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Identification of genes differentially expressed during early interactions between the stem rot fungus (*Sclerotium rolfsii*) and peanut (*Arachis hypogaea*) cultivars with increasing disease resistance levels

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ABSTRACT

Sclerotium rolfsii, a destructive soil-borne fungal pathogen causes stem rot of the cultivated peanut, *Arachis hypogaea*. This study aimed to identify differentially expressed genes associated with peanut resistance and fungal virulence. Four peanut cultivars (A100-32, Georgia Green, GA-07W and York) with increasing resistance levels were inoculated with a virulent *S. rolfsii* strain to study the early plant–pathogen interaction. 454 sequencing was performed on RNAs from infected tissue collected at 4 days post inoculation, generating 225,793 high-quality reads. Normalized read counts and fold changes were calculated and statistical analysis used to identify differentially expressed genes. Several genes identified as differential in the RNA-seq experiment were selected based on functions of interest and real-time PCR employed to corroborate their differential expression. Expanding the analysis to include all four cultivars revealed a small but interesting set of genes showing colinearity between cultivar resistance and expression levels. This study identified a set of genes possibly related to pathogen response that may be useful marker assisted selection or transgenic disease control strategies. Additionally, a set of differentially expressed genes that have not been functionally characterized in peanut or other plants and warrant additional investigation were identified.

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1. Introduction

Stem rot, caused by *Sclerotium rolfsii* is a major disease of peanut and occurs in all areas of peanut production, limiting maximal yields and causing production losses ranging from 25% to 80% (Bowen et al., 1996; Akgul et al., 2011). Peanut is the fourth most important oilseed crop worldwide, cultivated mainly in tropical, subtropical and warm temperate climates (Proite et al., 2007). During 1996–2000, approximately 70% of the total global peanut production was from China, India and the United States; the United Stated produced 1655 thousand metric tons (Revoredo and Fletcher, 2002). Stem rot is a major disease of peanut in the southeastern US (Brenneman et al., 1990; Punja, 1985). According to the 2011 Georgia Plant Disease Loss Estimates, peanuts were planted on approximately 475,872 acres in the state with an average yield of 3721 lb/acre for a total production valued at $586 million. Stem rot prevalence reduced crop value by 7.0%. The damage was estimated at $41 million and the associated cost of control was estimated at an additional $6.8 million (Kemerait, 2011).

Despite its major economic importance both in the United States and worldwide, peanut has lagged behind other major crop plant species in the development of genomic sequence databases due to inherent challenges (Paterson et al., 2004). These challenges include the overall large genome size of peanut and the fact that it is an allopolyploid (AABB) with duplicate genes from the two parental genomes (Pandey et al., 2012). With the increased availability and affordability of deep-sequencing tools, there has been a recent increased effort to use sequencing tools specifically for peanut genomic research (Guo et al., 2008; Pandey et al., 2012).

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Several databases have been developed for peanut. As of December 2014, there were 1,092,181 nucleotide and 254,541 EST sequences deposited into the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/). These include a SSR (simple sequence repeat)-based genetic linkage map (Varshney et al., 2009; Hong et al., 2010), a database for comparative molecular maps (Jesubatham and Burow, 2006), a microRNA database (Zhao et al., 2010) and expressed sequence tag (EST) sequence databases. One EST database includes a transcript assembly constructed using Sanger-sequenced ESTs (Nagy et al., 2010). PeanutBase (peanutbase.org) is a web-based resource providing access to information related to genetic, genomic and trait data from research and the Peanut Genomics Initiative; the site includes *Arachis hypogaea*, *Arachis duranensis* and *Arachis ipaensis*. There is a section that links to maps and markers, gene expression, cytology, transcriptomes and QTLs. There is a sequences BLAST search and QTL search function. PeanutDB is another web-based resource with a bioinformatics platform that provides access to a whole transcript assembly for peanut (*A. hypogaea*) that integrates Sanger reads, Roche 454 reads and Illumina reads and contains 32,619 contigs. The contigs have EC, KEGG and GO functional annotations (Duan et al., 2012).

Differential gene expression using sequencing technologies has been the focus of studies used to identify genes that are responsible for observed phenotypic variation. Guo et al. (2008) conducted a large-scale EST sequencing effort comparing gene expression between the peanut genotypes, GT-C20 (resistant to *Aspergillus parasiticus*) and Tifrunner (susceptible to *A. parasiticus).* Resistance-related genes with significant up-regulation were found in both cultivars (Guo et al., 2008). Schmid et al. (2010) used transcriptomic 454 sequencing to sequence two *S. rolfsii* transcriptomes under scleroglucan-producing and scleroglucan-nonproducing conditions. *S. rolfsii* cDNA populations were pooled and sequenced and the data was archived at the Gene Expression Omnibus (http://www/ncbi.nlm.nih.gov/geo). This study led to insights into scleroglucan and oxalate (the main scleroglucan by-product) production as well as the prediction of biosynthetic pathways of scleroglucan and oxalate synthesis (Schmid et al., 2010).

In addition to gene expression studies, there is evidence of differential activity of enzymes in the interaction between peanut and *S. rolfsii* . In 2010, Nandini et al. (2010) showed that *S. rolfsii* derived elicitors could be used to induce systemic acquired resistance in four different peanut varieties. The fungal elicitors were introduced to peanut plants in the form of fungal culture filtrate and mycelial cell wall fragments. These authors employed biochemical assays to observe the defense-related signal molecule, salicylic acid; marker enzymes, peroxidase, phenylalanine ammonia lyase, β-1,3-glucanase as well as the polymer lignin. There was a varied increase in the fold activity of the signal molecule and the marker enzymes between all four peanut varieties exposed to the fungal elicitors compared to control plants (Nandini et al., 2010). Identification of the pathogen responsive genes in peanut upon infection with *S. rolfsii* would add to the understanding of gene expression in peanut in response to this major pathogen.

Identification of important pathogen and pathogen responsive genes provides valuable sequences for future studies that can provide additional insight into this destructive pathogen. Smith et al furthered existing knowledge by conducting a histological study on the infection of host tissue by *S. rolfsii* using microscopic and histochemical techniques. They used 5 day old bean hypocotyls, excised leaves from 2 month old sugarbeets, petioles from 2 month old carrot plants and mature carrot roots as the host tissue. They observed the hyphae from germinating sclerotia ramifying over the host tissue within 24–48 h post inoculation. The hyphae typically coalesced to form mycelial aggregates, referred to as infection cushions. The plant cells below the infection cushion were sunken. The infection cushion appears to assist the infection process by releasing enzymes and oxalic acid that lead to tissue maceration and cell death prior to fungal penetration. Both inter and intracellular subcuticular hyphal growth from aggregates occurred 48 h post inoculation. Penetration hyphae were formed 36–48 h post inoculation from the aggregates. Penetration of host tissue occurred from penetration hyphae from the infection cushion, from appressoria on the surface of the host and from hyphal penetration through the stomata. Some of the tips of the penetration hyphae had fine infection pegs. Crystals, shown to be calcium oxalate were observed in cell layers 3–4 layers ahead of the hyphal growth. These observations suggested that the oxalic acid precipitated calcium from the middle lamellae to form oxalate crystals (Smith et al., 1986).

The objectives of this study were designed to test the central hypothesis that differential gene expression in early interaction between *S. rolfsii* and peanut cultivars with varying degrees of resistance will identify mechanisms of plant resistance and fungal virulence with implications for disease control. Cultivars with varying resistance levels were used and inoculated with a single virulent fungal strain. Total RNA was extracted for cDNA synthesis followed by 454 sequencing. Differential gene expression was assessed by means of the Audic–Claverie statistical method (Audic and Claverie, 1997), designed for comparing two libraries for differential gene expression, and the newly-developed unpublished Olman statistical method (Olman et al. unpublished), designed to assess differential expression across multiple libraries. Real-time PCR confirmed the upregulation of 15 test genes. Twelve genes identified as differentially expressed using the Audic–Claverie statistical method, and related to defense in the plants (7 genes), virulence in the fungi (3 genes), or of no known functional categorization (2 genes), were selected for differential expression verification using real-time PCR. Genes identified as differentially expressed using the Olman statistical method were assessed for correlation with cultivar resistance levels.

2. Materials and methods

2.1. Peanut cultivar selection and growth

Current peanut production employs a variety of cultivars, with varying resistance and agronomic traits. In this study, four peanut cultivars were selected based on varying resistance to *S. rolfsii* and included A100-32 (highly susceptible), Georgia Green (susceptible), GA-07W (moderately resistant) and York (highly resistant). A100-32 is currently an unreleased breeding line with the pedigree Tifton 8 × Tifrun and is highly susceptible to *S. rolfsii*. Tifton 8 is a large-seeded, Virginia-type peanut with spreading bunch growth (Coffelt et al., 1985). Tifrun is a released single runner cultivar (Knauft and Gorbet, 1989). Georgia Green is a runner market-type peanut (Branch, 1996). GA-07W is a high-yielding, large-seeded, runner-type peanut with moderate resistance to *S. rolfsii* (Branch and Brenneman, 2008). York is a runner market-type peanut with excellent resistance to *S. rolfsii* (Gorbet and Tillman, 2011). Peanut seeds used in this study were coated with the industry standard fungicide treatment, Dynasty PD® at 2.5 g/kg of seed (contains azoxystrobin, fludioxonil and mefenoxam). Although this product has some systemic activity in young plants, this effect was considered irrelevant in the experiment as all seed received the same treatment and resulting plants were not inoculated until 50 days after planting (DAP), the R3 development stage (Boote, 1982) based on optimal susceptibility (Sconyers et al., 2005). The R3 development stage is referred to as the beginning pod stage when half of the plants have an elongated peg and the ovary tip is swollen to at least twice the width of the peg (Boote, 1982).