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SHORT COMMUNICATION

Identification of candidate resistance genes of cotton against *Aspergillus flavus* infection using a comparative transcriptomics approach

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Abstract A comparative transcriptome analysis was performed using the genes significantly differentially expressed in cotton, corn and peanut in response to aflatoxin producing fungus *Aspergillus flavus* with an objective of identifying candidate resistance genes in cotton. Two-way analyses identified 732 unique genes to be differentially regulated by the fungus with only 26 genes common across all three crops that were considered candidate *A. flavus* resistance genes with an assumption that these genes have specific roles in conferring the resistance trait. Genes of membrane cellular component involved in DNA binding with involvement in defense responses were highly represented among the differentially expressed unique genes. Most (six) of these genes coded for 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily proteins. Genes encoding helix loop helix protein, alcohol dehydrogenase and UDP glycosylation transferase which were upregulated in response to both atoxigenic and toxigenic strains of *A. flavus*, could be potential resistance candidate genes for downstream functional manipulation to confer resistance.

Keywords Aflatoxin · Cotton · Differentially expressed genes · Resistance · Transcriptome

Introduction

Cotton belongs to the genus *Gossypium*, where *G. hirsutum* and *G. barbadense* (allotetraploids) and *G. herbaceum* and *G. arboreum* (diploids) are widely cultivated for textile fiber, edible cottonseed oil, and cottonseed meal for diary industry (Paterson et al. 2012). Necrotrophic toxin-producing fungi, viz. *Aspergillus flavus*, *A. parasiticus,* and *A. nomius* are considered highly devastating to the quality and vigor of produce (Ashworth et al. 1969). Under natural conditions, *A. flavus* infects cottonseed during boll development through abrasions/cuts and/or after boll maturity (Cotty 1991) and accumulates toxic secondary metabolites called aflatoxins (Payne and Brown 1998). *A. flavus* is the primary causal agent of aflatoxin contamination of cottonseed, peanut, and maize (Garber and Cotty 1997; Guo et al. 2011). The annual estimated economic losses due to aflatoxin contamination are > $100 million in the U.S. and > $400 million globally (Schmale and Munkvold 2009). The stringent regulations on permissible aflatoxin concentrations and large economic loss faced by the agriculture sectors producing cotton, peanut and maize calls for biotechnological and plant breeding interventions to develop *A. flavus*-resistant cultivars.

The role of several pathogenesis-related proteins, stress-responsive proteins, and reactive oxygen species (ROS)-scavenging enzymes in regulating *A. flavus* resistance has

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been delineated in peanut and maize using various omics approaches (Chen et al. 2006; Wang et al. 2013; Fountain et al. 2015). Resistant and susceptible germplasms of peanut and maize were used to develop insertion/deletions, single nucleotide polymorphisms, and microsatellite markers for discovery and characterization of quantitative trait loci (QTLs) for aflatoxin resistance (Guo et al. 2001; Zhang et al. 2016). Biomarkers and QTLs were successfully used in conventional breeding programs for generating *A. flavus*-resistant lines of peanut and maize (Nigam et al. 2009; Williams et al. 2014; Scully et al. 2016).

Genetic enhancement of aflatoxin resistance in cotton is limited due to narrow germplasm diversity with no known resistant varieties (Cary et al. 2011). Application of atoxigenic strains of *A. flavus* and/or *A. parasiticus* to outcompete toxigenic strains has been proven successful in reducing aflatoxin contamination by ~ 70–90% in cotton and peanut (Dorner 2008). Nevertheless, identification of the genes and QTLs controlling *A. flavus* resistance is important to facilitate breeding of cultivars with durable resistance. Transcriptome profiling of cotton variety "Coker 312" pericarp and seed at different time-points post-infection with toxigenic (AF13) and atoxigenic (AF36) strains of *A. flavus* identified infection-responsive differentially expressed genes (DEGs), which included genes interfering with fungal virulence and growth, cross-talk between jasmonic acid/ethylene and salicylic acid signaling pathway, defense signaling pathways, oxidative burst, and transcription factors (Lee et al. 2012; Bedre et al. 2015). The present study attempts to identify *A. flavus* resistance candidate genes in cotton using comparative transcriptome analysis with known resistance-associated genes of peanut (Guo et al. 2011) and maize (Dolezal et al. 2014).

Methods

The probe IDs of DEGs from microarray experiments of *A. flavus* infected maize (8374 genes) and peanut (8303 genes) were retrieved from gene expression omnibus (GEO) database (GSE27612, Guo et al. 2011; GSE57629, Dolezal et al. 2014). Microarray data were the only genome-wide information available on *A. flavus*-induced transcriptome of peanut and corn for use while this study was being conducted. The 922 *A. flavus*-responsive DEGs of cotton (NCBI SRA database Acc.# PRJNA275482; Bedre et al. 2015) were interrogated against the maize and peanut DEGs through BLAST analysis (e-value cut-off 1e-05, $\geq$ 80% sequence identity, and $\geq$ 80% alignment length). Circos tool (Krzywinski et al. 2009) was used to demonstrate the homologous relationship of the genes in three genomes.

Gene ontology (GO) of cotton DEGs showing significant hits with maize and peanut DEGs was analysed using Blast2GO v3.0.10 (Conesa et al. 2005). The sequences were BLASTP searched against NCBI non-redundant protein database followed by mapping, InterProScan, and annotation. GO enrichment analysis was performed using BiNGO plugin of Cytoscape v2.6 (Maere et al. 2005). Domain analysis was performed using HMMSCAN (https://www.ebi.ac.uk/Tools/hmmer/search/hmmscan), SMART, and Pfam (Cao et al. 2016). Candidate resistance genes of cotton were then physically mapped on chromosomes using Mapchart v2.30 (Voorrips 2002). A triplicated expression analysis of 10 selected genes was performed by real-time PCR (Bedre et al. 2015).

Results and discussion

Querying the 922 *A. flavus*-responsive cotton DEGs against maize and peanut DEGs showed significant hits for 719 and 39 genes of which 693 and 13 were unique candidate resistance genes, respectively (Fig. 1; Supplementary Table S1). But, only 26 genes were common across all three crops (Fig. 1).

Of the 732 genes, 726 mapped onto the thirteen cotton chromosomes with uneven distribution (Fig. 2). Six genes could not be mapped, which is possibly due to the lack of complete genome annotation. Maximum numbers of genes were mapped on chromosome 1 (96; ~ 13%) followed by chromosome 7 (88; 12%), whereas chromosome 12 had the minimum number (37; ~ 5%) of genes. The genes were clustered in the telomeric regions with very few genes present in the centromeric region, except chromosomes 7 and 9 that had uniform distribution pattern. The physical location of the candidate resistance genes will be useful for their future map-based cloning, identifying upstream regulatory elements, location-specific methylation patterns moderating their expression, etc.

Functional annotation of 732 candidate resistance genes defined their involvement in molecular function, biological process and cellular component (Fig. 3; Supplementary Table S2). Under molecular function, the proteins of identified genes were found to possess ion-, tetrapyrrole-, nucleic acids-, protein- and lipid-binding ability and lyase-, transferase-, oxidoreductase- and hydrolase-catalytic activity. These proteins also participate in antioxidant enzyme and transcriptional regulation activities. Under biological process, the proteins were involved in stress stimulus response and metabolic processes. Noteworthy, the proteins were predicted to be involved in defense response to various fungal and bacterial pathogens in addition to oxidative, osmotic, salt, and wounding stresses. A high proportion of these proteins were localized in the

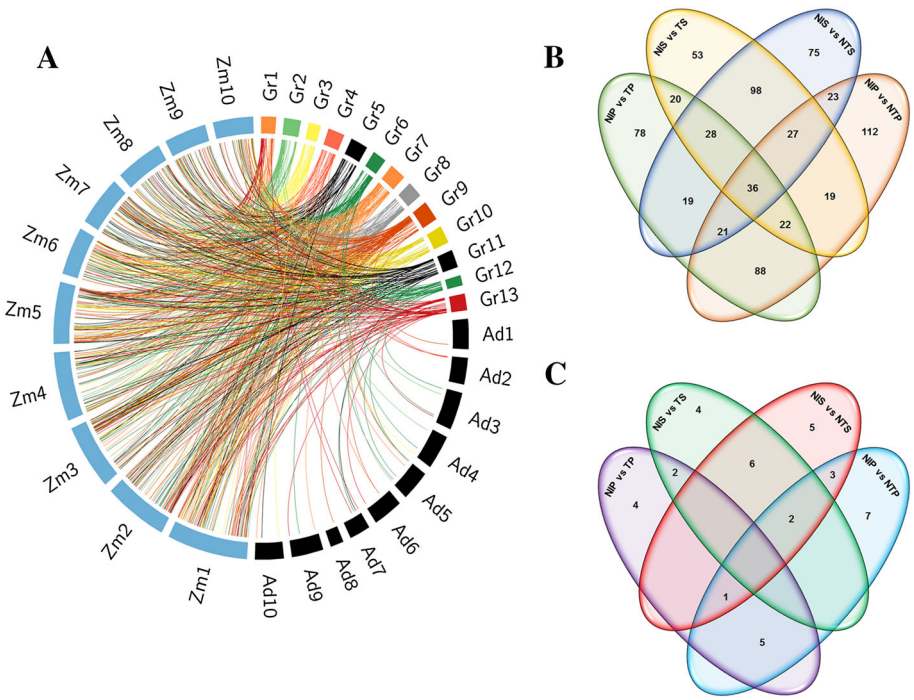

Fig. 1 Putative resistance genes conserved among cotton, maize and peanut. **a** Comparative map of putative resistance genes cotton (Gr), maize (Zm) and peanut (Ad); **b** venn diagram showing the unique and common genes between cotton and maize and **c** venn diagram showing the unique and common genes between cotton and peanut

membrane followed by extracellular matrix and nucleus. The GO information will help in the selection of proteins with defined functions relevant to pathogen response and identification of proteins with similar expression patterns to develop co-expression networks.

Functional annotation and domain composition of the 732 candidate resistance genes highlighted their roles as enzymes, signaling proteins, transcription factors, and defense-related proteins. Nearly 30% of the genes encoded enzymes, mainly kinases, such as phosphofructokinase (ATP-dependent 6-phosphofructokinase 3), protein kinase (CBL-interacting serine threonine-kinase 6, phytosulfokine receptor 2, rust resistance kinase Lr10, etc.) and protein tyrosine kinase (receptor-like serine threonine kinase SD1-8 isoform X1, G-type lectin S-receptor-like serine threonine kinase B120). Peroxidase, oxygenase, methyltransferase and amino acid lyase were also encoded by these genes. Additionally, a few genes encoded zinc-binding dehydrogenase, enoyl-CoA hydratase/isomerase, lipoxygenase, and serine acetyltransferase. Among transcription factors, bHLH, bZIP, HSF, LEA, MYB, WRKY, and different ZFPs were predominant. Several of these transcription factor super-families are reportedly stress-responsive, and therefore, are potential candidates for further functional characterization to delineate their role in mechanisms underlying *A. flavus* resistance.

The comparative transcriptome analysis also identified several proteins, which play a vital role in hormonal regulation. Auxin-responsive proteins (SAUR-like protein family), ethylene-responsive protein (ethylene-responsive protein kinase Le-CTR1), gibberellin-regulated protein (GAST1 protein homolog 1, peamaclein, gibberellin-regulated 4) and cytokinin oxidases present in the resistance gene pool underlines their putative involvement in hormone-regulated stress-responsive physiological and molecular mechanism. Genes encoding proteins of NAD(P)-binding Rossmann-fold superfamily, lactoylglutathione lyase glyoxalase I family, hydroxyproline-rich glycoprotein family, alpha 1,4-glycosyltransferase family, Glutaredoxin family, and lysophosphatidyl acyltransferase 2, pathogenesis-related 1, DNAJ heat shock protein, expansin A8, cysteine-rich RLK (receptor-like kinase), shikimate *O*-hydroxycinnamoyltransferase-like, and inter-alpha-trypsin inhibitor heavy chain-related proteins were also identified.

Twenty-six genes, identified from the three-way comparison among cotton, maize, and peanut, were considered candidate *A. flavus* resistance genes that may have specific roles in resistance response against *A. flavus* infection. Six genes coding for 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily proteins play roles in the biosynthesis of secondary metabolites, flavonoids and hydroxyproline rich proteins that are known to provide defense against invading pathogens (Gechev et al. 2005). In citrus, 2OG and iron-dependent dioxygenase showed several folds up-regulation in the tolerant cultivar during infection with *Candidatus* Liberibacter asiaticus (Albrecht and Bowman 2012). Four genes encoding UDP-glycosyltransferases that play significant roles in defense response (Jin et al. 2007) were also expressed in cotton, maize, and

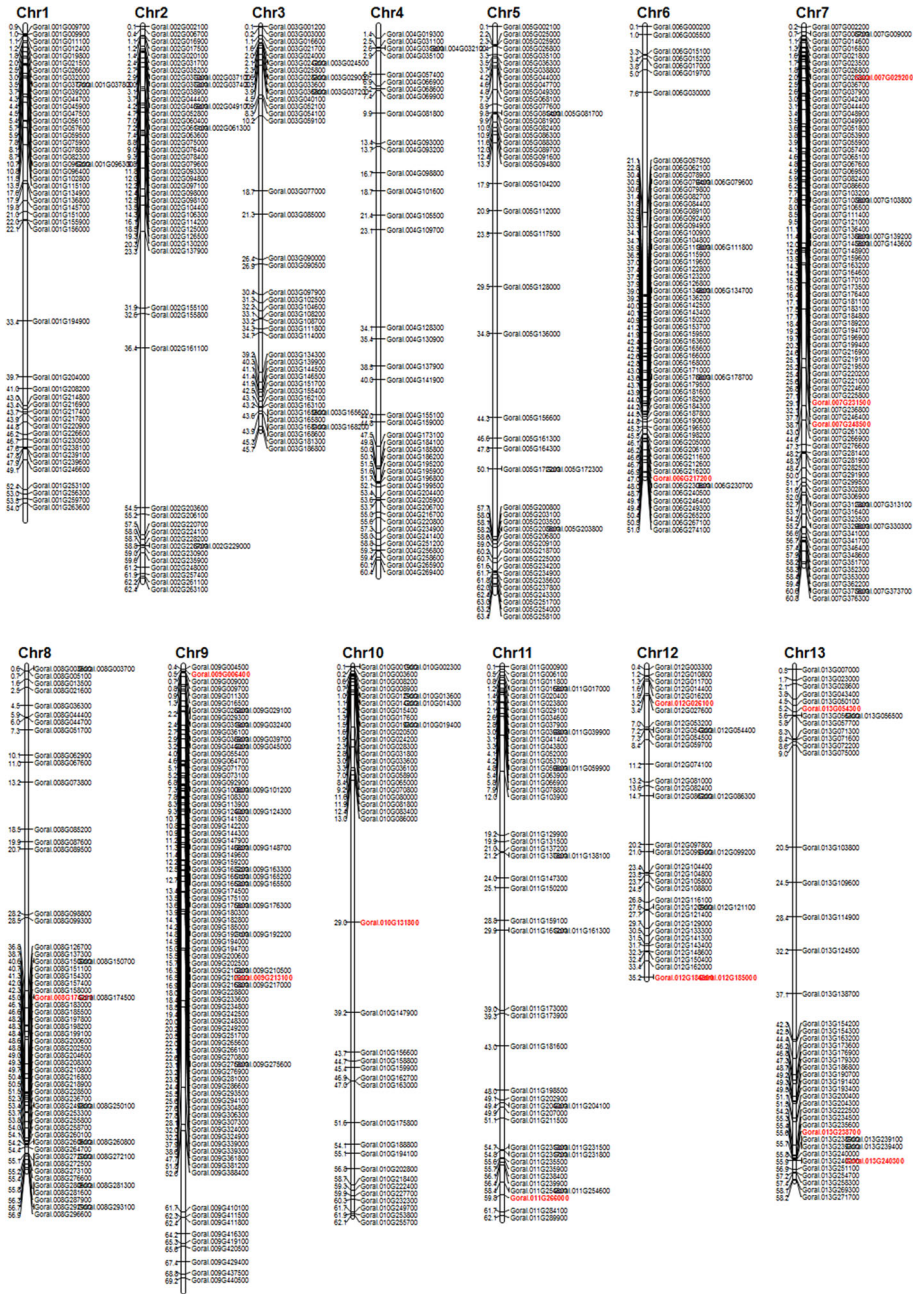

Fig. 2 Physical map of 732 putative *Aspergillus flavus* resistance genes. The bars represent the chromosomes with numbers at the left indicating the physical position (in Mb)

peanut. In *Arabidopsis*, lack of UDP-glycosyltransferase activity resulted in susceptibility to biotic and abiotic stresses (Blanco-Herrera et al. 2015). Transcription factors, namely basic helix-loop-helix (bHLH), heat shock factor (HSF), and homeobox-leucine zipper protein (HD-ZIP) were also expressed in all three crops in response to *A. flavus* infection. Though the molecular roles of these transcription factors have been elucidated in biotic stress responses, their roles in *A. flavus* defense response need to be ascertained.

Genes coding for bHLH, alcohol dehydrogenase (ADH), and UDP glycosylation transferase (UGT) were upregulated in response to both atoxigenic and toxigenic strains of *A. flavus*. In *Arabidopsis*, the bHLH transcription factor HBI1, mediated by hormonal, environmental and pathogen signals, played an important role in the trade-off between growth and immunity (Fan et al. 2014). ADHs are known to function in biotic stress response (Senthil-Kumar et al. 2010). Over-expression of a zinc-binding ADH from *Arachis diogoi* in tobacco showed hypersensitive response (HR) by *Phaeoisariopsis personata* infection (Kumar et al. 2016). Similarly, UGTs were involved in HR to *Pseudomonas syringae* infection in tomato and non-host resistance to the Asian soybean rust pathogen in *Arabidopsis*

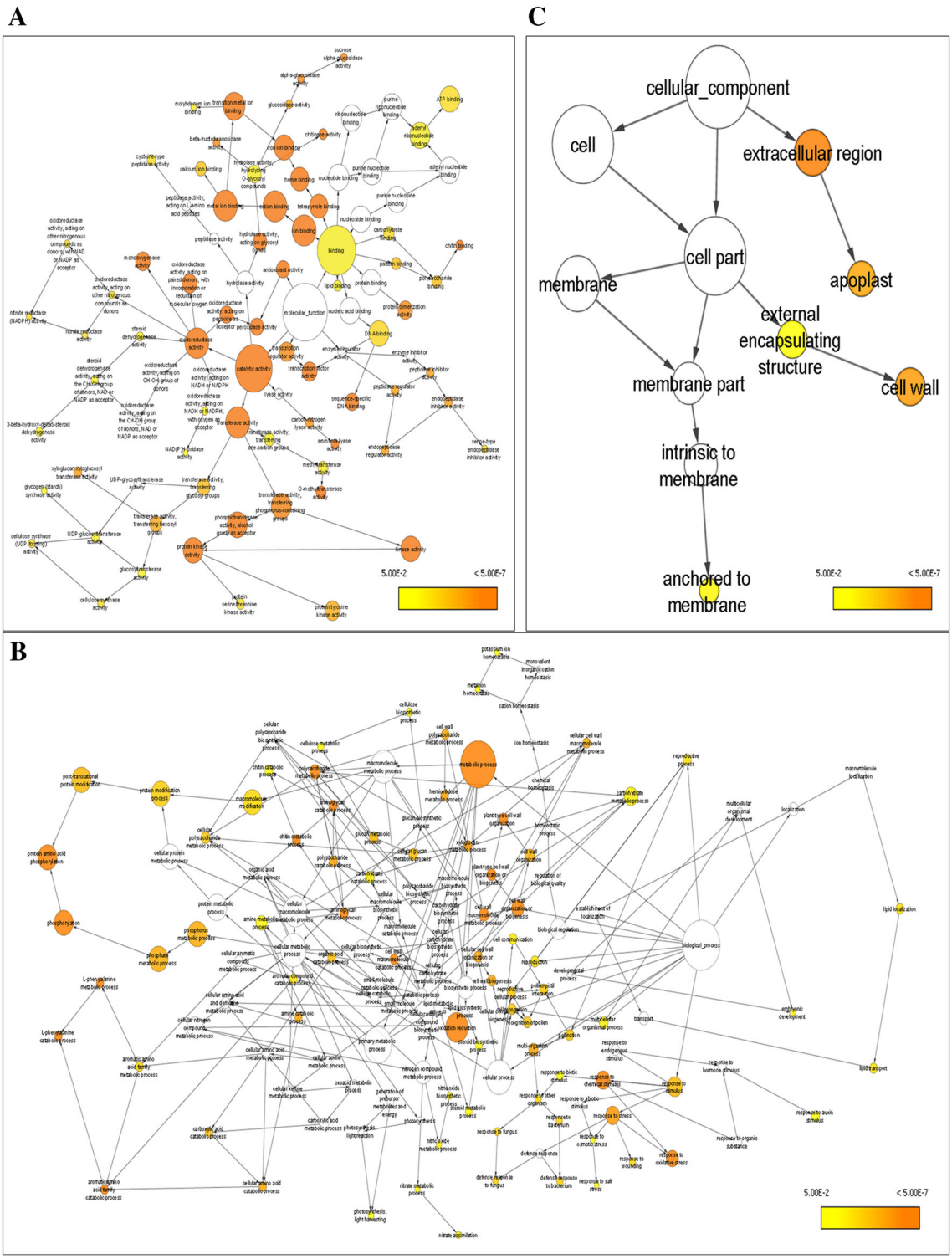

Fig. 3 Gene ontology (GO) analysis of 732 putative *Aspergillus flavus* resistance genes showing **a** biological process, **b** molecular function, and **c** cellular component. The number of genes in each GO category is directly proportional to the node size. The nodes are color shaded according to the significance level (corrected *P* value)

(Langenbach et al. 2013; Simon et al. 2014). The diverse molecular and physiological roles of these genes in stress–response and their upregulation in response to both atoxigenic and toxigenic strains of *A. flavus* accentuate them as potential candidates for downstream functional characterization to ascertain their roles in *A. flavus*

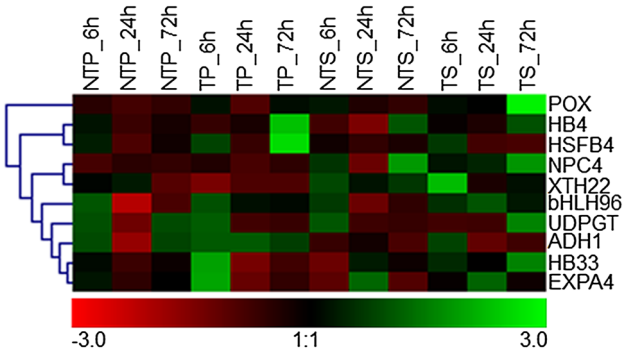

Fig. 4 Expression of 10 candidate *Aspergillus flavus* resistance genes in pericarp (P) and seed (S) tissues of cotton at different time points (6, 24, and 72 h) by non-toxigenic (NT) and toxigenic (T) strains. POX, peroxidase; HB4, homeobox domain containing protein 4; HSFB4, heat shock factor B4; NPC4, non-specific phospholipase C4; XTH22, Xyloglucan endotransglucosylase/hydrolase; bHLH, beta helix loop helix; UDPGT, UDP-glycosyltransferase 74 F1; ADH1, alcohol dehydrogenase; HB33, homeobox domain containing protein 33; EXPA4, expansin 4

resistance in cotton. Quantitative RT-PCR analysis of 10 selected genes also corroborated with the transcriptome results with their differential temporal expression in pericarp and seed tissues of cotton (Fig. 4).

Analysis of the promoter region of 26 genes commonly regulated in all three crops showed the presence of at least sixty *cis*-regulatory elements in the upstream region with 30 elements in all 26 genes (Supplementary Table S3).

Conclusion

In summary, comparative analysis of *A. flavus*-responsive transcriptome of cotton with peanut and maize identified 732 genes that might play putative roles in physiological and molecular responses to *A. flavus* infection. Twenty-six candidate resistance genes that were commonly regulated in all three crops are being functional characterized using functional genomics approaches. In addition to transgenic manipulation, sequence variations of these genes among three crops are being studied to identify targets for gene editing to improve *A. flavus* resistance in cotton.

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Conflict of interest The authors declare that they have no conflict of interest.

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