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Abstract

Differentially expressed genes help in exploring plant defense mechanism under variable stress conditions. In current investigation, RNA sequencing was executed to explore the differential gene expression in resistant and susceptible varieties of Cotton (*Gossypium hirsutum*), upon infection with *Aspergillus tubingensis*. Comparative RNA-Seq of control and infected plants was performed using Illumina HiSeq 2,500. Overall 79.84 G clean data was generated and 6,558 DEGs were identified in both varieties, in response to pathogen inoculation. Differentially expressed genes were found to be involved in defense, antifungal response, signaling pathways, oxidative burst and transcription. Genes involved in defense responses, MAPK signaling, cell wall fortification and signal transduction were highly induced in resistant variety. Real time PCR also revealed the up regulation of MAPKKK YODA like, L-ascorbate oxidase, late embryogenesis abundant protein (At1g64065) and flavonoid 3',5'-hydroxylase-like, in resistant variety. Elevated accumulation of such DEGs in resistant variety could function as the source for identifying biomarkers for breeding

and these can be used as potential candidate genes for transgenic manipulation. Their study also helped in understanding complex plant-fungal interaction and advanced the understanding of plant-microbe interaction. Inclusively, our findings provide an indispensable foundation for advanced understanding of the plant resistance mechanisms of cotton.

Keywords: RNA-Seq, DEGs, Cotton, *Aspergillus tubingensis*, MAPK

Introduction

Cotton is considered as one of the world's most treasured crops. It provides a significant part of planet's regular fiber to the international yarn production. Cotton fiber signifies about 90% of entire cotton economic worth, while further economic value is secured from seed and other related items. China, United States of America and India produce the greater part of the world's cotton. In year 2011, a joined aggregate of more than 15.9 million metric tons of cotton fiber and 30.4 million metric tons of cottonseed, with an estimation of 22.8 billion dollars and 6 billion dollars, respectively was reported ¹. Upland cotton is a characteristic allotetraploid species that rose after interspecific hybridization among diploid species *G. arboreum* (A-like genome) and *G. raimondii* (D-like genome) ².

With the developments in the field of transcriptomics, RNA-sequencing has emerged as a major “-omics” technology for reviewing inclusive expression of genes under various stresses ³. In cotton, stress mechanism had been studied under several biotic and abiotic factors. Currently, in Pakistan, transcriptomic analysis of *G. hirsutum* was carried out to investigate the DEGs in susceptible *G. hirsutum* variety in response to whiteflies attack ⁴. RNA-seq analysis of infested cotton floral buds has exhibited the differential expression of plant hormone signaling genes, transcription factors and kinase cascades ⁵. RNA-seq of *G. hirsutum* root tissues has also displayed differential genes expression in response to drought condition ¹. Comparative transcriptomic and reverse genetics have been employed for the identification of differentially expressed genes under the stress of *Verticillium dahliae* ⁶. RNA seq analysis divulges variances in the mechanisms of origination and elongation among long- fiber and short-fiber cotton lines ⁷. Transcriptomic analysis of *G. hirsutum* has also been applied for the detection of candidate genes in response to *Aspergillus flavus* ⁸.

Innate immune system of plants comprises of cells population and signaling pathways which constitutively function to provide quick response to pathogens, at diseased area ⁹. Both pathogen triggered immunity and effector triggered immunity respond to stress by using a joint set

of signaling components, which includes ROS, Ca²⁺ and multiple protein kinases. These signaling machineries regulate downstream regulatory protein activities including transcription factors, which then lead to vast transcriptional reprogramming and initiates the induction and accretion of various pathogenesis-related proteins, enzymes and pathogen infection-responsive metabolites ¹⁰. Rapid phosphorylation response against pathogens also involves some downstream mitogen-activated protein kinases (MAPKs) ¹¹ and calmodulin protein kinases ¹². Phytohormones play a central role in plant defense against pathogens. In *A. thaliana*, salicylic acid (SA) and ethylene can stimulate the expression of defensin-like (DEFL) genes ¹³. The phenylpropanoid pathway plays a central role in plant defense against *V. dahliae* ¹⁴. Furthermore, transcription factors such as ethylene response factors (ERFs) and WRKYs can be triggered by MAPKs, and are involved in modulating plant defense ¹⁵. *A. tubingensis* has been reported as a causal agent of leaf spot of cotton in Pakistan ¹⁶. Non-targeted metabolomics profiling of *G. hirsutum* infected with *A. tubingensis* has depicted ample accumulation of significant metabolites like carbohydrates, flavonoids, fatty acids, phenylpropanoids organic acids, terpenoids and fatty acids in tolerant cultivar ¹⁷.

This study was aimed to see the differential expression of different genes in tetraploid cotton, in response to *A. tubingensis* inoculation. Infected cotton cultivars were subjected to RNA-seq analysis and obtained sequences were subjected to data analysis, gene ontology, functional annotation and pathway enrichment analysis. We intended to distinguish key DEGs of cotton in response to *Aspergillus* infection to interpret its resistance mechanisms.

Results

Disease severity analysis

Infected cotton leaves showed symptoms at 4 dpi. Leaves of susceptible variety exhibited infection spots, which later spread on the whole leaf surface. Initial symptoms appeared on leaf veins. The tolerant variety showed reduced leaf spots (2 mm average size) which did not spread rapidly (Fig.1A, B). Diseased area of fungal inoculated leaves of both varieties was measured in cm (Fig.1C).

Figure 1. Phenotype of susceptible (A) and resistant cotton cultivar (B), after 4 days of infection with *A. tubingensis*. (C) Disease severity of susceptible and tolerant cultivars. (D) FPKM density

distribution reflects the protein-coding gene expression pattern of each sample. **(E)** The FPKM value box line **(F)** FPKM box-plot for each sample. The X-axis is the sample name, Y-axis is log10 (fpkm+1). The different colors in the figure represent different ranges of fpkm values, the abscissa is the sample, and the ordinate is the number of protein-coding genes.

Illumina Sequencing and Quality Control

The complete analysis was comprised of 12 transcriptomic sequencing samples and a total of 79.84 G clean data was obtained. The effective data volume of each sample was 6.19~6.82 G with 44.37% average GC content. The Q30 base distribution was found to be 91.62~93.46% (Table 1).

Table 1. Preprocessing results of sequenced data quality

Sample raw_reads	*Raw reads (M)	Raw bases (G)	*Clean reads	Clean bases (G)		Valid bases (%)	Q 30 (%)	GC (%)
Sample_R_ CON_1	49.90	7.49	47016742	6.74		90.08	91.88	44.13
Sample_R_ CON_2	49.42	7.41	46898960	6.71		90.54	92.43	44.54
Sample_R_ CON_3	46.11	6.92	43585100	6.19		89.48	91.87	44.12
Sample_R_ TRE_1	49.01	7.35	46053196	6.59		89.63	91.62	44.26
Sample_R_ TRE_2	49.39	7.41	46567842	6.66		89.94	92.13	44.22
Sample_R_ TRE_3	49.12	7.37	46329412	6.63		90.04	92.00	44.32
Sample_S_ CON_1	48.98	7.35	46575348	6.72		91.45	93.31	44.43
Sample_S_ CON_2	49.01	7.35	46730562	6.71		91.35	93.35	44.51
Sample_S_ CON_3	49.87	7.48	47505758	6.82		91.19	93.46	44.14
Sample_S_T RE_1	49.18	7.38	46478232	6.66		90.29	92.15	44.74

Sample_S_T RE_2	49.88	7.48	47342178	6.72		89.88	92.42	44.81
Sample_S_T RE_3	49.35M	7.40	46841020	6.69		90.36	92.68	44.26

Alignment to the Reference Genome

By comparing reads to the reference genome, the genomic alignment of each sample was obtained and the alignment rate was found to be 87.08~94.86%. Based on the alignment results, protein-coding gene expression levels were successfully analyzed (Supplementary Table 1).

FPKM density distribution

The FPKM density distribution reflected protein-coding gene expression pattern of each sample (Fig.1D). A density map of FPKM for all sample genes visually represented the FPKM distribution of genes in each sample. Because there were differences in the number and value of gene expression, each sample expression value (FPKM) was distributed into different intervals. The number of genes expressed in different expression interval samples was calculated and the stacked histograms were drawn (Fig.1E)

In FPKM value Box-whisker plot, five statistics i.e. minimum, first quartile (25%), median (50%), third quartile (75%) and maximum value were used to define information. It efficiently showed the symmetry of data and the degree of dispersion of distribution (Fig. 1F).

Assessment of variation among samples

PCA showed discrete behavior of resistant and susceptible variety and 74% total variance was contributed by all three PCs. PC1 showed 42.95% variance, while the variance of PC2 and PC3 was recorded to be 21.39% and 9.82 %, respectively (Fig. 2). Unsupervised hierarchical clustering of differentially expressed genes also provided useful information (Figure S1). Generally, the same types of samples were clustered together with similar biological functions. Constant with the prospects of the experimental conditions, pathogen infected samples in both varieties were clustered together. The closer was the correlation coefficient to 1; the elevated was the degree of similarity in expression patterns between samples (Figure S2).

Figure 2. 3D PCA exhibiting the variance of DEGs in all four data sets, with an overall variance of 74% contributed by all the three PC.

Differential gene expression statistics and profiling

According to the expression levels, protein-coding genes were found in four differential groups (Fig. 4). Numbers of differential protein-coding genes were detected to be 2,409 in group 1, 1,998 in group 2, 302 in group 3 and 1,849 in group 4.

Based on the alignment of the obtained cotton reads with reference genome (*G. hirsutum*), 6,558 DEGs was found in both varieties, using threshold of $FC > 2$ and $P < 0.05$. In susceptible treated and susceptible control (ST vs SC) treatment, 1,070 genes were upregulated while 1,339 were down regulated. In resistant treated and resistant mock (RT vs RC) treatment, 849 genes were upregulated and 1,000 were down regulated. In resistant treated and susceptible treated condition (RT vs ST), 1,426 genes were upregulated and 572 genes were down regulated. On the other hand, in resistant control and susceptible control (RC vs SC), 217 genes were

upregulated and 85 were suppressed, under similar conditions (Fig. 3A). Venn diagram showed common and unique DEGs in four data sets (Fig. 3B). Volcano plots also revealed the presence of up and down regulated genes among different treatments of resistant and susceptible varieties (Fig. 4).

Figure 3. Statistical representation of differentially expressed genes horizontal. (A) Bar graph in which horizontal axis is the comparison group; the vertical axis is the number of differential genes in the comparison group, where “Up” is the number of up-regulated genes with significant differences, and “Down” is the number of down-regulated genes with significant differences. (B) Venn Graph is showing common and unique DEGs between different comparison groups.

Figure 4

Figure 4. Differential expression by volcano map in four data sets. The gray color represents genes with a non-significant difference, while red and green are showing significantly up and down regulated genes. The horizontal axis is the display of log₂ fold change, and the vertical axis is -log₁₀ P value.

Validation by Real Time PCR

The transcriptomic data was confirmed using nine genes (with FC expression greater than 5), under inoculated conditions in both varieties. The results exhibited substantial up-regulation of mRNA, in response to *A. tubingensis* inoculation (Fig. 5).

Figure 5. Relative expressions of candidate genes (DEGs), in fungal inoculated resistant and susceptible varieties where X -axis shows the relative expression of DEGs and Y- axis shows the genes.

GO enrichment analysis of DEGs

Based on gene ontology enrichment analysis, DEGs were distributed in different functional groups including BP, MF and CC that were distinctly represented in resistant and susceptible variety in response to *Aspergillus* leaf spot. The DEGs were further distributed into 22, 13 and 13 sub-categories of BP, CC and MF, respectively, in resistant variety. DEGs were principally found to be involved in biological process including locomotion, signaling, response to stimulus, metabolic process and cellular process (Fig. 6A). The DEGs in susceptible cultivar post fungal inoculation were divided into 3 key subcategories, including biological process (15 DEGs), cellular process (13 DEGs) and molecular process (15 DEGs). The DEGs were mainly belonged to biological processes including localization, growth, biological regulation and cellular processes (Fig.6B). The DEGs for resistant and susceptible inoculated were presented in (Fig.6C). Detailed GO enrichment analysis of resistant cultivar (RT vs RC) has been provided in supplementary table (Table S2).

Figure.6 GO enrichment analysis of resistant variety (A), Susceptible variety (B) and

Resistant pathogen inoculated and susceptible pathogen inoculated (C) where green, blue and red color depicts biological, cellular and molecular functions respectively.

The top GO directed acyclic graph displayed the GO node (Term) and hierarchical relationship of differentially expressed genes. It involved graphical representation of the results of GO enrichment analysis of DEGs and the inclusion relationship of branches, from top to bottom. The scope of the defined functional descriptions became more specific and prominent with color transition from yellow to red. Top GO acyclic graph for RT vs RC have been provided in supplementary data (Figure S3, S4 and S5).

KEGG enrichment analysis of DEGs

KEGG pathways gathered information regarding the incorporation of various pathways (plant pathogen interaction, signal transduction and molecular processes). Moreover, pathways which were considerably enhanced in resistant variety were MAPK signaling pathway, plant–pathogen interaction pathway, plant hormone signal transduction pathway, linolenic acid metabolism pathway Isoquinoline alkaloid biosynthesis pathway, phytohormone signal transduction, phenylalanine metabolism pathway, cutin, suberine and wax biosynthesis pathway, and phenylalanine metabolism pathway (Fig. 7A). KEGG enrichment pathways for susceptible cultivar and RT vs ST were also shown in (Fig 7B,7C). DEGs found in various KEGG pathways along with their FC have also been described in Table S3. Results of this analysis indicated that 47, 97,106 and 116 KEGG pathways were embodied under RC vs SC; RT vs RC; RT vs ST; and ST vs SC, respectively. Significant KEGG network path diagrams of resistant variety have been presented in supplementary data (Figure S6, S7, S8).

Figure 7

Figure 7. KEGG enrichment top20 bubble diagram of RT vs RC (A) ST Vs SC (B) and RT vs ST (C). The X-axis is the enrichment score. The larger the bubble, the more the number of differential protein-encoding genes contained. The bubble color changes from purple-blue-green-red. With the color transition the scope of protein encoding genes become more well defined. Smaller enrichment p value represents the greater degree of significance.

Discussion

Plants use sophisticated constitutive and inducible defense mechanisms to cope with the attacks of pathogens ¹⁸. Different transcriptomic analyses have helped us to understand defense mechanism of cotton plant after the inoculation of *V. dahliae* ¹⁹. Presently, the whole gene profiling of three cotton species (*G. hirsutum*, *G. raimondii* and *G. arboreum*) is accessible ²⁰. This makes transcription profiling possible and the advancement of RNA-Seq provides insight into the cotton defense mechanism against pathogen infection and also aids in the detection of selected genes, imparting role in disease tolerance.

In the present investigation, defense response of two cotton (*G. hirsutum*) varieties was compared, following the infection of *A. tubingensis*. Obtained data was adequate for the quantitative analysis of gene expression. Over-all 6,558 genes were differentially expressed. DEGs involved in “plant hormone signal transduction” and ‘plant-pathogen interaction’ pathways were considerably enhanced in the leaves of tolerant cotton variety upon fungal inoculation. Consistent with our metabolomics data, in which primary metabolites (sugar, amino acids, fatty acids) and secondary metabolites (alkaloids, terpenoids, phenylpropanoid) exhibited higher accumulation in resistant cultivar ¹⁷.

Genes involved in defense signaling

In this study, 7 receptor-like protein genes were highly induced in resistant variety post fungal inoculation. In susceptible cultivar, LRR receptor-like serine/threonine-protein kinase RCH1, cysteine-rich repeat secretory protein 55-like (2 genes) and receptor-like protein 12 (8 genes) were down regulated, post inoculation. These receptors like protein kinases were not expressed in mock inoculated samples. In response to fungal inoculation, nine transcripts encoding mitogen associated protein kinases such as mitogen-activated protein kinase, kinase kinase 3-like, mitogen-activated protein kinase, NPK1-like kinase, mitogen-activated protein kinase, YODA-like kinase, mitogen-activated protein kinase (8-like) were specifically induced in resistant variety. In this study, cellulose synthase like protein (3 genes), caffeic acid 3-Omethyltransferase 1-like (2

genes), 4-coumarate--CoA ligase-like, and cinnamoyl-CoA reductase 1-like, were upregulated in resistant cultivar, post inoculation.

Plant receptor protein kinases (RPK) illustrate plant pattern-recognition receptors (PRRs) which perceive pathogen signal for prompting an inducible defense ²¹. Elevation of cytosolic Ca²⁺ is one of the prime events in plant intrinsic immunity. Under stress conditions, elevated calcium concentrations are recognized by calcium binding proteins. In plants, wounding and pathogen elicitors activate rapid initiation of Mitogen activated Protein kinase signaling cascade ²¹. Instigation of MAPK pathway is one of the earliest studied signaling events after pathogen attack ²². In this study, MAPK signaling cascade was not stimulated in susceptible leaf tissues, upon *Aspergillus* inoculation. The elicitation of signaling cascade modulates downstream transcription factors, which express the defense related genes and primarily result in improved and persistent defense response. It also provides resistance against invading fungus by directing the generation of antimicrobial proteins and reactive oxygen species ¹⁰. NDR1 has been reported to play significant role in defense signaling against pathogens, cell wall adhesion and cell physiology. NDR1 is required for proper activation of the MAPK signal cascade ²³. Specific up regulation of both NDR1 and MAPK cascade in our study shows their probable role in imparting resistance to the tolerant cultivar via signal transduction.

Genes involved in stress response

In this study, 10 genes of putative disease resistance protein RGA3 were highly induced in resistant cultivar and disease resistance protein RGA2-like was also up regulated in resistant variety. In response to the infection of *Phytophthora infestans*, resistant genes like TMV and RGA3 have been reported to be upregulated in potato ²⁴. RPP13 locus of *A. thaliana* has been described for wide range of resistance against *Peronospora parasitica* (downy mildew pathogen) ²⁵. In this study, hypersensitive-induced response protein (1-like) was up regulated in resistant cultivar. Hypersensitive Induced Reaction (HIR) plays role in development of impulsive HR in leaves, following pathogen attacks. During the spontaneous HR lesions, accumulation of development related genes was noticed in plant leaves ²⁶.

Late embryogenesis abundant (LEA) proteins were found to be expressed in vegetative tissues and these proteins have been reported to be induced by numerous stresses and abscisic acid ²⁷. Among them, calcium dependent signaling triggers LEA-type genes, which then possibly functions in plant defense and repair ²⁸. Results of current study revealed the up-regulation of three

genes encoding late embryogenesis abundant protein. At1g64065-like were up regulated in tolerant variety however in susceptible variety, LEA proteins were down regulated. Linoleate 13 S-lipoxygenase 3-1 (chloroplastic-like) genes were highly induced in resistant cultivar while in susceptible cultivar, these were suppressed. In plants, HR response is efficiently activated by LOX genes ²⁹. In cotton, expression of 9-LOX gene leads to the stimulation of HR-like cell death, in response to the infection of *Xanthomonas campestris* pv *malvacearum*. GhLOX1 is linked with the accumulation of salicylic acid during the HR of cotton to *X. campestris* pv *malvacearum* ³⁰.

Genes involved in phytohormone signalling

In response to pathogen invasion, differential gene expression of phytohormones had been observed in previous studies ⁵. *A. tubingensis* inoculation in *G. hirsutum* resulted in the upregulation of DEGs linked with phytohormone signaling pathways. In the present study, auxin, cytokinin, jasmonic acid, ABA, salicylic acid and gibberellin related genes were differentially up regulated in both varieties, highlighting their roles in plant defense responses. Allene oxide synthase (AOS) gene is involved in translating JA biosynthesis and it was observed to be upregulated in resistant cultivar, following pathogen inoculation. The existence of AOS transcript and JA concentration had been reported to be significantly high in viral and pathogen attacks ³¹. In the current study, abscisic acid 8'-hydroxylase 1-like (3 genes), auxin-induced protein 22D-like (2 genes), and auxin-responsive protein SAUR6 like (4 genes) were specifically induced in resistant variety, in response to *Aspergillus* infection. ABA plays significant role in plant defense responses via transcriptional reprogramming of plant cell metabolism ³². Two genes encoding jasmonic acid-amido synthetase (JAR1) and salicylate carboxymethyl transferase-like were upregulated in tolerant cultivar, after fungal inoculation. SA plays a basic role in plant defense and inducing SAR response ³³. JA amino synthetase has been specifically induced in resistant cultivar in post fungal inoculation. JAR1 is a bioactive molecule that converts a JA amino acid synthetase enzyme ³⁴.

Genes involved in Phenyl propanoid pathway

Plants accumulate proline-rich glycoproteins, lignins and phytoalexins, as a part of their defense mechanism against fungal pathogen infection ³⁵. Lignification strengthens plant cell-wall and thus confers resistance to pathogen invasion ³⁶. In this study, secondary metabolism and phenyl propanoid pathway related DEGs including cellulose synthase like protein (3 genes), 4coumarate-CoA ligase-like, xyloglucan endotransglucosylase/hydrolase protein 22-like, flavonoid 3',5'-hydroxylase-like (2 genes), flavonol sulfotransferase-like (2 genes), glucan endo1,3-beta-

glucosidase 13-like, pectin esterase-like and proline rich receptor like kinases (2 genes) were induced in resistant cultivar, post infection. Genes encoding pectinesterase/ pectinesterase inhibitor (PPE8B-like) were specifically up regulated in resistant variety upon fungal infection.

Studies have shown that pectin methylesterase-inhibiting protein (GhPMEI3) of cotton provides tolerance against *V. dahliae* infection ³⁷. The phenylpropanoid pathway plays role in plant defense by cell wall fortification, serving as an antioxidant and producing more SA (stress hormone) ³⁸. The DEGs involved in the phenylpropanoids metabolism exhibited differential expression in both tolerant and susceptible varieties. Phenylalanine ammonia lyase (PAL) was differentially regulated in both cultivars under *Aspergillus* infection. Genes encoding 4coumarate--CoA ligase 1-like was also up-regulated in susceptible cultivar. The enzymes chalcone synthase (CHS), cinnamoyl-CoA reductase 1-like, cinnamate-4-hydroxylase (C4H), caffeic acid 3-O-methyltransferase-like, and caffeoylshikimate esterase-like were down regulated in susceptible variety after fungal infection. Concurrent up-regulation of genes of phenylpropanoid pathway in response to fungal inoculation endorsed their coordinated production.

Walls are thin 1 (WAT 1) related protein were highly induced in the resistant cultivar while 8 genes of WAT 1 were down regulated in the susceptible cultivar. WAT 1 from *Arabidopsis* has been studied for its role in the deposition or thickening of secondary cell wall as well as in conferring resistance against vascular pathogens such as *Xanthomonas campestris* pv. *Campestris*, *Ralstonia solanacearum* and *V. dahliae* ³⁹. Six genes of cytochrome P450 including 71A1-like (Ghi00905), 86A22 like (Ghi00073), 77A4 like (Ghi00232, Ghi00380, Ghi00591), 84A1like (Ghi00940) and 82G1-like (Ghi00904) were up regulated in the resistant cultivar.

Studies have shown that numerous CYPs function in phenylpropanoid metabolism ⁴⁰.

Genes encoding Transcription factors

Transcription factors (TFs) in plants play part in diverse biological processes including plant innate immunity, pathogen defense response, synthesis of phytoalexin and hormone signaling pathways. In this study, WRKY 7, WRKY 47, WRKY 50, WRKY15, WRKY 53, WRKY46, WRKY 25, WRKY 40, WRKY 41, WRKY 33, and WRKY 57 were specifically induced in resistant cultivar, followed by pathogen inoculation. WRKYs functions in plant defense responses, in addition to various physiological and developmental processes ⁴¹. AtWRKY33 works as a positive regulator of tolerance against *Botrytis cinerea* and *A. brassicicola* ⁴². In *Arabidopsis*, AtWRKY53 positively modulates Systemic acquired response. In cotton, GhWRKY33 has been known to be associated with defense response against *V. dahliae* ⁴³. WRKY genes, for instance

GhWRKY11, GhWRKY15, GhWRKY40 and GhWRKY44 have been recognized in regulating plant defense responses ⁴⁴. Expression of ethylene responsive TF is regulated via phytohormones involving SA, JA, ABA, ET and CK ⁴⁵. Specific upregulation of large number of transcription factors in resistant variety depicts its possible involvement in resistance and their down regulation might be the cause of susceptibility of susceptible cultivar.

Genes involved in Oxidative burst

In plants, generation of reactive oxygen species (ROS) is a hallmark of positive recognition of infection and instigation of defense genes by signaling mechanism. Production of reactive oxygen species has been described under both plant triggered immunity and effector triggered immunity ⁴⁶. In tolerant variety, genes encoding glutathione S-transferase (GST) (2 genes) Ghi00480, L ascorbate oxidase (2 genes) and peroxidase (POX) (Ghi00940) were upregulated after fungal inoculation. These genes have been reported to be involved in the processing of ROS. L ascorbate oxidase and GST genes were down regulated in susceptible variety. Glutathione S-transferase is a major scavenger of ROS ⁴⁷. POXs (peroxidases) play role in physiological processes including wound healing, lignification, suberization and catabolism of auxin, and defense strategies against pathogen infection ⁴⁸. Our results suggested that *Aspergillus* infection triggered ROS-regulated defense response in leaf tissues of resistant cultivar.

Rationality of our Illumina sequencing data was validated by real time polymerase chain reaction of nine genes having FC 5 or above, under fungal treated condition in both varieties. The comparison of q-PCR expression and fold change values from sequenced data confirmed the credibility of our RNA-seq analysis.

KEGG enrichment analysis of DEGs

The alkaloid biosynthetic pathways (isoquinoline alkaloid biosynthesis) was highly enriched in resistant cultivar. Expression of various alkaloids is stimulated by fungal elicitors, UV radiation, osmotic shocks and heavy metal ions. The phenyl alanine metabolic pathway was highly enriched in resistant variety and it was also enriched in susceptible variety, in response to *Aspergillus* infection. The amino acid phenylalanine plays vital role in the interlinkage of primary and secondary metabolism in plants ⁴⁹.

In tolerant variety Linolenic acid metabolism pathway was also found to be enriched. Jasmonic acid and its products, produced from alpha-linolenic acid pathway, are primary modulators in plant resistance against necrotrophic pathogens ⁵⁰. Up-regulations of sugars, lipids and amino acids metabolisms in plants control signal transduction cascades in plant defense

strategies ⁵¹. Plant hormone signal transduction pathway was also highly enriched in tolerant cultivar. Phytohormones control complicated signaling networks and responses to stresses including biotic and abiotic stresses ⁵². MAPK signaling pathway was also specifically enriched in resistant cultivar, post inoculation. By modifying MAPK cascades, plant cells are able to respond to wounding, wide range of stresses and pathogen infections. Importantly, phytohormones are known to effect signaling through MAPK cascades ⁵³.

Plant hormone signal transduction pathway, plant pathogen interaction pathway, jasmonic acid, salicylic acid. WRKY family and MAPK specifically functions in plant resistance ^{54,55}. Linolenic acid acts as a precursor of jasmonic acid and intervenes new plant defense signaling pathways ⁵⁶. Consequently, it is thought-provoking to inspect DEGs in these three pathway. The pathways involved and enriched under *Aspergillus* infection in leaf tissues of cotton can be employed for stress resistance. Transcriptome analysis revealed that the genes linked with linolenic acid metabolism, ROS metabolism, phenylalanine metabolism, isoquinoline alkaloid biosynthesis, plant signal transduction and various transcription factors (WRKY, NAC, MAPK) were specifically induced high in resistant cultivar, upon fungal inoculation. Here, our results also offer an essential foundation for better understanding of the defense mechanisms of cotton plant, which in future might assist the breeders.

Materials and Methods

Preparation of fungus culture and disease assessment

Strain of *A. tubingensis* was grown on potato dextrose agar (PDA) at 30 °C for 6 days. Conidia were harvested by scrapping the mycelium from PDA after adding Czapek broth in the petri plates. The suspension was collected in a sterilized flask and adjusted to our desired concentration (10^4 conidia/ml). Cotton varieties (CIM-573 and NIA-Sadori) were grown in the greenhouse. Greenhouse grown samples were collected in compliance with relevant institutional, national, and international policies and legislation. Leaves of both varieties were used to make small holes and 10 µL of the conidial suspension was injected into each hole. Leaves inoculated with only Czapek broth (without fungal conidia) served as mock control. Four days post inoculation (dpi), symptoms were observed and the necrotic areas were measured. Inoculated and mock leaves were collected in liquid nitrogen and stockpiled at -80°C for RNA isolation. Randomized complete block design was employed which comprised of four treatments: RT (resistant variety treated with pathogen), RM (resistant variety with mock inoculation), ST (susceptible variety treated with pathogen) and

SM (susceptible variety with mock inoculation). For each treatment, four biological replicates were used and entire experiment was repeated three times, over a time interval of 3–4 days.

RNA extraction, library construction and sequencing

or chemical extraction, greenhouse grown specimens were collected in compliance with relevant institutional, national, and international guidelines and legislation. High quality total RNA of mock inoculated and pathogen treated cotton leaves were extracted using mirVana miRNA Isolation Kit (Ambion®). Integrity of extracted RNA was estimated with Agilent 2100 Bio-analyzer (Agilent Technologies, Santa Clara, CA, USA).

Samples having RNA Integrity Number (RIN) ≥ 7 were preceded for further analysis. The cDNA libraries were constructed using TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, CA, USA), according to the manufacturer's instructions. The obtained libraries were sequenced using Illumina sequencing platform (HiSeq™ 2500) and paired end reads (125bp/150 bp) were generated.

Bioinformatics Analysis

RNA Seq quality assessment and genome mapping

Raw data (raw reads) was processed and converted to clean reads via Trimmomatic software ⁽⁵⁷⁾. Raw reads and the obtained results were stored in FASTQ (referred as fq file format), which included sequencing. Reads having poly-N, adapter sequence and low quality reads were eliminated via Trimmomatic, to attain clean reads. By using FastQC, quality of both trimmed and untrimmed reads was assessed. Mapping and alignment of the obtained clean reads to the *G. hirsutum* reference genome was done using HISAT2 aligner ⁵⁸.

Gene-Level quantification

Cufflinks was employed for computing the expression level of protein coding gene (FPKM reads) ⁵⁹. Using htseq-count, the read counts of each protein coding gene were attained ⁶⁰.

Analysis of differentially expressed genes (DEGs), GO and KEGG enrichment

Differential expression of genes was evaluated using DESeq. Base mean value was used to determine levels of the expression of gene. The difference multiple was calculated and the significant difference test was performed on the number of reads using NB (negative binomial

distribution test). For the screening of differentially expressed genes, threshold of P value (less than 0.05) and fold change (more than 2) was set. In order to explore expression pattern of genes, hierarchical cluster analysis was done. To define key biological functions and pathways, all DEGs were mapped to terms in Kyoto Encyclopedia of Genes and Genomes (KEGG: www.kegg.jp/kegg/kegg1.html). Based on the hypergeometric distribution, gene ontology (GO) enrichment and KEGG pathway enrichment analysis of DEGs were correspondingly done, via R program. Cellular component (CC), molecular function (MF) and biological process (BP) enrichment analysis were performed on the differential genes between samples, using the fisher algorithm and a directed acyclic graph was drawn on the enriched term using topGO ⁶¹. To check the reliability and consistency of biological copies and differences among *Aspergillus* inoculated and control samples of both varieties, principal component analysis (PCA), hierarchical clustering and correlation among all samples was performed.

Real time PCR analysis

Using q-PCR, expression of selected candidate genes was determined for the authentication of our transcriptomic data. Ubiquitin was used as a housekeeping gene. Using Primer 3 software tool, forward and reverse primers were designed (Table 2) and synthesized (Sangon Biotech, Shanghai, China). For each sample, three technical replicates were used. Rather than cDNA, RNase-free water was employed as negative control. PCR was done in three replicates in an optical 96-well plate by 7500 Real-Time PCR System (Applied Biosystems), following a standard protocol ⁶². PCR mixture was prepared and thermal cycling was executed with an initial denaturation step of 95° for 1 min, followed by 40 cycles comprising of 95 °C for 15 s, 57 °C for 15 s and 72 ° C for 45 s.

Data analysis

Results were described as mean $\pm$ standard deviation (SD) and investigated by one way analysis of variance (ANOVA, $P < 0.05$).

Table 2. Primer sequences of selected genes for q-PCR

Sr. No	Gene ID	Gene Name	Primers
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1.	LOC107942464	Cytochrome P450 71A1-like	F TGAGCTTTTCAGCCATTGAC R AACCGGTGAAGAGAACCAAC
2.	LOC107932995	Peroxidase 45-like	F TGAGCTTTTCAGCCATTGAC R TGCAGCAACAGTTGAAATGA
3.	LOC107935490	WAT1-related protein At5g07050-like	F GAAAGTGAGATGCCAAGCAA R ATGGAGCCTTTGAACCAATC
4.	LOC107926776	Pathogenesis-related protein PR-4-like	F GGATTGTGATGGCAGTGAGTG R CTCCATTGCTGCATCTGTCT
5.	LOC107887492	Flavonoid 3',5'-hydroxylase-like	F CAATTGATATCGGCGAATTG R TTCCCCTCTTGATACCTTGG
6.	LOC107906829	Probable WRKY transcription factor 40	F CGTCAAGAAAAAGGTGCAAA R AGTGGGAGGTGAAGATTTGG
7.	LOC107932294	L-ascorbate oxidase homolog	F AGTAGAGGGGACCCACACTG R CAGTGGTGGAAGGATGTTG
8.	LOC107897904	Mitogen-activated protein kinase kinase kinase YODA-like	F CCAAATTGAACCGAGTGTTG R GTCACCCTTCTCAACCGAAT
9.	LOC10793713	Ethylene-responsive transcription factor 5-like	F ATTTGAGCTCCGATTCGTCT R TTTGAGCGATCTGAGGTTTG

Data availability

Data are available at <http://www.ncbi.nlm.nih.gov/bioproject/751446> of NCBI, accession number PRJNA751446.

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Author Contributions: M.K executed, analyzed and wrote the manuscript. J.S. designed this work and helped in the analysis of results. U.H. assisted pathogen related experiments. M.A. and F.L. helped in statistical and computational analyses. M.A formatted the manuscript. H.S, and A.K. helped in lab experiments. K.M. and H.J.C. prepared graphs and did data analysis. M.F.H.M. supervised the whole experiment.

Figure Legends

Figure 1. Phenotype of susceptible (A) and resistant cotton cultivar (B), after 4 days of infection with *A. tubingensis*. (C) Disease severity of susceptible and tolerant cultivars. (D) FPKM density

distribution reflects the protein-coding gene expression pattern of each sample. (E) The FPKM value box line (F) FPKM box-plot for each sample. The X-axis is the sample name, Y-axis is $\log_{10}(\text{fpkm}+1)$. The different colors in the figure represent different ranges of fpkm values, the abscissa is the sample, and the ordinate is the number of protein-coding genes.

Figure 2. 3D PCA exhibiting the variance of DEGs in all four data sets, with an overall variance of 74% contributed by all the three PC.

Figure 3. Statistical representation of differentially expressed genes. The horizontal axis is the comparison group; the vertical axis is the number of differential genes in the comparison group, where “Up” is the number of up-regulated genes with significant differences, and “Down” is the number of down-regulated genes with significant differences. (B) Venn Graph is showing common and unique DEGs between different comparison groups.

Figure 4. Differential expression by volcano map in four data sets. The gray color represents genes with a non-significant difference, while red and green are showing significantly up and down regulated genes. The horizontal axis is the display of $\log_2$ fold change, and the vertical axis is $-\log_{10}$ P value.

Figure 5. Relative expressions of candidate genes (DEGs), in fungal inoculated resistant and susceptible varieties where X axis shows the relative expression of DEGs and Y axis shows the genes.

Figure.6 GO enrichment analysis of resistant variety (A), Susceptible variety (B) and Resistant pathogen inoculated and susceptible pathogen inoculated (C) where green, blue and red color depicts biological, cellular and molecular functions respectively

Figure 7. KEGG enrichment top20 bubble diagram of RT vs RC(A) ST Vs SC(B) and RT vs ST. The X-axis is the enrichment score. The larger the bubble, the more the number of differential protein-encoding genes contained. The bubble color changes from purple-blue-green –red. With the color transition the scope of protein encoding genes become more well defined. Smaller enrichment p value represents the greater degree of significance.

Conflicts of Interest

We confirm that all the authors in our manuscript have no conflict of interest.

Figures

Figure 1

Phenotype of susceptible (A) and resistant cotton cultivar (B), after 4 days of infection with *A. tubingensis*. (C) Disease severity of susceptible and tolerant cultivars. (D) FPKM density distribution reflects the protein-coding gene expression pattern of each sample. (E) The FPKM value box line (F)

FPKM box-plot for each sample. The X-axis is the sample name, Y-axis is $\log_{10}(\text{fpkm}+1)$. The different colors in the figure represent different ranges of fpkm values, the abscissa is the sample, and the ordinate is the number of protein-coding genes.

Figure 2

3D PCA exhibiting the variance of DEGs in all four data sets, with an overall variance of 74% contributed by all the three PC.

Figure 3

Statistical representation of differentially expressed genes horizontal .(A) Bar graph in which horizontal axis is the comparison group; the vertical axis is the number of differential genes in the comparison group, where “Up” is the number of up-regulated genes with significant differences, and “Down” is the number of down-regulated genes with significant differences. (B) Venn Graph is showing common and unique DEGs between different comparison groups.

Figure 4

Figure 4

Differential expression by volcano map in four data sets. The gray color represents genes with a non-significant difference, while red and green are showing significantly up and down regulated genes. The horizontal axis is the display of $\log_2$ fold change, and the vertical axis is $-\log_{10}$ P value.

Figure 5

Relative expressions of candidate genes (DEGs), in fungal inoculated resistant and susceptible varieties where X-axis shows the relative expression of DEGs and Y-axis shows the genes.

R_Treatment-vs-R_Control(Total): Top 30 GO Term

Figure 7

Figure 7

KEGG enrichment top20 bubble diagram of RT vs RC (A)ST Vs SC (B) and RT vs ST (C). The X-axis is the enrichment score. The larger the bubble, the more the number of differential protein-encoding genes contained. The bubble color changes from purple-blue-green-red. With the color transition the scope of protein encoding genes become more well defined. Smaller enrichment p value represents the greater degree of significance.

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