for genetic engineering of cotton with manipulation of orthologous resistance-associated genes/proteins and availability of naturally tolerant genotypes in corn and peanuts provides a window to look for resistant genes and factors. Using antifungal proteins and synthetic peptides (*D4E1*), transgenic cottons capable of resisting various phytopathogens including *A. flavus* have been developed [18]. Various other approaches have also been suggested for genetic control of pre-harvest aflatoxin contamination, including the development and use of crops with resistance to insects, resistance to plant stress (especially for tolerance to drought and high temperatures).

The expressed sequence tag (EST) and oligonucleotide microarray strategies have been used to identify differentially expressing genes under *A. flavus* infection of resistant and susceptible peanut and maize cultivars [19,20]. Although there is a fairly good understanding of the aflatoxin biosynthetic pathway and pathway cluster genes, the complex interaction between the saprophytic *Aspergillus* and cotton plant (cottonseed, in particular) is poorly understood due to the unavailability of resistant cotton genotype(s). Availability of several small-scale differential mRNA imaging techniques and gene expression analysis also allows primary but rapid progress for identification and understanding of gene function related to phenotype. Our objective in this study was to identify differentially expressed genes in cottonseed and pericarp as a result of *Aspergillus* infection; and to this end we used an annealing control primer (ACP) system providing a suitable primer with annealing specificity, which specifically targets sequence hybridization to the template via a polydeoxyinosine poly (dI) linker [21,22].

2. Materials and methods

2.1. Fungal cultures

Toxin-producing *A. flavus* strain 13 (SRRC # 1532) was grown for 7 d at 30 °C on maltose extract agar (MEA) media before assay. Conidia were harvested by flooding a single plate with 9 ml of sterile potato dextrose broth (PDB) containing 0.01% (v/v) Triton X-100 solution and scraping the surface of mycelium with a sterile pipette. The conidial suspension was counted with the aid of a hemocytometer and adjusted to a concentration of 10^4 conidia/ml.

2.2. Inoculation of cotton bolls

Cotton bolls (28–30 dpa) on greenhouse-grown cotton plants (var. Coker 312) were wounded in the center of one of the locules to a depth of 5–10 mm with a 3 mm dia cork borer. A small aliquot (10 μl) of the *A. flavus* conidial suspension (10^4 conidia/ml) was pipetted into the hole. Control bolls received only PDB with no conidia. Bolls were harvested at 6, 24, 48 and 72 h after inoculation and were divided into several sub-samples for gene expression analysis. The sub-samples included pericarp and fiber-free seeds from both inoculated (L1) and non-inoculated, adjacent (Adj) locules. All the samples were quick-frozen in liquid nitrogen and stored at −80 °C for RNA isolation. Three bolls each from two different plants (biological replicates) were used for each treatment.

2.3. RNA isolation

Total RNA was isolated from boll of uninfected and *A. flavus*-infected cotton using Spectrum total RNA isolation kit (Sigma–Aldrich, St. Louis, MO) at different time points after infection. The RNA was independently extracted from cottonseed and pericarp from each infected locules (L1) and three adjacent/distal locules (Adj), respectively. Total RNA was quantified by an ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). The RNA integrity was also assessed by running 2 μl of total RNA in a 1.2% (w/v) agarose/formaldehyde gel, stained with ethidium bromide, and visualized under UV light. An aliquot of 1 μg RNA extracted from adjacent/distal locules (Adj) of cottonseed or pericarp was pooled for cDNA preparation.

2.4. cDNA preparation and ACP-based gene-fishing PCR

Three μg of the total RNA was reverse transcribed to first-strand cDNA using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) in a final reaction volume of 20 μl including 1 μl RNase H^+ Moloney murine leukemia virus-derived reverse transcriptase pre-blended with RNase inhibitor, and 4 μl of 5× reaction mix containing an optimized blend of oligo (dT) and random primers. The reaction was conducted as follows: one cycle at 25 °C for 5 min, followed by 42 °C for 30 min, and 85 °C for 5 min. The first-strand cDNAs were diluted 10-fold with nuclease-free water and stored at −80 °C for further use.

Second-strand cDNA synthesis and subsequent PCR amplification was performed in a single tube using the GeneFishing™ DEG premix kit manual (Seegene, Rockville, MD) as described earlier [22]. Briefly, second-strand cDNA synthesis was conducted at one cycle of first-stage PCR in a final reaction volume of 20 μl containing 3 μl (~50 ng) of the diluted, first-strand cDNA, 10 μl of 2× SeeAmp™ ACP™ master mix (Seegene, Rockville, MD), 2 μl of 5 μM arbitrary ACPs [22], 1 μl of 10 μM dT-ACP2. The tube containing the reaction mixture was placed in a preheated (94 °C) thermal cycler. The first-stage PCR profile was: 1 × 94 °C for 1 min followed by 50 °C for 3 min, and 72 °C for 1 min. The second-stage PCR amplification profile was as follows: 40 × 94 °C for 40 s, followed by 65 °C for 40 s, 72 °C for 40 s, and a 5 min final extension at 72 °C. The PCR products amplified by 20 ACP primers were resolved in a 2% agarose gel, stained with ethidium bromide, and visualized under UV and documented in a Kodak Gel Logic200 system (Carestream Health Inc, Rochester, NY).

2.5. Cloning and sequencing of differentially expressed genes (DEGs)

Cloning and sequencing of fragments corresponding to differentially expressed genes (DEGs), based on their intensity or presence/absence, between control and infected cottonseed and pericarp was performed following [23]. Thirty-two DEGs from both pericarp and cottonseed tissues were extracted with a Qiaquick gel extraction kit (Qiagen, Valencia, CA) and cloned into pGEM®-T Easy vector (Promega, Madison, WI). Plasmids were isolated from forty-four clones that were confirmed positive by colony PCR using M13F and M13R primers, and single-pass sequenced with T7 primer in an ABI 3730x1 genetic analyzer.

The vector backbone and the poly (A) tail were processed manually and clean, DNA sequences were searched against the non-redundant (nr) nucleotide and protein database of NCBI using BLASTN and BLASTX interface (http://www.ncbi.nlm.nih.gov/BLAST), respectively. Gene enrichment analysis of the DEGs was performed as described earlier [24].

2.6. (Semi)quantitative reverse transcription polymerase chain reaction analysis of DEGs

Transcript abundance of DEGs with known functional annotation (Table 1) was determined by (semi)quantitative reverse transcription PCR (sq/qRT-PCR) using first-strand cDNA and DEG-specific primers. DEG-specific primers (Table 2) were designed using Primer 3.0 web resource (http://frodo.wi.mit.edu/primer3) and synthesized by Integrated DNA Technologies (IDT Inc, Coralville, IA).

The sqRT-PCR was performed with 1 μl of first-strand cDNA using GoTaq DNA polymerase kit (Promega, Madison, WI) as described earlier [23]. The qPCR analysis was carried out in triplicate using SYBR green master mix (Bio-Rad, Hercules, CA), 2 μl of diluted (10×) first-strand cDNA and 3.25 pmol each of DEG-specific primer in a MyiQ Real-Time PCR analysis system (Bio-Rad, Hercules, CA) following method described in [23]. The relative quantitation method ($2^{-\Delta\Delta Ct}$) was used to compare the gene expression levels [25]. Independently, comparison of expression of DEGs was made between seed tissues versus pericarp tissues, among different time points, and in general between control and inoculated tissues. Cotton (*Gossypium hirsutum*) elongation factor (GhEF1α; GenBank Accession number DQ174250) was used as an internal reference gene.

The relative gene expression values were normalized and used for the heat map generation based on hierarchical clustering using Genesis software v 1.7.6 (http://www.tugraz.at). Heat map reflects the ranking of gene expression along the horizontal axis.

3. Results and discussion

3.1. Identification of differentially expressed genes

All 20 annealing control primers (ACPs) resulted in differentially expressed genes (DEGs) in either cottonseed or pericarp tissues in response to *A. flavus* infection (Fig. 1). Forty-four DEGs that were sequenced showed BLAST hit with plant-specific cDNA sequences. Thirty-one DEGs had functional annotation in the public database with more than one representation (GenBank Acc# JK818484 - JK818504). The remaining 13 sequences did not have any defined functional role and showed similarity to either hypothetical or expressed protein in the nr database. Four DEGs (legumin A, spot11 catalase, alpha globulin A&, B, and cytochrome c oxidase) had match with known genes in cotton. Gene enrichment analysis classified the DEGs according to their role in biological process or molecular/cellular function (Table 1). The DEGs belonging to different functional categories suggested that the fungus induced a complex and multi-factorial responses in the cotton plant. Eight DEGs (Table 2) that were abundantly represented in the library were selected for further expression analysis.

Table 2
Primers for sqRT-PCR and qRT-PCR analysis of differentially expressed genes in *Gossypium hirsutum* under infection of *Aspergillus flavus*.

Clone ID	Forward primer (5′→3′)	Reverse primer (5′→3′)	Product length (bp)
GhC1	aaatgtcattgccacaagca	gctgcttatctcccatctgc	172
GhC2	taaacgcgctgtacatggaa	gtcctgcggtcatctgtttc	162
GhC3	caatcaaaatcagggggatct	ccgccttgtaagacccttttc	160
GhC5	ggatgagtaaaggactgaaatgtaga	cggcatttattaattaggacca	153
GhC6	tcaacagcaacccacaagag	caaaacaagggtctcctcctt	188
GhC38	tagctgtccccactggaatc	cccagaatttgccaggacta	152
GhC39	caatccactggagctcatca	tttccctcccctcgagtatt	155
GhC43	ctgcgcagtgcttacagaat	caaaagcatgaagtggcaaa	173
GhCelf	cttgcgtttacccttggtgt	aaggagagtgggacccttgt	237

3.2. Transcript abundance analysis of DEGs

The genes that showed differential expression in the ACP-based mRNA display also showed differential expression in a tissue- and time-dependent manner in the cotton boll in response to *A. flavus* infection, as was seen from the (semi)quantitative RT-PCR. Further, the expression of the genes at the site of infection (L1) was also different than at the adjacent tissues (Adj). As shown in Fig. 2, most of the DEGs were upregulated in both pericarp and cottonseed at different time point with at the site (L1) of inoculation of *A. flavus* or in the adjacent tissues (Adj). The DEGs, in general, were very highly expressed as early as 6 h and reached their maximum content at 24 h after infection by the fungus in L1 and/or Adj tissues of the seed. Thereafter the mRNA accumulation of the genes were maintained or reduced to the basal level. Interestingly, all the DEGs showed downregulation of their transcript accumulation in the adjacent locules of the pericarp tissue immediately after the fungal infection i.e., at 6 h and but increased or remained constant at subsequent time points. The DEG GhC3, similar to legumin A, did not show any apparent change in its transcript abundance in both L1 and Adj tissues of seed (Fig. 2). The DEGs in L1 of the pericarp tissue showed a nearly similar trend of those in the seed tissue. In general, the qRT-PCR data (Table 3; Fig. 3) was in agreement with the sqRT-PCR data. The detail expression pattern of the individual DEGs is discussed below.

3.2.1. Genes involved in transcription regulation

Leucine-rich repeat receptor-like protein kinase (LRR-RLK; GhC3), was upregulated in both the seed and pericarp at the site of infection (L1) as early as 6 h and maintained its expression at least up to 72 h after infected by *A. flavus*. Its expression was significantly higher in seed in comparison to that in pericarp. However, its expression in the adjacent (Adj) tissues of pericarp and seed did not change significantly after infection. Plant LRR-RLKs are

Table 1
Differentially expressed genes in pericarp and seed tissues of *Gossypium hirsutum* under infection of *Aspergillus flavus*.

Clone ID	Similarity	Length (bp)	GO ID	E value	GO term	Reference
GhC1	Leucine-rich repeat receptor-like protein kinase (*Arabidopsis thaliana*)	793	GO:0004674	7e-49	Molecular function	FJ708669
GhC2	RING-H2 finger protein ATL3J (*Ricinus communis*)	810	GO:0008270	3e-28	Molecular function	EQ974625.1
GhC3	Legumin A protein mRNA (*Gossypium hirsutum*)	827	GO:0045735	3e-118	Cellular component	M73072
GhC5	clone Spotp11 catalase mRNA (*Gossypium hirsutum*)	823	GO:0004096	7e-77	Molecular function	FJ415187
GhC6	Alpha globulin A and B genes (*Gossypium hirsutum*)	817	GO:0045735	0.0	Molecular function	M18027
GhC38	Cytochrome c oxidase mRNA (*Gossypium barbadense*)	753	GO:0004129	5e-127	Molecular function	AF531373
GhC39	Seed maturation protein LEA4 (*Glycine tomentella*)	807	GO:0016020	3e-15	Cellular component	AAG37451
GhC43	MADS-box protein 4 (*Vitis vinifera*)	786	GO:0003700	3e-17	Molecular function	XM_002275669

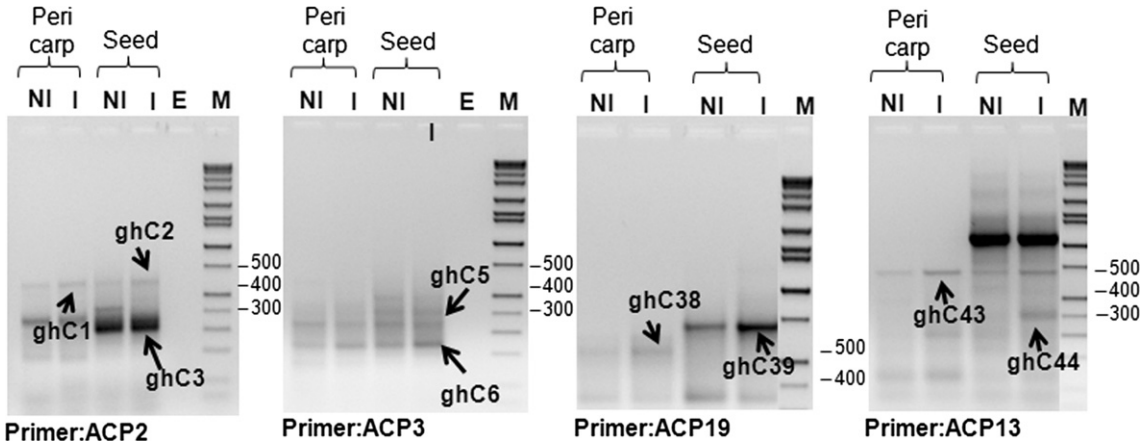

Fig. 1. ACP PCR showing differential expression of genes with ACP2, ACP3, ACP13 and ACP19 primers in pericarp and seed tissues of cotton upon infection with *Aspergillus flavus*. NI, not inoculated; I, inoculated; E, empty; M, 1 kb DNA size marker.

transmembrane proteins with putative extracellular domains and intracellular protein kinase domains, which can perceive external signals and initiate signal cascades [26]. Recently, Xu et al. [27] showed the upregulation of LRR-RLKs as a defense response of cotton against *Verticillium* wilt fungus. The identification of LRR-RLK suggested its putative role in mediating early events leading to plant defense such as hypersensitive reaction in response to A. *flavus* infection.

Different transcription factor family genes in various complex regulatory networks are known to be induced in plants in response to pathogen attack, which results in up/down-regulation of downstream defense-related genes [28]. The cDNA GhC2 similar to a transcription factor encoding RING-H2 finger protein showed comparatively similar level of upregulation at different time points in L1 tissues whereas in Adj tissues it was only upregulated after 6 and 24 h of fungal infection; the highest upregulation was observed at 24 h. However in the pericarp its expression was either down-regulated or remained unaltered after infection. Ring finger protein is a zinc finger-type protein, which is involved in many biological processes including the ubiquitination pathway as a ligase [29,30]. Ectopic/over-expression of a RING-H2 finger protein gene conferred resistance against rice blast fungus [31] or led to the upregulation of defense and/or cell death related genes in tobacco [32]. Thus it is possible that the protein interacting ring domain of this transcription factor may be involved in the oxidative stress response caused due to fungal pathogen infection. The cDNA GhC43, similar to MADS-box protein 4 (MADS4), showed very high expression in both pericarp and seed tissues under A. *flavus* infection; the upregulation was higher in seed compared to the pericarp. Genetic analysis of MADS-box genes has shown that most of them determine of flowering time, reproductive organ growth and vegetative growth [33]. However, expression analysis of the transgenic rice and *Arabidopsis* plants with MADS24 showed upregulation of genes in ethylene and reactive oxygen species biosynthesis. This further elucidates that ethylene and the ROS produced in response to pathogen attack act in the early signaling pathway that is regulated by the MADS-box transcription factor.

3.2.2. Stress responsive genes

The GhC5 had high similarity to cotton Spotp11 catalase gene, which codes for a ubiquitous hydrogen peroxide (H_2O_2)-scavenging enzyme. When a pathogen attacks, the plant rapidly produces reactive oxygen species (ROS) to strengthen the cell wall as a defense mechanism [34,35]. The significantly increased amounts of ROS including H_2O_2 can cause the cell damage. H_2O_2 plays an important role in plant defense mechanism as a signaling molecule and thus enzymes such as catalase or superoxide dismutase are induced. Upon *A. flavus* infection, the mRNA content of catalase increased highly (three- to nine-fold) in both the pericarp and seed tissues. The catalase gene induction was higher in the *A. flavus* inoculated corn immature embryos than non-inoculated ones and its level was shown to be positively correlated with the resistance of corn to the fungus [36].

Similarly, cytochrome c oxidase (COX) is known to be involved in the mitochondrial respiration chain and enzyme is a major contributor of reactive oxygen species (ROS) during the

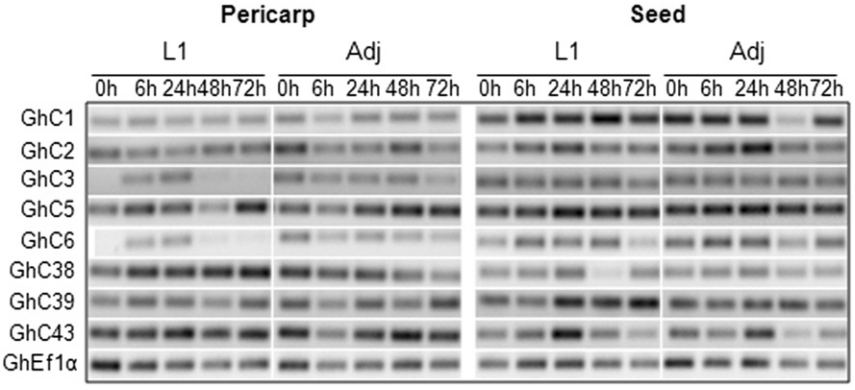

Fig. 2. Reverse transcription (RT) PCR analysis of differentially expressed genes in pericarp and seed tissues of cotton at different time points (0 h, 6 h, 24 h, 48 h and 72 h) post inoculation with *Aspergillus flavus*. Cotton elongation factor (GhEF1α) was used as the endogenous control.

Table 3
Fold-change in the expression of differentially expressed genes at different time points in pericarp and seed tissues of cotton in response to *Aspergillus flavus* infection relative to non-infected control.

			GhC1	GhC2	GhC5	GhC6	GhC38	GhC39	GhC43
Pericarp	L1	6 h	3.8	−1.2	3.0	1.4	4.1	3.3	1.9
		24 h	1.7	−2.0	3.1	32.6	3.2	2.6	3.6
		48 h	1.5	1.1	1.6	77.1	5.7	1.9	1.6
		72 h	2.1	−1.0	6.0	2.9	12.6	2.1	2.8
	Adj	6 h	−0.6	−0.6	1.3	1.7	−1.6	−1.6	−0.8
		24 h	1.1	−0.4	2.3	1.2	−1.1	1.1	−0.4
		48 h	1.1	1.0	5.9	1.0	−1.3	−0.9	1.0
		72 h	−0.9	−0.2	5.1	1.5	−2.0	3.9	3.3
Seed	L1	6 h	6.1	2.7	1.8	3.2	2.4	−1.4	2.4
		24 h	5.8	3.4	8.5	2.8	3.4	16.1	18.3
		48 h	8.9	2.5	4.0	2.3	−2.7	5.8	2.8
		72 h	3.3	2.4	3.1	1.0	2.5	19.7	1.8
	Adj	6 h	1.3	2.9	2.0	3.3	1.6	−1.6	−1.2
		24 h	−0.9	5.5	4.1	2.6	2.8	4.9	3.1
		48 h	−0.4	1.1	2.5	−1.2	1.3	−1.9	−1.7
		72 h	0.8	−0.9	1.6	1.7	1.5	−1.1	−1.2

L1 = Locule 1, site of infection; Adj = adjacent locules.

hypersensitive reaction [37]. Mitochondria are also a potential source of superoxide and peroxide, being produced when electron transport through the cytochome pathway is restricted by stress-induced due to wounding, thereby leading to the increased accumulation of oxidase enzymes [38]. The GhC38 similar to cox gene was upregulated in both seed and pericarp tissues; the upregulation was expectedly higher at the wounding site i.e., L1 of pericarp and seed. On the other hand, it showed downregulation in the Adj locules of the pericarp. This indicated the transcription of catalase and cox mRNA was upregulated along with the oxidative bursts in order to help maintain the reactive oxygen species homeostasis in the infected and adjacent tissues. LRR-RLK and Ring-H2-protein, catalase, and oxidase are also differentially regulated under drought stress and it has already been shown from proteomics research that resistant corn genotypes show increased protein levels of stress associated proteins [39].

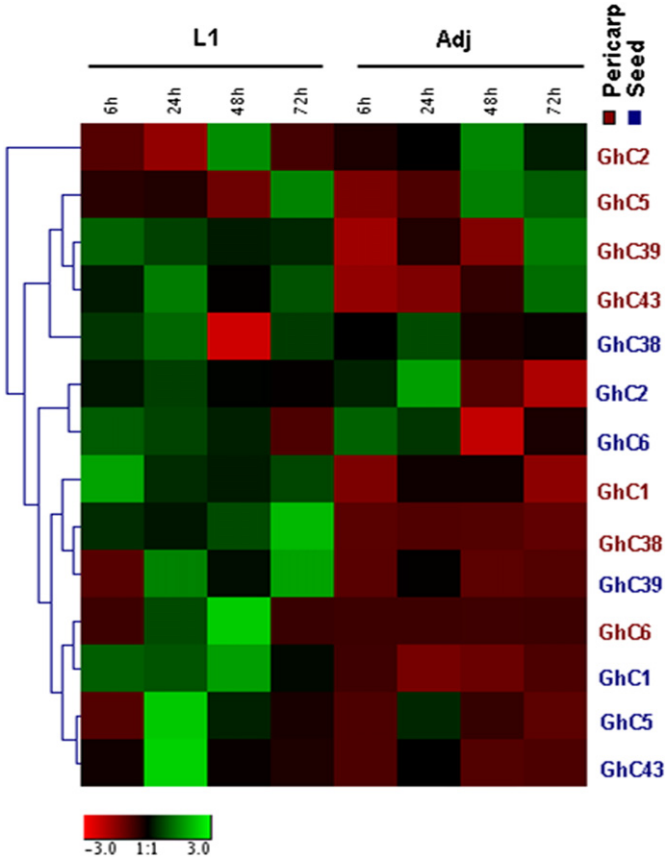

Fig. 3. Heat map of differentially expressed genes as analyzed by quantitative reverse transcription (qRT) PCR in pericarp and seed tissues of cotton at different time points (6 h, 24 h, 48 h and 72 h) post inoculation with *Aspergillus flavus*. Cotton elongation factor (GhEF1α) was used as the endogenous control.

3.2.3. Storage protein genes

Storage protein genes have been implicated in plant's defense response to diseases. In corn hydrophilic storage proteins such as alpha globulin A&B and late embryogenesis abundant protein (LEA4) were highly induced upon infection with *A. flavus* [40,41]. In our study alpha globulin A&B (GhC6) was induced in the pericarp at the site of infection (L1) only after 6 h and reached its highest expression at 48 h of infection by *A. flavus*; its upregulation in the Adj locules of pericarp was not as high compared to the L1 locule (Fig. 2, Table 3). Transcript accumulation of GhC6 in seed was higher until 48 h (L1) and 24 h (Adj) before being down-regulated or dropped down to basal level. The LEA gene (GhC39) was highly upregulated in seed in comparison to the pericarp. LEA proteins were shown to be regulated under multiple stresses such as desiccation, cold, and high salinity [42] and their transgenic manipulation has resulted in higher water and salt stress tolerance in rice [43]. The role of the storage proteins in peanut's defense response to *A. flavus* was shown very recently [19] where the authors, from an EST and microarray-based gene expression profiling, observed consistent upregulation of globulin and LEA genes upon fungal infection.

Both up- and down-regulated expression patterns of the genes implicated putative multiple pathways recognized by the cotton tissues to *A. flavus* infection. It suggests that the infected cells selectively turn on or off their transcription machinery for a broad range of metabolic functions, such as producing molecules against *A. flavus*, or reducing growth-related activities to minimize energy consumption as a part of its defense strategy.

4. Conclusions

In the absence of genetic resistance against *A. flavus* in cotton gene pool, identification of genes induced/regulated in response to the fungus will provide clues to the molecular network underlying the defense mechanism of cottonseed. This will help develop multiple strategies to control the aflatoxin contamination in cottonseed. The present data showed that certain genes or group of genes are activated or deactivated in the process of *A. flavus* infection; in addition to the storage protein genes, elucidation of responses of leucine-rich repeat receptor-like kinases (LRR-RLKs), stress-related genes, and transcription factors elucidated the interplay of signals in fine-tuning the defense response of cottonseed. Since several genes work in harmony toward to a common response reaction, there will be other genes of known and unknown function operating in coordination with the DEGs that were observed in this study. Hence, gene discovery through genome-wide transcriptome sequencing will unravel many more genes involved in key regulatory pathways. Further, this will also expound the gene clusters involved in the cross-talk between abiotic stress, such as drought, salinity and heat, and *A. flavus* infection response. The differentially expressed genes, identified using this approach, can be used to genetically engineer cotton to provide effective resistance against *A. flavus*. In addition, this will lead to the development of genic functional markers for use in marker-assisted breeding to mitigate aflatoxin contamination in cottonseed.

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