Transcriptomic analyses reveal the expression and regulation of genes associated with resistance to early leaf spot in peanut

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Abstract
Objective: Early leaf spot (ELS) caused by Cercospora arachidicola (Hori) is a serious foliar disease in peanut worldwide, which causes considerable reduction of yield. Identification of resistance genes is important for both conventional and molecular breeding. Few resistance genes have been identified and the mechanism of defense responses to this pathogen remains unknown.

Results: We detected several genes involved in disease resistance to ELS through transcriptome analysis. Using RNA-seq technology, one hundred thirty-three differentially expressed genes (DEGs) were identified between resistant and susceptible lines. Among these DEGs, coiled-coil nucleotide binding-leucine rich repeat (NLR) type resistance genes were identified as duplicated R genes on the chromosome B2. Peanut phytoalexin deficient 4 (PAD4) regulator of effector-triggered immunity mediated by NLR resistance proteins and polyphenol oxidase (PPO) genes play important roles in early leaf spot resistance. Our study provides the useful information on plant response to C. arachidicola infection in peanut. The results suggest that a few major genes and several factors mediate the resistance to ELS disease, showing the characteristics of quantitative trait in defense responses.

Keywords: Defense response, Differentially expressed gene, Gene regulation, RNA-seq, Transcriptome

Introduction
Peanut (Arachis hypogaea L.), originated in South America, is a major oilseed crop and is cultivated in tropical and subtropical regions of the world. Peanut production around the world totaled ~ 38 million tons in 2015 [1]. However, peanut productivity is severely constrained by several biotic and abiotic stresses. Amongst those stresses, early leaf spot (ELS) caused by Cercospora arachidicola Hori and late leaf spot (LLS) caused by Cercosporidium personatum (Berk & Curtis) are serious foliar diseases in peanut worldwide. Peanut yield losses may reach up to 50% worldwide due to these two diseases [2] and their management is highly dependent on multiple fungicide applications. The best strategy to avoid the significant economic loss is to develop resistant cultivars.

The genetic nature of resistance to early and late leaf spot diseases is quantitative and inherited independently [3, 4]. Resistance to ELS is associated with resistance components, such as longer latent period, reduced sporulation, smaller lesion diameter, and lower infection frequencies [5]. Dominant gene action controls resistance components and epistasis played a major role in the inheritance of resistance components [6]. Sequencing of the genome of C. arachidicola provides relevant information for the advancement of resistant cultivars and is a
useful resource to aid in the selection of target resistance genes for enhanced disease control [7].

RNA sequencing (RNA-seq) is an effective technology for understanding of metabolic processes, genome-wide quantification of gene expressions, and identification of key genes associated with the traits of interest. Recently, RNA-seq analysis provides insights into transcriptome profiles associated with several traits in peanut. Transcriptomes have revealed the mechanism of salinity resistance [8, 9], nodulation [10], pod development [11, 12], aflatoxin resistance [13, 14], defense related genes to *Ralstonia solanacearum* [15], and resistance to LLS [16] in peanut. However, transcriptomic profiling analysis of resistance to ELS has been limited. To explore the mechanism of resistance to ELS in peanut, the transcriptional profiles of two sister inbred lines, resistant (R) and susceptible (S) lines to ELS, from a F₉ RIL population were investigated under *C. arachidicola* infection. The objectives of this study were to (1) compare the differentially expressed genes (DEGs) between R and S lines, (2) understand the possible roles of DEGs in plant defense response, (3) identify resistance genes to ELS disease.

**Main text**

**Materials and methods**

*Plant materials and inoculation*

Two peanut lines 904 and 1006 selected from a F₉ RIL population [17] derived from a cross of Florida-07 × GP-NC WS 16 were used in this study. The parent GP-NC WS 16 is resistant and Florida-07 is susceptible to ELS, respectively. Early leaf spot disease was evaluated in the E.V. Smith Research Center, Tallassee, Auburn University (2016 and 2017). The inbred line 904 was selected due to its higher resistance level similar to the resistant parent and the sister line 1006 demonstrated higher susceptibility to ELS compared to the other lines in the F₉ RIL population. Nine seeds of each line were individually planted in 40 Magenta GA-7 plant culture boxes containing a potting mix.

*Fungus culture and inoculation*

A total volume of 0.5 ml *C. arachidicola* conidial suspension was applied to the foliage of each plant by spraying. The inoculated plants were kept at room temperature with 100% humidity in the closed culture box. Four leaves from each plant were collected at three time points, T1 (4 h), T2 (24 h), and T3 (44 h) after inoculation, with three biological replicates. RNA isolation from 18 samples, including 9 samples from each plant at 3 time points, 4 biological replicates, were used for transcriptome analysis. All clean reads were subjected to genome mapping. On average, 83.42% reads were mapped to the reference genome and 66.48% reads of each sample were mapped to only one location of the reference genome. The uniformity of the mapping results for each sample suggested that the samples were comparable.

**Identification of differentially expressed genes (DEGs)**

A total of 1711 DEGs were detected from all three time points between two lines. Among these DEGs, 595 were up-regulated and 1116 were down-regulated. The resultant data showed that the number of down-regulated DEGs was significantly higher than those of up-regulated DEGs in both lines (Additional file 1: Figure S1). Due to gene expression dynamically changing between T1 (4 h) and T3 (44 h), the comparison of DEGs focused on T1 vs T3. When comparing R and S lines at T3, 133 DEGs including 52 up and 81 down-regulated were identified according to gene expression level with fold change ≥2 (Additional file 2: Table S1). The summary of DEGs was illustrated in the Fig. 1.

**Gene ontology and KEGG enrichment analysis of DEGs**

Gene ontology (GO) enrichment analysis was performed to analyze the functions of DEGs. Metabolic process with 137 and 25 genes were dominant in the biological process category in R and S lines, respectively. Catalytic activity (181 genes) and binding (102 genes) were significant active responses in the molecular function category in
the R line, whereas only 33 and 25 genes with the same function in the S line, respectively, indicating there was a higher number of expressed genes in R line than in S line between T1 and T3 (Additional file 3: Figure S2).

To further explore biological functions and gene interaction, Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was used. For the R line, comparison of DEGs with T1 vs T3 showed 11 KEGG pathways were significantly enriched, including biosynthesis of secondary metabolites, phenylpropanoid biosynthesis, MAPK signaling pathway-plant, flavonoid biosynthesis, plant-pathogen interaction, circadian rhythm-plant, isoflavonoid biosynthesis, etc. While in the S line, 4 KEGG pathways including photosynthesis, plant-pathogen interaction, oxidative phosphorylation, MAPK signaling pathway-plant were significantly enriched for DEGs between T1 and T3 (Fig. 2).

**DEGs related the resistance to ELS**

There were 133 DEGs with 52 up and 81 down-regulated genes identified between R and S lines (Additional file 2: Table S1). Some DEGs were crucially involved in plant responses to the pathogen infection. For instance, nine of such genes were significantly up-regulated in the S line

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**Fig. 1** Comparison of DEGs after inoculation. Scatter plot a) T1 (4 h) vs T3 (44 h) in the line 904 (R), b) T1 vs T3 in the 1006 line (S), c) lines 904 (R) vs 1006 (S)

**Fig. 2** Comparisons of enriched KEGG pathway of DEGs. a) T1 (4 h) vs T3 (44 h) in 904 R line, b) T1 vs T3 in 1006 S line, and (c) 906 R vs 1006 S line. Dot size and color refer to gene number and the corrected P value, respectively.
while down-regulated in the R line based on the log2 fold change, including two nucleotide binding-leucine rich repeats (NLRs), Interleukin-1 receptor-associated kinase 4, ATP-dependent RNA helicase, Nuclear pore complex protein Nup85, and E3 ubiquitin-protein ligase. On the other hand, 10 different DEGs including phytoalexin deficient 4 (PAD4), Interleukin-1 receptor-associated kinase 4, ATP-dependent RNA helicase, ATP-binding cassette, Serine/threonine-protein kinase, Pectinesterase, Auxin responsive GH3 gene family, and Polyphenol oxidase were significantly up-regulated in the R line but down-regulated in the S line (Table 1). The sequence of putative NLRs were showed in Additional file 4: Table S2.

**Discussion**

The development of disease-resistant plants is important in mitigating the losses of productions. Understanding the mechanism of plant-pathogen interactions and searching for resistant resources is a prerequisite in plant breeding. Plant responses to pathogen attack are involved in several defense strategies against pathogens. The first barrier for pathogen invasion is the uses of plant reprogramming the cell wall damaged by virulent pathogens, resulting in pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) [19, 20]. This basal resistance layer is controlled by lipase-like protein phytoalexin deficient 4 (PAD4) either in combination with enhanced disease susceptibility 1 (EDS1) or independent of EDS1 [21, 22]. PAD4 is required for activation of responses during some other gene-for-gene resistance responses [23] and plays the potential role of defense signaling genes in quantitative disease resistance [22]. Several reports have demonstrated the increasing resistance against pathogens via expressing PAD4, such as in Arabidopsis [24]. In this study, PAD4 was significantly down-regulated in the S line compared to the R line, suggesting that the lower expression level of PAD4 might affect the resistance to ELS in the susceptible genotype.

Because PAD4 is an important component of the downstream signaling in a different immune pathway, the reduced expression of PAD4 may also have a negative effect on resistance pathways. Several other genes participated in various pathways responding to ELS pathogen challenges were identified in this study. These genes encoded various enzymes to conspire in biological processes. For instance, expression of pectinesterase gene was significantly reduced in the S line that could result in weak cell walls due to degradation of pectin, a critical cell wall component. In plants, pectinesterase plays a role in plant response to pathogen attack by influencing numerous physiological processes [25]. However, the repressed expression of pectinesterase gene led to degrade cell wall integrity, thus increase *C. arachidicola* colonization of leaf tissue and enhance disease development. [26]

| Table 1 Key DEGs related with the resistance to early leaf spot |
|---|---|---|---|---|---|---|---|
| Gene ID | Length | 904 line expression<sup>a</sup> | 1006 line expression<sup>a</sup> | Log2 fold change | PPEE | Up/down | Kegg orthology |
| Araip.IU5QT | 612 | 33.7176 | 5.4789 | −2.5519 | 0.0272 | Down | Phytoalexin deficient 4 (PAD4) |
| Araip.JWB3B | 1659 | 16899.7185 | 571.6533 | −4.8849 | 4.33E−06 | Down | Interleukin-1 receptor-associated kinase 4 |
| Araip.S6EUJ | 816 | 1003.7531 | 22.1262 | −5.4828 | 4.09E−08 | Down | Interleukin-1 receptor-associated kinase 4 |
| Araip.IK389 | 2844 | 321.6808 | 132.3308 | −1.2793 | 3.66E−15 | Down | ATP-dependent RNA helicase DHX36 |
| Araip.JZ9DY | 1208 | 23.2084 | 1.8579 | −3.4296 | 5.59E−09 | Down | ATP-binding cassette, subfamily A |
| Araip.Z7XRR | 1629 | 34.2064 | 7.2808 | −2.1825 | 6.23E−12 | Down | ATP-dependent RNA helicase DHX36 |
| Araip.KORJZ | 820 | 255.9979 | 37.3803 | −2.7651 | 1.96E−12 | Down | Serine/threonine-protein kinase ULK4 |
| Araip.V8NJN | 1773 | 272.7235 | 33.1558 | −3.0277 | 0.0206 | Down | Auxin responsive GH3 gene family |
| Araip.XC3V5 | 1413 | 665.8651 | 61.8233 | −3.4221 | 0.0046 | Down | Pectinesterase |
| Araip.AO5GR | 1758 | 542.5663 | 6.3732 | −6.3405 | 2.18E−11 | Down | Polyphenol oxidase |
| Araip.UOYME | 2421 | 26.8468 | 79.3904 | 1.5527 | 0.0003 | Up | CC-NBS-LRR |
| Araip.JM4Y4 | 2472 | 8.9355 | 24.9109 | 1.4462 | 0.0018 | Up | CC-NBS-LRR |
| Araip.A99BF | 438 | 6.3517 | 31.0765 | 2.2334 | 0.0002 | Up | Interleukin-1 receptor-associated kinase 4 |
| Araip.BV0ZS | 1467 | 17.1560 | 136.8627 | 2.9722 | 0.0413 | Up | Interleukin-1 receptor-associated kinase 4 |
| Araip.CR951 | 2301 | 0.7055 | 11.2789 | 3.4915 | 1.96E−12 | Up | ATP-dependent RNA helicase PIF1 |
| Araip.XOYWA | 795 | 41.0504 | 139.8412 | 1.7602 | 1.73E−09 | Up | ATP-dependent RNA helicase DBP3 (DEAD-box) |
| Araip.DV5FU | 390 | 17.1582 | 35.0932 | 1.0185 | 0.0006 | Up | Nuclear pore complex protein Nup85 |
| Araip.XE6AC | 339 | 1.4038 | 11.285 | 2.7271 | 4.39E−07 | Up | Nuclear pore complex protein Nup85 |
| Araip.ZNVXB | 240 | 6.4672 | 24.2974 | 1.8578 | 0.0421 | Up | E3 ubiquitin-protein ligase RNF38/44 |

<sup>a</sup> Line 904 is resistant and line 1006 is susceptible to early leaf spot.
described that the impact of pectin methyl esterification on plant-pathogen interactions and on the dynamic role of its alteration during pathogenesis. If these passive defenses were breached, the plant innate immune system would launch active defenses [27]. Nevertheless, due to the down-regulation of PAD4 in the S line, it lost the functions of the signaling accumulation for downstream activities.

Besides PAD4, polyphenol oxidase (PPO) genes also significantly down-regulated in the S line. Normally, PPO is up-regulated by abiotic and biotic stresses though the responses to stresses varies within PPO gene families by plant species [28]. Overexpression of PPO displayed enhanced resistance to pathogen in wheat, chickpea, tomato, and populus [29–33]. Conversely, down-regulation of PPO resulted in increased disease susceptibility. For instance, silencing of PPOs leads to increased susceptibility to disease in tomato [34] and to insects in tomato and cotton [35, 36]. [37] studied the interactions of host with fungi in pearl millet and reported that the resistant genotypes showed high, rapid accumulation of localized levels of PPO while susceptible genotypes failed to accumulate PPO even after consideration time. In this study, significant down-regulation of PPO in the S line could enhance susceptibility to ELS disease.

In addition to PTI, another type of defense response is activated by resistance proteins as sensors that indirectly or directly recognize specific effectors from many different pathogens, leading to effector-triggered immunity (ETI). The resistance genes, NLR with N-terminal Toll-interleukin-1-receptor domain or coiled coil domain encoding resistance proteins, are a major class of resistance genes that evolved to intercept these effectors. Two putative NLRs were identified as DEGs between the R line and S line in this study. Two R genes possessed the same DNA sequences with several SNPs and were closely located in the B2 chromosome. Higher R gene expression at the earlier reaction period in the susceptible genotype implied that resistance genes triggered by pathogen infection responded quicker in the susceptible than the resistant genotype. [16] observed the same scenario in a different peanut population responding to LLS disease. The susceptible genotype was evolutionally failed in defense response might be due to the lower expression of PAD4. Overexpression of PAD4 would allow us to have further insight into the plant response in the susceptible genotype.

**Limitations**

The resistance genes identified from differentially expressed genes still need to validate by functional study, such as loss of function mutation targeting on these genes.

**Supplementary information**

**Supplementary information** accompanies this paper at https://doi.org/10.1186/s13104-020-05225-9.

**Additional file 1: Figure S1.** Summary of DEGs among different groups. X-axis represents comparison of DEGs between each group and Y-axis refers to the number of DEG. Red color indicates up-regulated DEGs and blue color for down-regulated DEGs.

**Additional file 2: Table S1.** List of 133 differentially expressed genes identified between the R line and the S line.

**Additional file 3: Figure S2.** Gene ontology (GO) enrichment analysis showed DEGs involved in three function categories. A. T1 (4 h) vs T3 (44 h) in R line; B. T1 vs T3 in S line; C. R vs S lines.

**Additional file 4: Table S2.** DNA sequences of resistance genes to early leaf spot disease in peanut.

**Abbreviations**

DEG: Differentially expressed genes; FDR: False discovery rate; FPKM: Fragments per kilobase of exon per million mapped reads; GO: Gene ontology; KEGG: Kyoto encyclopedia of genes and genomes; MYB: Myeloblastosis; PAD4: Phytoalexin deficient 4; PDA: Potato dextrose agar; PPEE: Posterior probability of being equivalent expression; PPO: Polyphenol oxidase; ROS: Reactive oxygen species.

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**Authors’ contributions**

GH Conceptualized the idea for this study. LG performed the fungus culture and inoculation. AH conducted phenotyping in field. LG, SH, MY, and XM analyzed the date. GH, LG, and SH prepared original draft. GH, LG, SH, MY, XM, and AH reviewed and edited the manuscript. All authors read and approved the final manuscript.

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**Availability of data and materials**

All raw fastq sequences for these eighteen libraries were deposited in the National Center of Biotechnology Information (NCBI). The BioProject number is PRJNA494675 (https://www.ncbi.nlm.nih.gov/sra/PRJNA494675).

**Ethics approval and consent to participate**

Peanut seeds of two lines were obtained from Auburn University. After germination, only seedlings were used in the lab for this research.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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