IDENTIFICATION OF GENES DIFFERENTIALLY EXPRESSED DURING EARLY INTERACTIONS BETWEEN THE STEM ROT FUNGUS (*SCLEROTIUM ROLFSII*) AND PEANUT (*ARACHIS HYPOGAEA*) CULTIVARS WITH VARYING DISEASE RESISTANCE LEVELS

by

ANSUYA JOGI

(Under the Direction of SCOTT E. GOLD)

ABSTRACT

Sclerotium rolfsii, a fungal pathogen, causes stem rot of peanut. This study aimed to identify differentially expressed genes associated with peanut resistance and fungal virulence. Four peanut cultivars with increasing resistance levels were inoculated with a virulent fungal strain to study the early plant-pathogen interaction. 454 sequencing generated 225,793 high-quality reads with an average length of 311bp; normalized read counts and fold changes were calculated. Statistical analysis identified differentially expressed genes and genes of interest were selected based on functions of interest. Differential expression was confirmed using real-time PCR. There were 274 differentially expressed genes between the most susceptible and resistant cultivars. Analysis of all four cultivars revealed little colinearity between the cultivar resistance and gene expression. This study provides defense-related genes potentially useful in transgenic disease control strategies. Differentially expressed genes with no matches to genes of known function may be worthy of additional investigation to characterize their function.

INDEX WORDS: *Arachis hypogaea*, *Sclerotium rolfsii*, 454 sequencing, differential gene expression, real-time PCR, PR proteins

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DEDICATION

To my amazing parents, Bharatkumar and Jaya Jogi, the world's greatest sister, Anusha Jogi, my wise boyfriend, Ellis Wright, my aunt and uncle, Rajendrakumar and Jasu Jogi, my cousin, Elesh Jogi, and last but not least my late grandparents, Morar Ranchod, Ratan Pema Ranchod, Chotu Govan Jogi and Shanti Ravla Jogi and my sweet grandmother, Mani Nana Ranchod.

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CHAPTER 1

INTRODUCTION

Among oilseed crops, peanut is placed fourth worldwide as a source of edible vegetable oil. In addition to its role as a major source of oil, peanut is also the third most important source of vegetable protein and is used as a supplement to alleviate protein malnutrition (Singh and Singh, 1991). Production is widespread in most of the tropical to subtropical regions of the world with more than 90% of the production in Asia and Africa. Between 1996 and 2000, nine of the fifteen major peanut producing countries used more than 55% of the crop for oil and six of these countries consumed more than 60% for food. The United States ranks third in peanut production, behind China and India; peanuts produced in the US are mainly used as food (Sampson, 2002, Tillman and Stalker, 2009). The environmental conditions, including those found in the southeastern United States, of a prolonged growing season, warm temperatures and adequate moisture availability are especially conducive to growing peanuts (Franke et al., 1998). Unfortunately these conditions also favor the development of several diseases on peanuts.

Sclerotium rolfsii causes a disease of peanut known as stem rot or white mold. For many years, *S. rolfsii* has been categorized as a major pathogen of peanut in the regions of the southern United States where high temperatures prevail. Disease prevalence is maximized in warmer regions because maximum growth and formation of the sclerotia, the overwintering structures, occurs at 27ºC to 30ºC (Punja, 1985).

The primary objective of this study was to identify genes in the peanut and *S. rolfsii* genomes that are differentially expressed during the early contact phase of the plant-pathogen

interaction using cultivars of varying resistance, in order to identify plant resistance genes and genes related to fungal virulence. The central hypothesis of this study was that the genes related to disease resistance and fungal virulence would be up-regulated in the most resistant cultivar compared to the most susceptible cultivar. A second tier hypothesis was that genotypes with intermediate resistance would show intermediate levels of gene expression for resistance critical genes.

To expand our understanding of the defense-related gene expression profile of peanut and the virulence-related gene expression profile of *S. rolfsii* during the initial plant-pathogen interaction, we used high throughput sequencing technology to identify differentially expressed genes in cultivars with varying resistance levels. Four peanut cultivars of increasing resistance levels, A100-32, Georgia Green, Ga-07W and York, were inoculated with a single virulent *S. rolfsii* strain. 454 sequencing without library normalization was performed on infected peanut tissue. Using statistical analysis, differential expression of genes related to host plant resistance and fungal virulence was assessed. Normalization of the read counts and fold changes of these genes were calculated. Genes of interest were up-regulated in the highly resistant cultivar compared to the highly susceptible cultivar and included several PR proteins including PR-3, 4, 5 and 10, several other defense-related genes, virulence-related fungal endopolygalalcturonases, oxaloacetate acetylhydrolase, glucoamylase and unknown genes. This differential expression was confirmed using real-time PCR.

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CHAPTER 2

LITERATURE REVIEW

Peanut origin and background

The domesticated peanut evolved from the hybridization of two diploid species to generate an allotetraploid approximately 3500 years ago. *Arachis hypogaea L.* is the only domesticated species in the genus (Tillman and Stalker, 2009). The center of origin of the genus *Arachis* is southwest Mato Grosso do Sul in Brazil or northeast Paraguay; the most ancient species of this genus are still found growing as weeds in that region (Simpson et al., 2001). The primary center of diversity of the species is the Chaco region, located between southern Bolivia and northwest Argentina. In the 16th century, the crop was grown by the natives of the New World. During the European colonial expansion, peanut was taken to Europe, Asia, Africa and the Pacific Islands (Putnam et al., July 1991). Peanut is now grown throughout the tropical and warm temperature regions of the world and was introduced into the southeastern United States in the 1700s (Grichar et al., 2004).

Peanut is one of the world's major sources of food and vegetable oil (Singh and Singh, 1991). In 2010/2011, according to the United States Department of Agriculture databases, the cultivated peanut area was 21.44 million hectares worldwide, the average yield was 1.67 metric tons per hectare and total production was 35.88 million metric tons (http://www.fas.usda.gov/psdonline/). According to the 2010 Georgia Farm Gate Value Report, the Farm Gate Value of peanut has increased from $232,193,157 in 2002 to $470,504,152 in 2010 (Wolfe and Luke-Morgan, 2011).

The cultivated peanut plant is unusual in that produces flowers above ground and pods containing seeds below ground (Arenas-Huertero et al., 2009). Mature plants range in height from 15 to 60 cm, growing either upright or prostrate (Chenault et al., 2009). The cultivated peanut is comprised of four market types from two subspecies of *A. hypogaea*. *A. hypogaea hypogaea* includes the Virginia and runner market types. *A. hypogaea fastigiata* includes the varieties *vulgaris* (the Spanish market type peanut) and *fastigiata* (the Valencia market type). Virginia types have the largest pods and elongated seeds. Runner market types are medium-sized varieties of Virgina type. Spanish market types have small rounded seeds and the seeds of Valencia types are intermediate in size. Runner types comprise 70% of the production for food in the United States (Knauft and Gorbet, 1989, Putnam et al., July 1991). Peanuts have a well-developed tap root and multiple lateral roots (Tajima et al., 2008) and grow optimally between 25°C to 30°C (Prasad, 2003). It requires large amounts (50-75 cm) of rainfall during production to ensure optimal growth and seed maturity (Beasley and Padgett, 1997).

Diseases affecting peanut in the southeastern United States

As the production of peanut increased, there was an increase in the prevalence of diseases (Kokalis-Burelle et al., 1997). Early leaf spot (caused by *Cercospora arachidicola*), late leaf spot (caused by *Cercosporidium personatum*), stem rot (caused by *S. rolfsii*), *Rhizoctonia* limb rot (caused by *Rhizoctonia solani*) and Cylindrocladium black rot (caused by Cylindrocladium parasiticum) are the economically important fungal diseases of peanut in the southeastern United States (Woodward et al., 2008). In Georgia, these diseases account for annual losses in excess of $65 million (Kemerait, 2004) with stem rot is considered the most damaging in recent years (Brenneman et al., 1990).

Disease cycle of *Sclerotium rolfsii* on peanut

The genus *Sclerotium* differs from other sclerotium-producing fungi with respect to the sclerotia. Sclerotia from this genus are small, tan to dark-brown or black, spherical in shape with the rind, cortex, and medulla displaying internal differentiation. Differentiated sclerotia and sterile mycelia are characteristic of this fungus (Sarma et al., 2002). Sclerotia are the primary overwintering structures and also the primary inoculum for this disease (Aycock, 1966). Sclerotia are usually found in the soil or on plant debris. If they are buried deeply in the soil sclerotia can survive for up to a year, and in water they survive greater than two years. Sclerotia near the surface of the soil remain viable and when they encounter dying plant debris releasing either alcohols or volatiles, they will germinate (Porter et al., 1984).

High soil moisture from excessive irrigation and high planting density contribute to increased infection rates (Aycock, 1966). During favorable warm, moist conditions, the sclerotia germinate and form mycelial fans. The mycelium comes into contact with healthy plants and fluffy white mycelium is formed on the stem, crown and soil. Infection of peanut by *S. rolfsii* occurs at the crown, pegs, pods, and roots. Colonization occurs during peg and pod development. Factors including increased soil moisture, dense plant canopies and frequent irrigation increase infection and mycelial growth within and between plants (Augusto et al., 2010a). The mycelium of *S. rolfsii* continues to grow and forms white mycelia mats at and near the soil on the infected plant. The secondary cycle takes place here. Although rarely encountered, basidiospores may be produced on the mats. Sclerotial development begins 4-7 days after initiation of mycelial growth (Brenneman et al., 1997).

Economic impact of stem rot on peanuts in Georgia

Stem rot caused by *S. rolfsii* has a tremendous impact on peanut production. The disease is most prevalent in wet seasons but, there also have been severe outbreaks during dry seasons (Augusto et al., 2010b). Warm soil conditions favor the development of stem rot, which has been the most important peanut disease for Georgia since losses to TSWV have subsided in recent years. According to the 2008 Georgia Plant Disease Loss Estimates, peanuts were planted on approximately 690,000 acres in the state. The average yield was 3,400 kg/acre for a total production valued at $475 million. In 2008, stem rot prevalence reduced crop value by 8.0%. The damage was estimated at $38 million and the associated cost of control was estimated at $22.7 million. According to the 2009 Georgia Plant Disease Loss Estimates, peanuts were planted on approximately 529,293 acres. The average yield was 3,766 lb/acre for a total production valued at $401 million. This is more than double the worldwide average of 1 490lb/acre (1.67 metric tons per hectare) for 2010/2011 (http://www.fas.usda.gov/psdonline/). In 2009, stem rot prevalence reduced crop value by 6.0%. The damage was estimated at $24.1 million and the associated cost of control was estimated at $17.4 million (Kemerait, 2006, Kemerait, 2007, Kemerait, 2008, Kemerait, 2009).

Disease management for *Sclerotium rolfsii* on peanut

There are several methods for the management of stem rot on peanut. Research on peanut cultivars with resistance to stem rot began in 1917 (Brenneman et al., 1990). The late maturing cultivars, Southern Runner, Florida MDR 98 and C-99R, have partial resistance to *S. rolfsii*; however they are unfavorable to peanut producers. The Georgia-07W cultivar was released in 2007. In field evaluations over multiple years, this cultivar showed a high level of resistance to stem rot (Gorbet et al., 2004). The York cultivar was approved for release in 2006. York has

excellent resistance to white mold. York should be among the most white-mold resistant of the cultivars currently available to growers in the Southeast (Gorbet and Tillman, 2011). Control involves reduction of inoculum in the system by means of deep plowing (Brenneman et al., 1997). Crop rotation is also recommended for this disease. Two important pathogens of peanut are managed in part by rotation; these are *Meloidogyne arenaria*, the peanut root-knot nematode, and *S. rolfsii* (Timper et al., 2001). However, for stem rot control there is still a heavy reliance on fungicides by growers. Tebuconazole is recommended for stem rot; labeled formulations include Orius 3.6F, TriSum 3.6F and Integral 3.6F. The recommended amount per acre is 7.2 fl oz applied four times a season. Evito T (fluoxastrobin + tebuconazole), applied at 9.0-11.0 fl oz per acre is recommended for control of stem rot. Provost (tebuconazole + prothioconazole) is recommended for control of stem rot at a rate of 8.0 - 10.7 fl oz per acre (Kemerait et al., 2012). Tebuconazole + prothioconazole, when applied to the foliage may reduce disease in lower regions of the plant that have not been treated (Augusto and Brenneman, 2012).

Recent advancements in available sequencing tools for genomic studies

With the increased availability and affordability of deep-sequencing tools, the number of peanut databases has greatly increased. The 454 sequencing system was introduced in 2005. This pyrosequencing technology enables the execution of millions of sequencing reactions in parallel, producing an abundance of data rapidly (Wall et al., 2009). Next generation high throughput sequencing, including 454 and Solexa technologies, provides a powerful tool for the development of genomic sequence databases (Zhao et al., 2010).

Development of genomic research on peanut and the increasing availability of sequence databases

Genomic research has seen improvements in the availability of research tools to identify genes, their functions and their effects on plants. Exploitation of these tools is expensive and therefore funding has largely focused on major model crops. Despite its major economic importance both in the United States and worldwide, peanut had previously 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 establishment of sequence databases for peanut, this crop can now be more easily studied for solutions to diseases affecting peanut production (Paterson et al., 2004)

Sequencing advancements in *Sclerotium rolfsii*

Sclerotium rolfsii is a prevalent pathogen on several diverse agricultural crops grown globally; using 454 sequencing technology, researchers have developed a comprehensive transcriptome sequence database of *S. rolfsii* whilst conducting studies on its scleroglucan production. The sequencing yielded approximately 350,000 reads, assembled into 21,937 contigs and 171,833 singletons from which 6,951 were significantly matched in publically available protein databases (Schmid et al., 2010).

Differential gene expression studies

Differential gene expression has been the focus of studies used to identify genes that are responsible for observed phenotypic variation. A study by Guo et al. compared gene expression between two peanut genotypes, GT-C20 and Tifrunner, in response to *Aspergillus parasiticus*

infection. For the purpose of identifying resistance-related genes with significantly differential expression, researchers employed statistical analysis to estimate expressed sequence tag frequencies. Unique EST sequences were identified and were selected for examination of temporal gene expression patterns; GT-C20 is resistant while Tifrunner is susceptible to *Aspergillus* infection. Thirty six and forty seven unique EST sequences from libraries of GT-C20 and Tifrunner, respectively, were selected for examination of temporal gene expression patterns, based on EST frequencies. Nine and eight resistance-related genes with significant up-regulation were obtained in GT-C20 and Tifrunner libraries, respectively (Guo et al., 2008).

A study on *S. rolfsii* involving Suppressive Subtraction Hybridization PCR (SSHP) and real-time PCR (RT-PCR) revealed that differential gene expression occurs during sclerotial differentiation in *S. rolfsii*. Fifty EST-contigs were described. More than half of the contigs showed similarity to proteins found in public sequence databases. Genes that were most highly differentially expressed in the late stage as compared to the early stage had a predicted function of sclerotium germination but not mycelial growth. The most highly differentially expressed gene from the late stage encodes a lectin which had previously been shown to have 1,000-fold higher specific activity during sclerotium formation compared to mycelial growth. Several protein synthesis and processing genes were shown to be more highly expressed during initial sclerotium development but downregulated in mature sclerotia, indicating that differential gene expression occurs between stages of sclerotial development (Takach, 2009).

A study on the induction of systemic acquired resistance in peanut by means of *S. rolfsii* derived elicitors showed that plant defense responses were induced in four different peanut varieties. The fungal elicitors were introduced to the peanut in the form of fungal culture filtrate and mycelial cell wall fragments. The defense-related signal molecule salicylic acid (SA),

marker enzymes including peroxidase (POX), phenylalanine ammonia lyase (PAL), β-1,3-glucanase and the plant cell wall component, lignin, were measured. The authors found a significant fold increase in POX, PAL, SA, β -1,3-glucanase and lignin content in all four peanut varieties compared to that of control plants. This study indicated that *S. rolfsii* derived components induce systemic resistance in the varieties studied (Nandini et al., 2010).

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CHAPTER 3

IDENTIFICATION OF GENES DIFFERENTIALLY EXPRESSED DURING EARLY INTERACTIONS BETWEEN THE STEM ROT FUNGUS (SCLEROTIUM ROLFSII) AND PEANUT (ARACHIS HYPOGAEA) USING CULTIVARS OF VARYING RESISTANCE[1]

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ABSTRACT

Sclerotium rolfsii is a destructive soil-borne fungal pathogen that causes stem rot in the cultivated peanut (*Arachis hypogaea*). The purpose of this study was to identify resistance-related genes in peanut and to a lesser degree, virulence in the fungus. In order to identify differentially expressed genes in the fungal and peanut genomes related to recognition events and cell signaling mechanisms, infected peanut plants were studied with a focus on the early phase of the plant-pathogen interaction. Four peanut cultivars with different resistance levels, A100-32, Georgia Green, Ga-07W and York, were inoculated with a virulent *S. rolfsii* strain, Sr-18. Transcriptomic 454 sequencing was performed on infected tissue collected at 4 days post inoculation; this generated 225,793 high quality sequences. The first objective was to employ the Audic-Claverie statistical method to identify differentially expressed genes between the highly resistant cultivar, York, compared to the highly susceptible cultivar, A100-32. Normalized read counts and fold changes of these genes were calculated. Twelve genes of immediate focus for confirmation of differential expression were chosen from those up-regulated in York, compared to A100-32. These genes included several peanut PR proteins, defense-related genes, virulence-related fungal genes and plant genes of unknown function. Differential expression was confirmed using real-time PCR. The second objective involved expanding this study to all four cultivars. A novel statistical method developed by Victor Olman (University of Georgia; Kerry et al. in prep.) was used to assess differentially expressed contigs across all four cultivars. Contigs showing a pattern of increasing and decreasing gene expression related to resistance levels in the peanut cultivars were assessed. This study identified a set of genes that may be related to pathogen response which may be useful in potential marker assisted selection or transgenic disease control strategies. This set includes differentially expressed genes that have

not been functionally characterized in peanut or other plants and. therefore warrant additional investigation in future studies.

INTRODUCTION

Stem rot, caused by *Sclerotium rolfsii* is a major disease of peanut and occurs in all areas of peanut production, limiting the attainment of 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 in the world, 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 1,655 thousand metric tons (Revoredo and Fletcher, 2002). Stem rot is a major disease of peanut in the southeastern US (Brenneman et al., 1990). According to the 2008 Georgia Plant Disease Loss Estimates, peanuts were planted on approximately 690,000 acres in the state with an average yield of 3,400 lb/acre for a total production valued at $475 million. Stem rot prevalence reduced crop value by 8.0%. The damage was estimated at $38 million and the associated cost of control was estimated at $22 million (Kemerait, 2008).

Despite its major economic importance both in the United States and worldwide, peanut had previously 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 is a 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 an increased effort to use sequencing tools specifically for peanut genomic research (Pandey et al., 2012, Guo et al., 2008).

As of June 2012, there have been 192,077 nucleotide sequences and 253,274 EST sequences deposited into the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/). Several databases have been developed for peanut. These include an 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).

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. 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. 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 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. 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, as well as marker enzymes, peroxidase, phenylalanine ammonia lyase, β-1,3-glucanase and lignin. There was a varied increase in the fold activity of the signal molecule and the marker enzymes in 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 and could identify valuable sequences useful in potential future marker-assisted selection or transgenic disease control strategies. Understanding the different genetic and biochemical pathways expressed early in the peanut infection process would potentially provide new targets to slow or stop infection by this significant peanut disease-causing organism.

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 of varying resistance types were used and inoculated with a single virulent fungal strain. Total RNA was extracted for cDNA synthesis and then 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 (Audic and Claverie, 1997). Twelve genes of initial interest, assessed to be differentially expressed using the Audic-Claverie statistical method, related to defense in the plant and virulence in the fungus as well as genes with no known functional categorization were identified and selected for

verification using real-time PCR. Genes assessed to be differentially expressed using the Olman statistical method were assessed for trends in gene expression based on the hypothesis that there would be increasing gene expression with increasing levels of resistance. Real-time PCR was performed to confirm the differential expression.

MATERIALS AND METHODS

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 x Tifrun and is highly susceptible to *S. rolfsii*. Tifton 8 is a large-seeded, Virginia-type peanut with a spreading bunch growth (Coffelt et al., 1985). Tifrun is a released cultivar. Georgia Green in a runner market-type peanut (Branch, 1996). Ga-07W is a high-yielding, large-seeded, runner-type peanut with 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 standard Dynasty PD ® fungicide treatment. Although this fungicide has some systemic activity in young plants, this effect was considered irrelevant in the experiment as all seed received the same treatment and inoculation was 50 DAP, the R3 development stage (Boote, 1982).

Twenty pots (15 cm diameter) of each peanut cultivar (two seeds each) were prepared in potting soil. The potting medium was composed of three parts composted pine bark to one part vermiculite and was fertilized with lime, phosphate, calcium nitrate, gypsum and potassium

nitrate. The soil was sterilized by autoclaving for 30 min on 2 consecutive days before planting. Plants were grown in growth chambers with a 12 hr, 29°C day and 12 hr, 21°C night cycle (Banks, 1976). Pots were thinned to one plant/pot at 21 days after planting (DAP).

A previous study focusing on stem rot incidence found that there was a significant plant age effect. Disease incidence was high for the 50 DAP inoculations, moderate for the 70 DAP inoculations, and slightly less for the 90 DAP inoculations (Sconyers et al., 2005). Thus to generate a high level of disease, plants in this study were inoculated at 50 DAP.

***Sclerotium rolfsii* isolate information, plant inoculation and growth conditions**

The virulent *S. rolfsii* strain SR-18 was isolated from an infected field grown peanut plant in Georgia. This fungal strain has been used in a previous study exploring inoculation techniques with *S. rolfsii* (Shokes et al., 1996, Woodward and Brenneman, 2008). One cm diameter mycelial plugs from potato dextrose agar (Difco, Franklin Lakes, NJ) plates supplemented to 2% agar (2PDA) were cut using a number 5 sterile cork borer and stored in sterile water at room temperature. Mycelial plugs were routinely used to start fresh cultures on 2PDA. The fungus was grown for approximately one month at which point sclerotia had formed and matured. The mature sclerotia were placed on 2PDA plates and allowed to germinate and produce mycelial colonies. One cm diameter agar plugs with germinated sclerotia and 1 cm diameter plugs with only mycelium (no sclerotia) were cut with a number 5 cork borer from fresh 2-3 day-old 2PDA plates and were used to inoculate the peanut plants using sterile forceps (Figure 1). The plugs were placed at the crown of the plant during inoculation (Figure 2). Since the plugs were removed from the 2PDA plate and placed immediately onto the plant, they were moist and adhered easily to the plant surface.

Each plant received one germinated sclerotium agar plug and two mycelial plugs, with no germinated sclerotia. Previous field studies involving inoculation of peanut with *S. rolfsii* utilized a single agar plug with one germinated sclerotium, resulting in almost all of the plants being infected. In recent field studies no difference between infections initiated with mycelial plugs with or without a germinated sclerotium was observed (Brenneman, T. B., personal communication). Preliminary studies verified this finding and multiple plugs were used to ensure uniform infection. Twelve plants of each cultivar were inoculated as described at 50 DAP. Field-grown peanut plants produce a dense root system and thick canopy which creates a warm humid environment around the base of the plant. This microenvironment is conducive to initial fungal infection and establishment. To mimic the high humidity conditions in the field, plants were covered with clear plastic bags after inoculation for 48 hr to maintain high humidity and promote disease establishment (Shokes et al., 1996) (Figure 3). Shade was simulated during this 48 hr period by turning off 50% of the growth chamber lights. The bags were held up and away from the plants using bamboo stakes. Noninoculated plants of each cultivar were treated similarly and retained as controls (Shokes et al., 1996). Developing immature sclerotia were seen at 4 days post inoculation (DPI) and symptom development on several plants was observed at this time. In order to study early interactions, plant tissue from all four cultivars was harvested at 4 DPI. The agar plugs were removed and the underlying crown and adjacent healthy stem tissue was collected from each cultivar. The samples collected consisted of the diseased crown tissue as well as the adjacent healthy stem tissue in an approximate 1:1 ratio. Samples were collected, frozen in liquid nitrogen, and stored at -80°C for later use.

Total RNA isolation, cDNA synthesis and library construction

The samples collected consisted of the diseased crown tissue as well as the adjacent healthy stem tissue in an approximate 1:1 ratio. Using a mortar and pestle, tissue from each plant sample was ground to a fine powder under liquid nitrogen. The collected sample was divided into diseased crown tissue and healthy adjacent tissue; the tissue ground was the diseased crown tissue of the sample collected. RNA was isolated from tissue using the Spectrum Plant Total RNA kit (Sigma-Aldrich, St. Louis, Mo). Samples were run on a 1.6% formaldehyde gel to determine RNA quality and were quantified by measuring the sample absorbance at 260 nm. For subsequent steps, six samples from each cultivar were pooled; the sample was from the diseased crown. The four cultivar specific pooled samples of total RNA were submitted to the Georgia Genomics Facility (GGF, at the University of Georgia, Athens, GA) as the starting material for complementary DNA (cDNA) synthesis, library preparation and 454 high-throughput sequencing. cDNA synthesis was performed by GGF using the Evrogen MINT-Universal cDNA synthesis kit (Axxora, San Diego, CA) which is designed to synthesize full-length-enriched double stranded cDNA from total RNA. PCR adapters were incorporated at both ends of the cDNA to allow replication of the sample to a sufficient quantity for sequencing. Downstream processing and sequencing of the MINT cDNA library was performed with standard Roche protocols for 454 Titanium chemistry employing a modified oligo (dT) primer. The primer was the same as that described in Beldade et al. in which the first strand synthesis primer [5'-AAGCAGTGGTATCAACGCAGAGTAC(T)4G(T)9C(T)10VN-3'] was used (Beldade et al., 2006). To barcode the cultivar specific samples different Roche MID-tag linkers were added to each.

454 Sequencing, sequence cleaning and processing

The sequencing was designed to increase the sequence output generating the maximum number of raw sequence reads which would then require validation and further processing. The four bar coded libraries were pooled. The tagging and pooling approach can lead to variation in the number of reads generated for each tagged library within the pooled sample. The pooled barcoded samples were run together as a $1/4^{th}$ 454 run on a Titanium sequencing plate with an expectation of generating about 200,000 total sequence reads. To retain differential gene expression information, the four cDNA libraries were not normalized. Seqclean <http://compbio.dfci.harvard.edu/tgi/software/> was used for automated trimming and validation of sequences by screening for various contaminants, low quality and low-complexity sequences. The resultant sequences are considered 'cleaned.'

Assembly and assessment of differentially expressed contigs using Roche Newbler and the Audic-Claverie statistical method in NG-Magic software

Assembly of the sequence reads generated contigs that were of greater length than the individual sequence reads; differential expression analysis was then performed on these longer assembled contigs as well as a determination of the origin of the contigs (whether they are peanut or *S. rolfsii*). The assembly and assessment of the differential expression of sequences was performed. The sequences were assembled using the Roche GS *De Novo* Assembler (Roche, Indianapolis, IN). The expression assessment was performed using the NG-Magic software program, developed by John Kerry in the Leebens-Mack lab (Plant Biology, UGA). Peanut sequences were identified by BLASTing the transcript assembly against the NCBI peanut EST databases (http://www.ncbi.nlm.nih.gov/nucest/?term=arachis) and nucleotide databases (http://www.ncbi.nlm.nih.gov/nuccore/?term=arachis). Similarly, *S. rolfsii* sequences were

identified by querying the complete set of transcriptional raw data archived at Gene Expression Omnibus (http://www. ncbi. nlm. nih. gov/geo). This method allowed the designation of each contig as of either peanut (*Ah*) or *S. rolfsii* (*Sr*) origin as well as putative function. The Audic and Claverie statistical method was used to assess the differentially expressed reads in the comparison between A100-32 (highly susceptible) and York (highly resistant) cultivars. This is a sequence tag based method that is specifically designed for comparison of two libraries. The p-value max was designated as 0.05 and the rarity probability as 0.10%. The smaller the rarity probability, the more differentially expressed the gene is between the two libraries (Audic and Claverie, 1997). Differential gene expression targets are represented by the contigs comprised of overlapping cDNA sequences.

Normalization and fold change of contigs

The normalized read count was calculated as:

Normalized read count = (actual clean read count for contig/total clean reads in cultivar library) x total count of clean reads in all libraries (225,793).

Contigs with fold changes below 2 were removed from further analysis. Contigs with fold changes where the read count from one of the cultivars was 0 were designated as presence/absence (P/A).

Assessment of differentially expressed contigs using the Olman statistical method in NG-Magic Web Stat

NGMagic Web Stat is a web application layered on top of the original NGMagic database model that specializes in discovering differential expression among paired samples. The Olman method (unpublished) is built into the web-based program and was used to determine differential expression of the sequenced contigs. It is specifically designed for comparison of multiple

libraries. Functional categorization was performed on the contigs designated as significantly differentially expressed between cultivars by both statistical methods. Olman p-values <0.05 are designated as significantly differentially expressed contigs. Contigs with a p-value of -1 were removed as this indicated that the contig was not detected with enough frequency to reliably estimate differential expression (Figure 1). The contig with the most number of reads was assessed for differential expression using the two statistical methods.

Trends in gene expression across all four peanut cultivars

Following normalization of the read counts, the differentially expressed contigs were analyzed for increasing and decreasing expression patterns. The hypothesis that gene expression correlates directly proportionally with the level of resistance was statistically tested. The read counts were sorted based on their normalized reads.

Functional categorization of contigs

BLAST searches using default parameters against the NCBI non-redundant nucleotide database and the protein database using a translated nucleotide query were employed to functionally categorize differentially expressed contigs. To assist with determination of putative gene function, searches were performed on the databases for *A. hypogaea*, *Lotus japonicus*, *Glycine max* and *Arabidopsis thaliana* which have more functionally annotated genes. Defense-related peanut sequences and virulence-related *S. rolfsii* sequences were of primary interest.

Verification of differential expression using real-time PCR analysis comparing cultivars A100-32 (highly susceptible) and York (highly resistant)

In order to validate the differential expression detected using the 454 sequence data, a verification using real-time PCR was performed. The growth and inoculations of the peanut was repeated in different growth chambers than had previously been used. Samples were collected

from six plants from all four cultivars (24 samples collected) at 4DPI. Each sample from a plant of a particular cultivar represents a biological replicate for that cultivar. Total RNA was extracted from the diseased crown tissue using the Spectrum Plant total RNA kit (Sigma, St. Louis MO). RNA samples were treated with DNAse and quantified by measuring sample absorbance at 260 nm. A 3 µg sample was run on a 1.6% formaldehyde agarose gel to ensure sample integrity. For each tissue sample, 1 µg RNA was reverse transcribed to cDNA using a Superscript III First Strand cDNA synthesis kit (Invitrogen Carlsbad, CA). Real-time PCR measured relative gene expression using the SYBR-GREEN Supermix kit (Bio-Rad, Hercules, CA, USA) using a reference gene. An actin gene, with the top BLAST hit description of *Glycine max* actin-101-like, was used as the reference gene. Actin has been found to be a stable control for peanut real-time PCR experiments (Jiang et al., 2011). Normalization and fold change calculations indicate that the actin gene selected for use in the real-time PCR experiments showed fold changes of <2 comparing all cultivars. All reactions were performed on a Cepheid Smartcycler I (Cepheid, Sunnyvale, CA) machine with the following thermal cycling parameters: an initial denaturation step of 94ºC for 4 min followed by 40 cycles of two-step PCR, 94ºC for 30 s, and 60ºC for 30 s. Data was collected during the annealing step. The relative expression level of each target gene was calculated by $2^{-\Delta\Delta CT}$ method (Pfaffl, 2001). The fold changes values are average Ct values of three biological replicates with two technical replicates each, normalized against the actin reference gene, (contig00089).

Standard error values were calculated as:

Standard error = standard deviation/(square root (n)

n=3 as there were three biological replicates

Normalization of fold change data from real-time PCR

The normalization method was described by Livak and Schmittgen (Livak and Schmittgen, 2001). Table 8 shows the calculations performed on contig03022. Table 9 shows the $2^{-\Delta\Delta Ct}$ value and the corresponding standard error values as well compared to the fold change using the NRC from the 454 data for the genes of interest. Figure 9 is a graphical representation of this data.

RESULTS

Sequencing output and cleaning

The expected sequence output from the 454 sequencing was approximately 200,000 reads. There were 260,390 total reads generated from sequencing the four cultivar samples. For current 454 sequencing technology, the average read length generated is 300-400 bp. The average read length of the sequences generated in this study was 311 bp. The raw reads generated were 29,967, 46,354 and 34,380 for A100-32 (highly susceptible), Georgia Green (susceptible) and York (highly resistant) repectively. The read count for the moderately resistant cultivar Ga-07W was higher; there were 149,689 reads generated for Ga-07W. The raw sequences were cleaned using the Seqclean program. The program trimmed the sequences and removed low quality and low-complexity sequences. The resultant clean reads had been filtered according to the percentage of undetermined bases, polyA tail removal, low complexity and terminal matches with adaptors. There were 225,793 total clean reads generated from the Seqclean processing. There were 88%, 89%, 86% and 87% valid reads from the A100-32, Georgia Green, Ga-07W, York sample, respectively (Table 1).

Assembly of the sequences using the Roche GS *De Novo* Assembler

Prior to the assembly process, the reads from the individual libraries were labeled with the name of the specific cultivar from which those reads were generated. The reads were then compiled into a single file. Assembly of the sequences, using the Roche Newbler Assembler, generated 6742 contigs. There was an average of 28.82 reads per assembled contig. There were 10 contigs, each represented by a single raw read. The most abundant contig (contig06476) contained 2073 reads; this contig had no significant similarity from blastn and blastx searches. This contig was predicted to be differentially expressed using both the Audic-Claveire and Olman statistical method (Figure 4). The average contig size was 898 bp and the range of lengths was 100 to 3212 bp. Reads below 100 bp were removed from the sequence data. Figure 5 shows the read length of the contigs generated from the assembly for each contig used for the analyses (Figure 5).

Assessment of differentially expressed contigs using Audic-Claverie statistical method in NG-Magic

Differentially expressed reads were assessed using statistical methodology. Using the Audic-Claverie statistical model built into the NG-Magic program, the highly susceptible cultivar A100-32 compared against the highly resistant York cultivar . There were 274 contigs assessed to be differentially expressed in the comparison between A100-32 and York (Figure 6). The BLAST descriptions and information for the contigs for peanut and *S. rolfsii* were loaded into the NG-Magic program. This facilitated the designation of the origin of the contig as either peanut, *S. rolfsii* or other (Figure 7). Genes of interest with functional categorization related to defense in the peanut, virulence in the fungus and unknown function were selected from the contigs that were assessed to be significantly differentially expressed. Table 2 show the genes of

interest selected for verification by real-time PCR as well as the fold changes calculated using the NRC of the 454 data and the fold changes from real-time PCR (Table 2).

Assessment of differentially expressed contigs using Olman statistical method in NG-Magic Web Stat

The Olman statistical method is more inclusive than the Audic-Claverie statistical method (Figure 8). There were 1747 contigs assessed to be differentially expressed across all four cultivars and 385 assessed to be differentially expressed between the cultivars A100-32 and York. There were genes of interest assessed to be differentially expressed using the Olman method that were selected for verification by real-time PCR; these were not predicted using the Audic-Claverie statistical method. They included contig05790, contig03219 and contig00427 (Table 4). Fold changes for York/A100-32 were calculated using real-time PCR data. Of the 1747 differential contigs there were 12 assessed to be differentially expressed that showed a pattern of increasing gene expression, with A100-32 < Georgia Green < Ga-07W < York. Two contigs (contig01183 and conti05264) were also found to be differentially expressed using the Audic-Claverie statistical method. There were 27 contigs assessed to be differentially expressed that showed the opposite, a pattern of decreasing gene expression, A100-32 > Georgia Green > Ga-07W > York (Table 6 and 7).

Expression of genes encoding PR-proteins are up-regulated in the resistant cultivar

Genes assessed to be differentially expressed by the Audic-Claverie statistical method that were of peanut origin were selected based on defense-related functions as genes of interest. Initially, in order to search for genes based on defense-related functions, the properties of PR-proteins were used to search the BLAST annotations. The PR-proteins selected for verification were PR-3 (chitinase type I-II, IV-VII), PR-4 (chitinase type I and II), PR-5 (thaumatin-like) and

PR-10 (ribonuclease-like) (van Loon and Strien, 1999). Table 3 lists the fold changes comparisons calculated using NRC from 454 data and real-time PCR data for the genes of interest assessed to be differentially expressed using the Audic-Claverie statistical method (Table 3). Contig02763 was BLASTed against the nucleotide nr/nt database at NCBI; no significant similarity was found from this search. The contig was then BLASTed against the blastx database. The description for the top hit was predicted protein [*Populus trichocarpa*]. The description for the second hit was class IV chitinase [*Galega orientalis*] with an e-value of 3e-76. The fold change for the highly resistant cultivar York compared to highly susceptible cultivar A100-32 (Y/A), when calculated using the normalized read count (NRC) from the 454 data, was 2.69-fold. Verification using real-time PCR indicated a fold change of 8.00-fold. Although this fold change was 3 times higher than the fold change calculated using the 454 sequence data, both fold changes showed an upregulation of PR-4 in the highly resistant cultivar. Contig01183 was similarly BLASTed against the blastn database and retrieved as the top hit an *Arachis diogoi* thaumatin-like protein mRNA with an e-value of 0. The fold change from the 454 data for Y/A calculated using NRC was 5.28-fold. Verification using real-time PCR generated a fold change of 11.84-fold. The fold change was 2 times higher than the fold change calculated using the 454 sequence data. Contig03022 had a fold change that was 3 times higher than the fold change calculated using the 454 sequence data. All three contigs 02763, 1183 and 3022 all showed a fold change that were higher using real-time PCR than the fold changes calculated using the 454 data. Contig06098 had a top hit of *A. hypogaea* pathogenesis-related class 10 protein (PR10). The fold change of Contig06098 using real-time PCR was highly similar to the fold change calculated using the 454 data for this contig.

Expression of defense-related genes is up-regulated in the resistant cultivar

In addition to PR-proteins, there are other defense-related genes that were of interest and were up-regulated in the most resistant cultivar. Contig00281 was BLASTed against the nucleotide nr/nt database on NCBI. The description for the top hit was *Phaseolus vulgaris* pgip2, encoding a putative polygalacturonase-inhibiting protein precursor with an e-value of 9e-97. The fold change for Y/A calculated using NRC was 5.06 -fold. Verification using real-time PCR generated a fold change of 41.02-fold. Contig05792 was BLASTed against the nucleotide nr/nt database on NCBI. The top hit BLAST description for contig05792 was *A. diogoi* glutathione S-transferase mRNA and for contig05451 was *A. hypogaea* resveratrol synthase mRNA. The fold change from 454 data for contig05792 and contig05451 was P/A as there were no reads in the A100-32 cultivar. Verification using real-time PCR generated a fold change of 8.26-fold for contig05792 and 1.49-fold for contig05451.

Virulence-related genes selected for verification by real-time PCR

As the origin of the contigs was assessed, it became possible to select fungal virulence-related genes for verification of differential expression. A well-known virulence-related compound produced by *S. rolfsii* is oxalic acid; the synthesis of oxalic acid is catalyzed by oxaloacetate acetylhydrolase. The top BLAST description from a blastx search of contig04719 was *Serpula lacrymans* var. *lacrymans* hypothetical protein. The third hit had a BLAST description of *Coprinopsis cinerea* okayama7#130 oxaloacetate acetylhydrolase. There was a fold change of P/A from the 454 data and 2.34-fold from the real-time PCR verification. *S. rolfsii* has been shown to produce polygalacturonase which has been determined to be responsible for tissue maceration in the host (Bateman, 1972). One significantly differentially expressed contig (contig00240) was found to have a top BLAST hit with the description of

precursor of endopolygalacturonase (EndoPG) 1. Contig00240 was calculated to be 4.40-fold up-regulated in the York cultivar compared to the A100-32 and showed a presence/absence fold change as there were no reads in A100-32. The fold change from real-time PCR was 5.58-fold for contig00240. A differentially expressed contig with the functional classification of glycoside hydrolase was found and selected for verification. Contig00332 was BLASTed against the nucleotide nr/nt database on NCBI. The description for the top hit was *Corticium rolfsii* (*Sclerotium rolfsii*) mRNA for glucoamylase G2. The fold change from the 454 data was 14.08 and the fold change from real-time PCR was 1.61-fold.

An additional virulence-related gene was assessed to be differentially expressed and selected for verification after using the less restrictive Olman statistical method on the four cultivars. Contig00427 had a top blast hit description of *A. rolfsii* endoPG 2 precursor. There was a P/A expression using 454 data for the Y/A calculation and the fold change using real-time PCR was 1.99-fold (Table 4 and 5).

Genes with no assigned functions selected for verification by real-time PCR

Contigs with no assigned function or low e-values from BLAST searches were considered unknown and a subset selected for verification. The contigs selected for verification all had a P/A expression from the 454 data as there were no reads in the A100-32 cultivar. Contig02782 was BLASTed against the nucleotide nr/nt database on NCBI parameters. The description for the top hit was *Pisum sativum* mRNA for putative glycine rich protein precursor (*grp1* gene) with an e-value of 6e-06. The fold change for Y/A calculated using real-time PCR generated a fold change of 7.47-fold. Contig03219 had a top blastn description of *Dugesia japonica* gene for Piwi homologue; however only 31 bases matched with the subject sequence and the e-value was 6.00E-06. There was no significant similarity from blastx searches.

Contig05264 had a top blastx description of expressed protein [*Schizophyllum commune* H4-8] with an e-value of 1e-05. Real-time PCR data showed a 0.76-fold upregulation in York compared to A100-32. The fold change for Y/A calculated using real-time PCR generated a fold change of 2.40-fold. Contig05790 had a top BLAST description of *Myceliophthora thermophile* glycoside hydrolase family 7 protein. The fold change from real-time PCR was 6.02-fold for Y/A (Table 4 and 5).

Statistical analysis of real-time PCR data revealed that the majority of the genes of interest had unreliable fold-change values

Statistical analysis for the real-time PCR data included the calculation of the standard error associated with fold change values. The graphs in Figures 9 and 10 contain depictions of the error bars. If these error bars overlap, then the fold change information is not reliable. Contig00281 and was found to have reliable fold changes using the Livak and Smittengen normalization method. Further experimentation is required on more biological replicates to obtain more reliable real-time PCR data.

DISCUSSION

In this report, we studied the differential expression of genes associated with resistance in peanut and virulence in *S. rolfsii*. By focusing on the early phase of the interaction in this study, we expected to identify novel targets including plant resistance genes and genes related to fungal virulence that represent potential targets for enhancing plant resistance.

Variation in number of sequences generated from each cultivar

In order to maximize sequence output from the 454 sequencing, the samples were barcoded and pooled. Pooling of samples maximizes sequence output; however it may also

result in varied numbers of sequences generated from each sample as certain samples are preferentially sequenced.

Genes encoding select PR proteins are up-regulated in cultivar York compared to cultivar A100-32

Pathogenesis-related proteins (PR) are induced in response to pathogen attack. The PR proteins accumulate at the site of infection and are also systemically induced via systemic acquired resistance (SAR) thus reducing further infection (van Loon and Strien, 1999). Most PRs are induced through the signaling compounds salicylic acid, jasmonic acid, or ethylene. PR proteins may have antimicrobial activities through cell wall hydrolytic activity, toxicity, and defense signaling. This suggests that they play an important role in defense to pathogen attack (van Loon et al., 2006).

Hydrolytic enzymes include, amongst others, chitinases and glucanases that may be induced upon fungal attack by pathogen derived chitin wall fragments. In this study, two chitinases PR-3 (contig02763) and PR-4 (contig03022) were both found to be up-regulated in the resistant peanut York cultivar compared to A100-32 in response to *S. rolfsii*. Chitinases are thought to provide the plants with anti-fungal activity since their substrate, chitin, is an integral component of fungal cell walls. PR-3 includes chitinase type I, II, IV, V, VI and VII (van Loon et al., 2006). *Rhizoctonia solani* was the first fungus shown to be suppressed in transgenic tobacco and canola overexpressing a PR-3-type chitinase from bean (Brogue et al., 1991). Hejgaard et al. showed the induction of a PR-4 protein in the leaves of barley following inoculation with *Erysiphe graminis.* Antifungal activity *in vitro* has been shown for proteins from barley leaf and grain; the proteins are homologous to the PR-4 proteins from tobacco and tomato (Hejgaard et al., 1992).

Similar to the inducible plant defense protein chitinase, several additional defense-related genes are involved in antifungal activity. Some thaumatin-like proteins (PR-5) are highly inducible following pathogen attack (Vigers et al., 1992). Antifungal activities of PR-5 proteins have been reported against such fungi and oomycetes as *Fusarium oxysporum*, *Alternaria solani* and *Phytophthora infestans* (Kitajima and Sato, 1999). In this study, contig01183, contained a gene encoding a thaumatin-like protein (PR-5), was 5.28-fold up-regulated based on 454 sequence and 33.90-fold up-regulated using real-time PCR data. The thaumatin-like proteins belong to a family of proteins that can permeabilize fungal membranes. They are thought to form transmembrane pores and are also referred to as permatins (Fritig et al., 1998). Structural studies on maize PR-5 proteins showed that they have an electrostatically polarized surface which may have antifungal activity through interaction with a membrane ion channel, a water channel or an osmotic receptor (Batalia et al., 1996). Antifungal activity has also been shown in peanut due to PR-10, which has 'ribonuclease-like' activity. Contig06098 was designated as a PR-10 and was shown to be up-regulated in the York cultivar compared to A100-32 cultivar. Chadha and Das have reported that recombinant AhPR10 has antifungal activity against the peanut pathogens *Fusarium oxysporum* and *R. solani*. They linked antifungal activity with RNase activity (Chadha and Das, 2006). PR-10 proteins with RNase activity may increase plant defense during programmed cell death surrounding infection sites or may directly act on the pathogens (Liu and Ekramoddoullah, 2006).

Select genes encoding defense-related or unknown proteins are up-regulated in the resistant (York) vs the susceptible (A100-32) cultivar, in response to *S. rolfsii*

In addition to PR-proteins there are other defense-related proteins that were of interest as they were not classified as PR protein but were classified as defense-related genes.

Glutathionine S-transferases (GST), polygalacturonase-inhibiting proteins (PGIPs) and resveratrol synthase genes have roles in defense response in plants. GST (contig05792), PGIP (contig00281) and resveratrol synthase (contig05451) were found to be up-regulated in York compared to A100-32 in response to *S. rolfsii*. GSTs play roles in metabolism as well as in the detoxification of a wide variety of compounds. A GST subclass has been suggested to have roles in stress responses, including pathogen attack (Marrs, 1996). *S. rolfsii* produces endopolygalacturonase (PG) activity likely involved in host tissue maceration (Bateman, 1972). PGIPs inhibit a variety of PGs with different kinetics. Common bean, *P. vulgaris* PvPGIP2 binds the N-terminal portion of *Botrytis cinerea* PG1 and partially covers its active site thereby decreasing affinity for its substrate (Sicilia et al., 2005). Resveratrol, 3,5,4'-trihydroxystilbene is synthesized by resveratrol synthase. Resveratrol is a phytoalexin with antifungal activity and is produced upon induction by a pathogen (Chung et al., 2003). Resveratrol synthase genes are induced by fungal infection, elicitors and UV exposure in cultured peanut cells and *in planta* (Chung et al., 2003, Lanz et al., 1990).

Fungal virulence-related genes are more highly up-regulated in the intereaction with resistant cultivar, York, than in the susceptible cultivar, A100-32 in response to *S. rolfsii*

In 1965, Bateman and Beer proposed that oxalic acid, pectic enzymes, and cellulose act together in the destruction of host tissue by *S. rolfsii* (Bateman and Beer, 1965). In this study, the virulence-related enzyme encoding genes, oxaloacetate acetylhydrolase (contig04719) and endopolygalacturonase (EndoPG) (contig00240 and contig00427), were found to be up-regulated in York compared to A100-32. Oxalic acid is secreted by the phytopathogens *Sclerotinia sclerotiorum* and *S. rolfsii* (Ferrar and Walker, 1993). It is involved in the pathogenicity of certain necrotrophic fungi (Hegedus and Rimmer, 2005). It has been shown that *S. sclerotiorum*

oxalic acid deficient mutants were non-pathogenic on *P. vulgaris* (Godoy et al., 1990). Oxalic acid synthesis in *S. sclerotiorum* is proposed to be catalyzed by oxaloacetate acetylhydrolase (Rollins and Dickman, 2001). Oxalic acid is found to increase toxicity by means of acidification of the environment inside the middle lamellae, sequestering calcium and working with cell wall depolymerizing enzymes to reduce the integrity of the host cell wall. Many fungal pectinolytic enzymes, including polygalacturonases show optimal activity in acidic, low calcium environments, created by oxalic acid secretion (Hegedus and Rimmer, 2005). EndoPG enzymes work in combination with oxalic acid and are likely involved in tissue maceration of their host (Bateman, 1972). Another virulence-related gene of interest in this study included a glucoamylase; glucoamylases belongs to the glycosyl hydrolase 15 family. Glycosyl hydrolases are a widespread group of enzymes that cleave the glycosidic bond of a variety of polysaccharides. Contig00332, encoding a putative glucoamylase was found to be up-regulated in York compared to A100-32 in response to *S. rolfsii*. Cellulolytic fungi usually secrete a cellulase mixture, composed mainly of glycoside hydrolase cellulases (cellulose-hydrolyzing enzymes) during cellulose degradation (Suzuki et al., 2009). *Corticium rolfsii* (the teleomorph of *S. rolfsii*) was reported to produce a raw starch-digeseting glucoamylase (Nagasaka et al., 1998). Martel et al. suggested that the presence of a glucoamylase secreted by a phytopathogen could provide energy to the fungus during infection by means of starch degradation (Martel et al., 2002).

The selected genes of interest with low e-values show differential upregulation in the York cultivar in comparison to the A100-32 cultivar. These contigs (contig02782, contig03219 and contig05264) were selected for verification using real-time PCR. Contig02782 and contig03219 may be of interest in characterization studies.

Differences between fold changes from 454 sequence data and real-time PCR

The factors that may have contributed to the differences between the fold changes calculated using the normalized 454 data and real-time PCR data are the depth of sequencing, the tissue used for RNA extractions to generate the cDNA used in real-time PCR, the selection of the endogenous control, the normalization method used to calculate the NRC or the normalization method used on the real-time PCR data and the use of different growth chambers for the 454 sequencing experiment and the real-time PCR experiments. The depth of sequencing may have affected the fold change values calculated from the 454 data. Since the fold change was calculated based on read counts, deeper sequencing may have generated different read counts which would have affected the fold change calculations for the 454 data. Normalization of real-time PCR data is dependent on the stability of the endogenous control; the choice of the endogenous control would alter the fold change calculations for the real-time PCR data.

Trends of gene expression across four cultivars using Olman statistical method

As the Olman statistical method is more inclusive than the Audic-Claverie statistical method, we expected to find more differentially expressed genes. There were 1747 contigs assessed to be differentially expressed across all four cultivars. It was unexpected that there were so few contigs (12 contigs) that showed a pattern of increasing gene expression, with the York cultivar showing the highest gene expression and the A100-32 cultivar showing the lowest gene expression and only 27 contigs assessed to be differentially expressed that showed a pattern of decreasing gene expression, with the A100-32 cultivar showing the highest gene expression and the York cultivar showing the lowest gene expression. This reveals that the gene expression profile related to defense in peanut in response to *S. rolfsii* is complex and that the cultivars may attain resistance by varied mechanisms.

Outcomes of this study

The hypothesis of this study was that there would be a differential expression of genes associated with resistance in the peanut and virulence in the fungus that showed expression patterns related to their resistance levels. This is the first 454 sequencing study performed on the early interaction between peanut and *S. rolfsii*. Sequence data produced from this study was used to explore the defense profile of peanut in response to pathogen attack by *S. rolfsii* as well as the gene expression profile of *S. rolfsii* upon infection of cultivars of varying resistance. The number of contigs assessed to be differentially expressed was much lower than expected. This may have been due to the time point at which the samples were collected. Defense-related genes show an expression profile that varies with time following attack by a pathogen. There were very few contigs that showed a trend in gene expression related to their resistance levels. This may be due to the complex nature of defense profiles for the different cultivars. Further experimentation is needed on an increased set of biological replicates and an increased set of genes of interest.

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Table 1. Read counts from 454 sequencing and Seqclean processing. The read count for the moderately resistant cultivar Ga07W was higher likely due to preferential sequencing following pooling of individually barcoded libraries. Seqclean processing produced cleaned valid high-quality reads. The number of raw reads, valid reads, trimmed reads, trashed reads and the percentage of valid reads are indicated.

Sample	Resistance type	Raw Reads	Valid	Trashed	% Valid
A100-32	Highly Susceptible	29,967	26,362 (2,489 trimmed)	3,605	88%
Georgia Green	Susceptible	46,354	41,131 (4,759 trimmed)	5,223	89%
Ga07W	Moderately Resistant	149,689	128,340 (18,722 trimmed)	21,349	86%
York	Highly Resistant	34,380	29,960 (3,989 trimmed)	4,420	87%

Table 2. Genes of interest from contigs assessed to be differentially expressed using the Audic-Claverie statistical method. Genes were selected for verification using real-time PCR. The Audic-Claverie statistical method was used to compare the highly susceptible cultivar against the highly resistant cultivar. The p-value max was designated as 0.05 and the rarity probability as 0.10. The data shows the raw unnormalized read counts and the NRC (normalized read counts) as well as the fold change calculated using the 454 data. The fold change was designated as presence/absence (P/A) when there were no reads for that contig from the A100-32 library.

First library = A100-32, Second library = York										
Contig	Raw Reads			NRC		0<A<Min	Max<A<Inf	Fold change		Description of blastn/blastx hit
	Y-A	in Y	in A	Y	A			Y/A	A/Y	
Defense-related genes of interest in peanut										
03022	43	47	4	354	34	23	57	10.34	0.10	*A. hypogaea* PR-4A
06098	53	60	7	452	60	32	70	7.54	0.13	*A. hypogaea* PR-10
01183	40	48	8	362	69	24	58	5.28	0.19	*A. diogoi* thaumatin-like
00281	19	23	4	173	34	8	33	5.06	0.20	*P. acutifolius* pgip2
02763	39	58	19	437	163	30	68	2.69	0.37	*G. orientalis* class IV chitinase
05792	9	9	0	68	0	1	17	P/A	0.00	*A. diogoi* GST
05451	8	8	0	60	0	1	16	P/A	0.00	*A. hypogaea* resveratrol synthase
Virulence-related genes of interest in *S. rolfsii*										
00332	15	16	1	121	9	4	25	14.08	0.07	*C. rolfsii* glucoamylase
00240	12	15	3	113	26	4	24	4.40	0.23	*A rolfsii* EndoPG 1 precursor
04719	9	9	0	68	0	1	17	P/A	0.00	*C cinerea* oxaloacetate acetylhydrolase
Gene of interest considered to be unknown in peanut										
02782	20	20	0	151	0	7	29	P/A	0.00	*P. sativum* grp1
Gene of interest considered to be unknown in *S. rolfsii*										
05264	8	8	0	60	0	1	16	P/A	0.00	*S. commune* expressed protein

Table 3. Fold changes comparisons calculated using NRC from 454 data and real-time PCR data. The contig numbers and the fold changes from the 454 data and real-time PCR experiments are shown for Y/A. The descriptions from the BLAST results using either blastn or blastx are listed. The final column shows the e-value from BLAST searches. P/A is a presence/absence fold change and is when there are no reads in the A100-32 cultivar. The fold changes values from the real-time PCR experiments are average Ct values of three biological replicates with two technical replicates each, normalized against the actin reference gene, (contig00089)

Contig	Fold change from 454	Fold change from real-time PCR	Description of blastn/blastx hit	e-value
	Y/A	Y/A		
Endogenous control				
00089			*G. max* actin	0
Defense-related genes of interest in peanut				
03022	10.3389	51.2121	*A. hypogaea* PR-4A	2.00E-175
06098	7.5421	15.5280	*A. hypogaea* PR10	2.00E-100
01183	5.2794	33.9033	*A. diogoi* thaumatin-like	0
02763	2.6860	17.5801	*G. orientalis* class IV chitinase	3.00E-76
00281	5.0595	53.5994	*P. acutifolius* pgip2	9.00E-97
05792	P/A	22.7921	*A. diogoi* GST	4.00E-63
05451	P/A	4.1402	*A. hypogaea* resveratrol synthase	4.00E-129
Virulence-related genes of interest in *S. rolfsii*				
00332	14.0785	10.5619	*C. rolfsii* glucoamylase	0
00240	4.3995	19.3563	*A rolfsii* EndoPG 1 precursor	0
04719	P/A	6.7617	*C cinerea* oxaloacetate acetylhydrolase	4.00E-40
Genes of interest considered to be unknown in peanut				
02782	P/A	13.2527	*P. sativum* grp1	1.00E-04
Genes of interest considered to be unknown in *S. rolfsii*				
05264	P/A	1.2830	*S. commune* expressed protein	1.00E-05

Table 4. Select contigs assessed to be differentially expressed using the Olman statistical method and selected for verification of differential expression using real-time PCR. The contigs were assessed to be differentially expressed using the Olman statistical method. The table shows the contig numbers, origin of the contig (Ah/Sr or other), normalized read count (NRC) and fold changes (FC) for York/A100-32 calculated using the NRC from the 454 data are shown. The descriptions from the BLAST results using either blastn or blastx are also shown. The first group of contigs are genes that are defense-related in peanut that are up-regulated in York compared to A100-32. The second group of contigs are genes that are virulence-related in *S. rolfsii*. The third group of contigs are genes that are considered unknown in peanut and the last group of contigs are genes that are considered unknown in *S. rolfsii*.

Contig	Olman P-Value	Ah/ Sr	NRC				FC	Description of blastn/blastx hit	e-value
			A	G	W	Y	Y/A		
Defense-related genes of interest in peanut									
03022	2.35E-12	Ah	34	351	146	354	10.34	Arachis hypogaea clone Gsi76 PR protein 4A mRNA, partial cds	0.00E+00
06098	0.00E+00	Ah	60	324	100	452	7.54	Arachis hypogaea pathogenesis-related class 10 protein (PR10) mRNA, complete cds	1.00E-105
01183	2.51E-08	Ah	69	82	255	362	5.28	Arachis diogoi thaumatin-like protein mRNA, complete cds	0.00E+00
02763	0.00E+00	Ah	163	538	144	437	2.69	Populus trichocarpa predicted protein, mRNA	2.00E-15
00281	8.27E-13	Ah	34	198	33	173	5.06	Phaseolus acutifolius pgip2 gene for putative polygalacturonase-inhibiting protein precursor, biomaterial PA-PI 321638	6.00E-20
05792	5.85E-07	Ah	0	5	5	68	P/A	Arachis diogoi glutathione S-transferase mRNA, partial cds	1.00E-66
05451	2.52E-03	Ah	0	27	11	60	P/A	Arachis hypogaea clone AHRES 2-1 resveratrol synthase gene, partial cds	7.00E-75
Virulence-related genes of interest in *S. rolfsii*									
00332	3.33E-16	Sr	9	159	9	121	14.08	Corticium rolfsii mRNA for glucoamylase G2, complete cds	0.00E+00
00240	6.07E-05	Sr	26	104	30	113	4.40	Athelia rolfsii EndoPG 1 mRNA for endopolygalacturonase 1 precursor, complete cds	0.00E+00
04719	3.50E-10	Sr	0	104	7	68	P/A	Coprinopsis cinerea okayama7#130 SeqLit oxaloacetate acetylhydrolase, mRNA	7.00E-14
00427	2.22E-06	Sr	0	82	11	53	P/A	Athelia rolfsii EndoPG 2 mRNA for endopolygalacturonase 2 precursor, complete cds	2.00E-25
Genes of interest considered to be unknown in peanut									
02782	1.51E-11	Ah	0	60	14	151	P/A	Pisum sativum mRNA for putative glycine rich protein precursor (grp1 gene)	6.00E-06
03219	1.01E-02	Ah	0	71	26	30	P/A	Dugesia japonica iwi gene for Piwi homologue	6.00E-06
Genes of interest considered to be unknown in *S. rolfsii*									
05264	3.20E-04	Sr	0	5	11	60	P/A	Schizophyllum commune H4-8	1.00E-05
05790	4.96E-09	Sr	0	0	0	53	P/A	Myceliophthora thermophila ATCC 42464 glycoside hydrolase family 7 protein (MYCTH_42937) mRNA, complete cds	3.00E-05

Table 5. Comparison of fold changes for genes of interest assessed to be differentially expressed using the Olman statistical method. The contig numbers and the fold changes from the 454 data and real-time PCR experiments are shown for Y/A. The descriptions from the BLAST results using either blastn or blastx are listed. The final column shows the e-value from BLAST searches. P/A is a presence/absence fold change and is when there are no reads in the A100-32 cultivar.

Contig	Fold change from 454	Fold change from real-time PCR	Description of blastn/blastx hit	e-value
	Y/A	Y/A		
Defense-related genes of interest in peanut				
03022	10.34	51.21	*A. hypogaea* PR-4A	2.00E-175
06098	7.54	15.53	*A. hypogaea* PR10	2.00E-100
01183	5.28	33.90	*A. diogoi* thaumatin-like	0
02763	2.69	17.58	*G. orientalis* class IV chitinase	3.00E-76
00281	5.06	53.60	*P. acutifolius* pgip2	9.00E-97
05792	P/A	22.79	*A. diogoi* GST	4.00E-63
05451	P/A	4.14	*A. hypogaea* resveratrol synthase	4.00E-129
Virulence-related genes of interest in *S. rolfsii*				
00332	14.08	10.56	*C. rolfsii* glucoamylase	0
00240	4.40	19.36	*A rolfsii* EndoPG 1 precursor	0
04719	P/A	6.76	*C cinerea* oxaloacetate acetylhydrolase	4.00E-40
00427	P/A	3.40	*A rolfsii* EndoPG 2 precursor	2.00E-25
Genes of interest considered to be unknown in peanut				
02782	P/A	13.25	*P. sativum* grp1	6.00E-06
03219	P/A	2.60	*D. japonica* gene for Piwi homologue	6.00E-06
Genes of interest considered to be unknown in *S. rolfsii*				
05264	P/A	1.28	*S. commune* expressed protein	1.00E-05
05790	P/A	20.26	M thermophila glycoside hydrolase family 7	3.00E-05

Table 6. Contigs showing a pattern of increasing gene expression A<G<W<Y. There are 12 contigs that are differentially expressed according to the Olman-statistical method that show a pattern of increasing gene expression. Contigs with fold changes listed as P/A are calculated with 0 reads in one cultivar.

Contig	Olman P-Value	Ah/ Sr	NRC				Y/A	Y/G	Y/W	Description of blastn/blastx hit	e-value
			A	G	W	Y	FC = NRC1 /NRC2				
							Fold change>2				
06738	0	Ah	1045	1790	1833	2736	2.62			Pisum sativum mRNA for putative glycine rich protein precursor (grp1 gene)	1.20E+00
01183	2.51E-08	Ah	69	82	255	362	5.28	4.39		Arachis diogoi thaumatin-like protein mRNA, complete cds	0.00E+00
05171	4.23E-07	Ah	180	412	436	701	3.90			Puccinia graminis f. sp. tritici CRL 75-36-700-3 histone-lysine N-methyltransferase, mRNA	2.00E-01
05379	2.38E-05	Ah	0	5	99	106	P/A	19.22		Arachis hypogaea clone AHF-205D04 NBS-LRR gene cluster, complete sequence	3.00E-15
05264	3.20E-04	Sr	0	5	11	60	P/A	10.98	5.71	Schizophyllum commune H4-8	1.00E-05
05483	5.84E-04	Ah	505	511	746	874				Nematostella vectensis Hl2 (Hl2) mRNA, complete cds	1.10E-02
05234	0.003376	Ah	308	532	544	708	2.30			Nematostella vectensis Hl2 (Hl2) mRNA, complete cds	3.00E-03
01595	0.022963	Ah	26	49	104	128	4.99	2.59		Glycine max uncharacterized LOC100499875 (LOC100499875), mRNA gb|BT089925.1| Soybean clone JCVI-FLGm-3M13 unknown mRNA	0.00E+00
04873	0.027625	Ah	9	22	26	75	8.80	3.43	2.86	PREDICTED: Glycine max 60S ribosomal protein L30-like (LOC100806260), mRNA	2.00E-57
02879	0.037055	Ah	34	44	97	128	3.74	2.92		Millettia pinnata mitochondrion, complete genome	0.00E+00
03929	0.03999	Ah	0	5	26	53	P/A	9.61		Lotus japonicus cDNA, clone: LjCa-005-AH12, HTC	2.00E-42

Table 7. Contigs showing a pattern of decreasing gene expression A>G>W>Y. There are 27 contigs determined to be differentially expressed by the Olman-statistical method that show a pattern of decreasing gene expression. Contigs with fold changes listed as P/A are calculated with 0 reads in one cultivar.

Contig	Olman p-Value	Ah/ Sr	NRC				FC = NRC1 /NRC2 Fold change>2			Description of blastn/blastx hit	e-value
			A	G	W	Y	A/G	A/W	A/Y		
05698	3.39E-08	Ah	77	11	5	0	7.02	14.61	P/A	PREDICTED: Glycine max 60S ribosomal protein L7-4-like (LOC100813342), mRNA	3E-55
00001	3.82E-07	Ah	274	132	86	75	2.08	3.18	3.64	Millettia pinnata mitochondrion, complete genome	0
00521	5.25E-05	Ah	394	274	218	121			3.27	PREDICTED: Glycine max uncharacterized protein LOC100792196 (LOC100792196), mRNA	3E-34
00034	7.23E-05	Ah	111	33	28	8	3.38	3.96	14.77	PREDICTED: Glycine max eukaryotic translation initiation factor 5-like (LOC100777906), mRNA	0
01784	4.00E-04	Ah	60	16	9	0	3.64	6.82	P/A	Glycine max serine/threonine-protein phosphatase PP1-like (LOC100783178), mRNA gb|BT095835.1| Soybean clone JCVI-FLGm-25I20 unknown mRNA	0
00031	6.60E-04	Ah	137	71	42	30		3.25	4.55	Glycine max cDNA, clone: GMFL01-17-K15	2E-73
01107	7.47E-04	Ah	43	11	4	0	3.90	12.17	P/A	PREDICTED: Glycine max uncharacterized protein LOC100791985 (LOC100791985), mRNA	1E-149
02922	7.47E-04	Ah	43	11	4	0	3.90	12.17	P/A	PREDICTED: Glycine max succinate-semialdehyde dehydrogenase, mitochondrial-like (LOC100808318), mRNA	0
02103	0.00111376	Ah	69	16	16	0			4.55	Arachis hypogaea germin-like protein subfamily 3 member 3 precursor (GLP1) mRNA, complete cds	0
03647	0.0012712	Ah	240	143	134	53				Cicer arietinum isolate C962-8 actin mRNA, complete cds	0
01007	0.00301172	Ah	660	527	503	339	2.73	4.26	P/A	Oryza glaberrima clone OG_BBa0011M11, complete sequence	7E-90
01215	0.00548686	Ah	60	22	14	0	3.12	4.87	P/A	Glycine max phosphopantothenate--cysteine ligase 2-like (LOC100777777), mRNA gb|BT097377.1| Soybean clone JCVI-FLGm-26M13 unknown mRNA	1E-136
04411	0.007258	Ah	51	16	11	0			3.10	PREDICTED: Glycine max trafficking protein particle complex subunit 2-like (LOC100781378), mRNA	1E-154
01258	0.00946943	Ah	257	165	160	83		2.25	4.55	Glycine max cDNA, clone: GMFL01-14-O16	0
03019	0.01451137	Ah	103	88	46	23		2.70	11.36	Glycine max extensin (EXT) mRNA, complete cds	2E-24
00297	0.01527665	Ah	86	44	32	8		3.10	P/A	PREDICTED: Glycine max ALA-interacting subunit 3-like (LOC100793838), mRNA	0
00371	0.01828628	Ah	60	38	19	0	2.23	3.25	3.79	PREDICTED: Glycine max glutathione reductase, cytosolic-like (LOC100810846), mRNA	0
00315	0.02170587	Ah	86	38	26	23	2.60	4.87	P/A	Glycine max OAS-TL3 cysteine synthase (OAS-TL3), mRNA gb|EF584898.1| Glycine max OAS-TL3 cysteine synthase (OAS-TL3) mRNA, complete cds	0
01939	0.02223541	Ah	43	16	9	0	2.60	4.87	P/A	Arachis hypogaea plasma membrane mannitol transporter (MaT2) mRNA, partial cds	0
02307	0.02223541	Ah	43	16	9	0		3.10	P/A	PREDICTED: Glycine max putative cyclin-A3-1-like (LOC100801988), mRNA	9E-64
00958	0.02231633	Ah	60	33	19	0		4.06	P/A	PREDICTED: Glycine max uncharacterized protein LOC100778076 (LOC100778076), mRNA	1E-149
03062	0.02932673	Ah	43	11	11	0	2.34	4.17	6.82	Glycine max uncharacterized LOC100800393 (LOC100800393), mRNA gb|BT097247.1| Soybean clone JCVI-FLGm-26G19 unknown mRNA	1E-148
02630	0.03162226	Ah	43	27	11	0				Glycine max glycogen synthase kinase-3 (GSK-3), mRNA gb|BT093874.1| Soybean clone JCVI-FLGm-24B1 unknown mRNA	1E-141
04301	0.04036263	Ah	51	22	12	8	3.90	4.87	5.68	Lupinus angustifolius cytosolic malate dehydrogenase (MDH1) mRNA, complete cds	1E-139
00439	0.04090707	Ah	394	280	262	219				Arachis hypogaea clone 1P23 glutamine synthetase GS56 mRNA, partial cds	0
02934	0.04297725	Ah	43	11	9	8	3.9006	4.86837	5.68242	Glycine max uncharacterized LOC100815998 (LOC100815998), mRNA gb|BT096190.1| Soybean clone JCVI-FLGm-20D3 unknown mRNA	2E-95
00481	0.04533524	Ah	60	33	16	15		3.78651	3.9777	PREDICTED: Glycine max uncharacterized protein LOC100817904 (LOC100817904), mRNA	0

Table 8. Normalization method for real-time PCR data as described by Livak and Schmittgen (Livak and Schmittgen 2001). The fold change of contig00240 in York is 5.58-fold up-regulated relative toA100-32, with a standard error of 13.05. Expression of A100-32 is set to 1.00-fold, with a standard error of 8.45. The designations of the acronyms are: R = resistant, S= susceptible, Ct = the fold change measured for contig03022 in the resistant cultivar, Cc = the fold change measured for contig03022 in the susceptible cultivar, Crt = the fold change measured for the endogenous control, actin, in the resistant cultivar and Crc = the fold change measured for the endogenous control, actin in the susceptible cultivar, BR = biological replicate, TR = technical replicate, ΔΔCt for R is calculated as (Ct-Crt)-2.40(average(Cc-Crc)), ΔΔCt for S is calculated as (Cc-Crc)-2.40(average(Cc-Crc)), SD = standard deviation, CV = coefficient of variation, SE = standard error, CI = confidence interval.

Endogenous Control = Actin												
Treatment = York - R												
Contorl = A100-32 - S												
contig00240=*A. rolfsii* EndoPG1 precursor												
R												
	Ct 00240 (R)		Crt Actin (R)			ΔCt=(avCt-avCrt)		ΔΔCt=(avΔCt-acΔCt(S))		$2^{-\Delta\Delta Ct}$ Normalized 00240 amount relative to S ± SE		
BR	TR	BR	TR	BR								
1	21.09	21.11	22.2	22.2		1.92		-2.48		5.58 ± 13.05		
	21.13		22.16									
2	26.55	26.48	20.25	20.3								
	26.41		20.35									
3	21.13	21.38	20.59	20.7								
	21.63		20.87									
Average (av)		22.99		21.07								
S												
	Cc 00240 (S)		Crc Actin (S)			ΔCt=(avCc-avCrc)		ΔΔCt=(avΔCt-acΔCt(S))		$2^{-\Delta\Delta Ct}$ Normalized 00240 amount relative to S ± SE		
BR	TR	BR	TR	BR								
1	30.12	30.12	23.76	23.5		4.40		0.00		1.00 ± 8.45		
	30.12		23.29									
2	29.26	29.22	22.33	22.3								
	29.17		22.34									
3	22.62	22.53	22.7	22.8								
	22.44		22.91									
Average (av)		27.29		22.89								

Table 9. Results of the Livak and Schmittgen normalization method used on the real-time PCR data. Expression of A100-32 is set to 1.00-fold. The fold changes calculated using the normalized read counts from the 454 data are shown in the final column. SE = standard error.

		2-ΔΔCt values nomalized to A100-32				
Contig	Description of blastn/blastx hit	$2^{-\Delta\Delta Ct}$ York	SE	$2^{-\Delta\Delta Ct}$ A100-32	SE	NRC FC
Defense-related genes of interest in peanut						
03022	PR-4A	31.41	34.50	1.00	9.56	10.34
06098	PR10	7.50	11.97	1.00	12.74	7.54
01183	thaumatin-like	11.84	29.48	1.00	2.47	5.28
02763	class IV chitinase	8.00	13.02	1.00	18.07	2.69
00281	pgip2	41.02	27.88	1.00	7.53	5.06
05792	GST	8.26	17.13	1.00	13.34	P/A
05451	resveratrol synthase	1.49	2.87	1.00	29.97	P/A
Virulence-related genes of interest in *S. rolfsii*						
00332	glucoamylase	1.61	5.89	1.00	1.62	14.08
00240	EndoPG 1 precursor	5.58	13.05	1.00	8.45	4.40
04719	oxaloacetate acetylhydrolase	2.34	3.91	1.00	6.64	P/A
00427	EndoPG 2 precursor	1.99	1.55	1.00	10.08	P/A
Genes of interest considered to be unknown in peanut						
02782	grp1	7.47	9.69	1.00	3.21	P/A
03219	gene for Piwi homologue	0.92	1.98	1.00	2.34	P/A
Genes of interest considered to be unknown in *S. rolfsii*						
05264	expressed protein	0.76	0.58	1.00	2.05	P/A
05790	glycoside hydrolase family 7	6.02	18.28	1.00	2.66	P/A

Figure 1. Agar plugs used for inoculation. Germinated mature sclerotia on 2PDA agar plugs used for peanut inoculation are shown with one agar plug removed. Sclerotia were germinated on 2PDA plates for 48 hr and 1cm plugs with a germinated sclerotia in the center of the plug were removed with a cork borer (1cm diameter) and used for inoculation. Mycelial plugs were removed from these plates and used for inoculation.

Figure 2. Inoculation of crown using agar plugs. Inoculation of peanut 50 days after planting with mycelial plugs of *Sclerotium rolfsii*. Peanut plants were inoculated with one agar plug with one germinated sclerotia in the center of the plug and two mycelial plugs. The plugs were pressed onto the crown of the peanut plants.

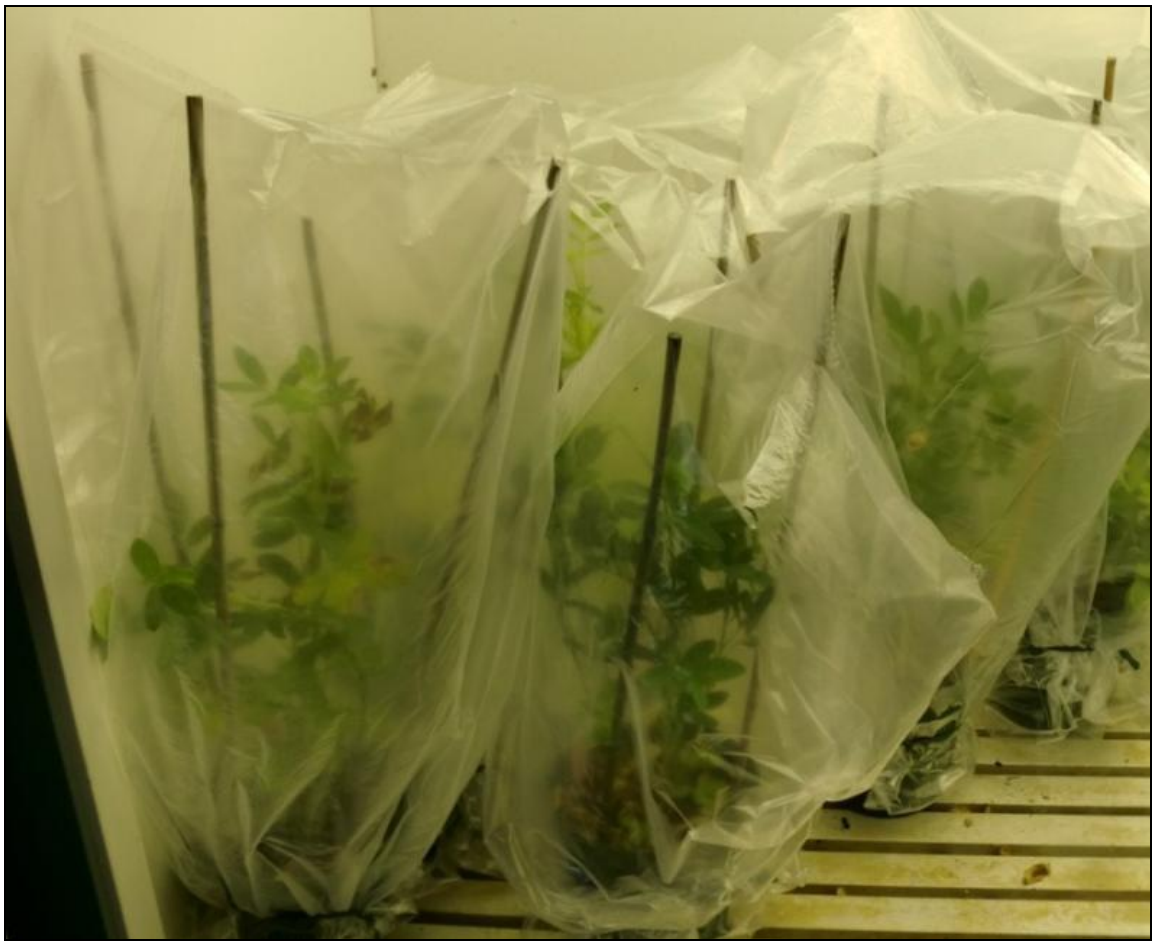


Figure 3. Plastic bags on plants used to generate high humidity conditions. Freshly inoculated peanut plants in the growth chamber were covered with plastic bags to increase humidity and promote disease establishment. Bamboo stakes were used to support bags. Bags were removed prior to tissue collection.

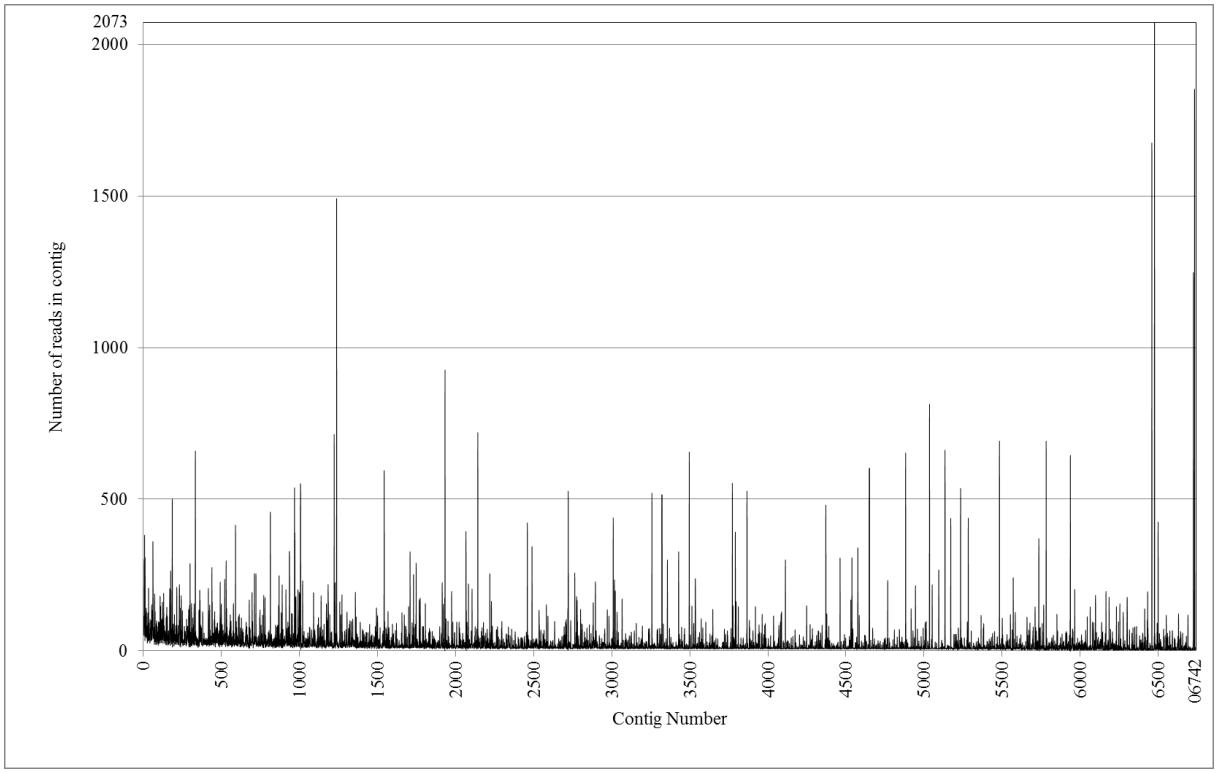


Figure 4. Number of reads in each contig for the contigs assembled using Roche GS *De Novo* Assembler. There was an average of 28.82 reads comprising each contig. The minimum and maximum number of reads per contig were 1 and 2073, respectively. Contig06476 contained 2073 reads; this contig had no significant similarity from blastn and blastx searches. This contig was predicted to be differentially expressed using both the Audic-Claveire and Olman statistical method

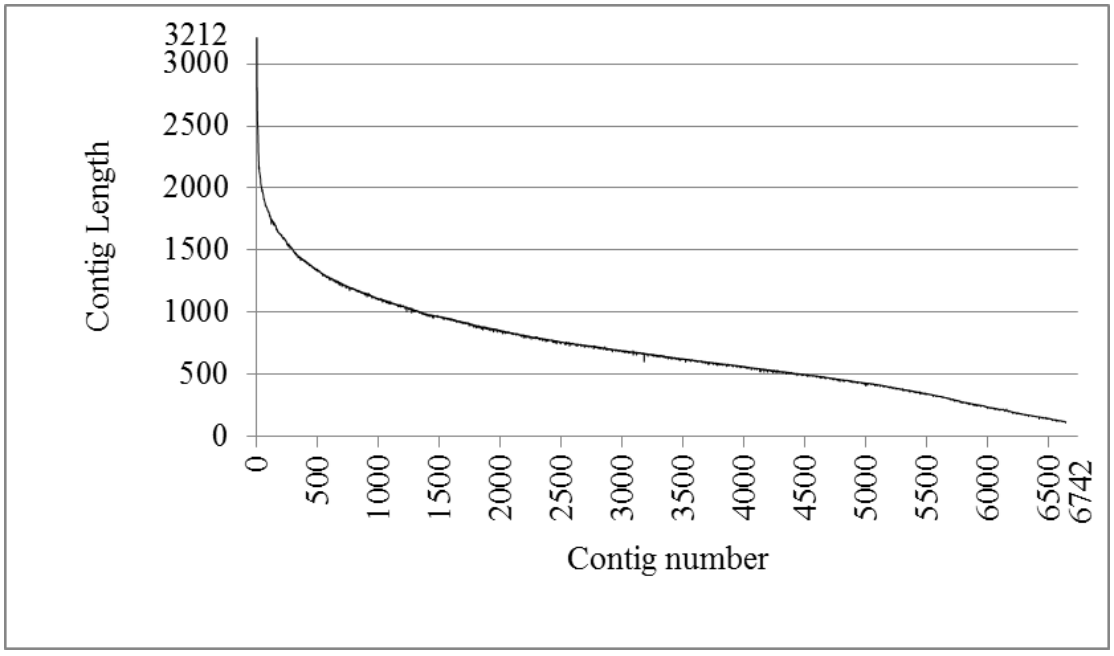


Figure 5. Length of the contigs assembled using Roche GS *De Novo* Assembler. Contigs below 100 bp were removed. The average contig size was 898 bp and the range of lengths was 100 to 3212 bp.

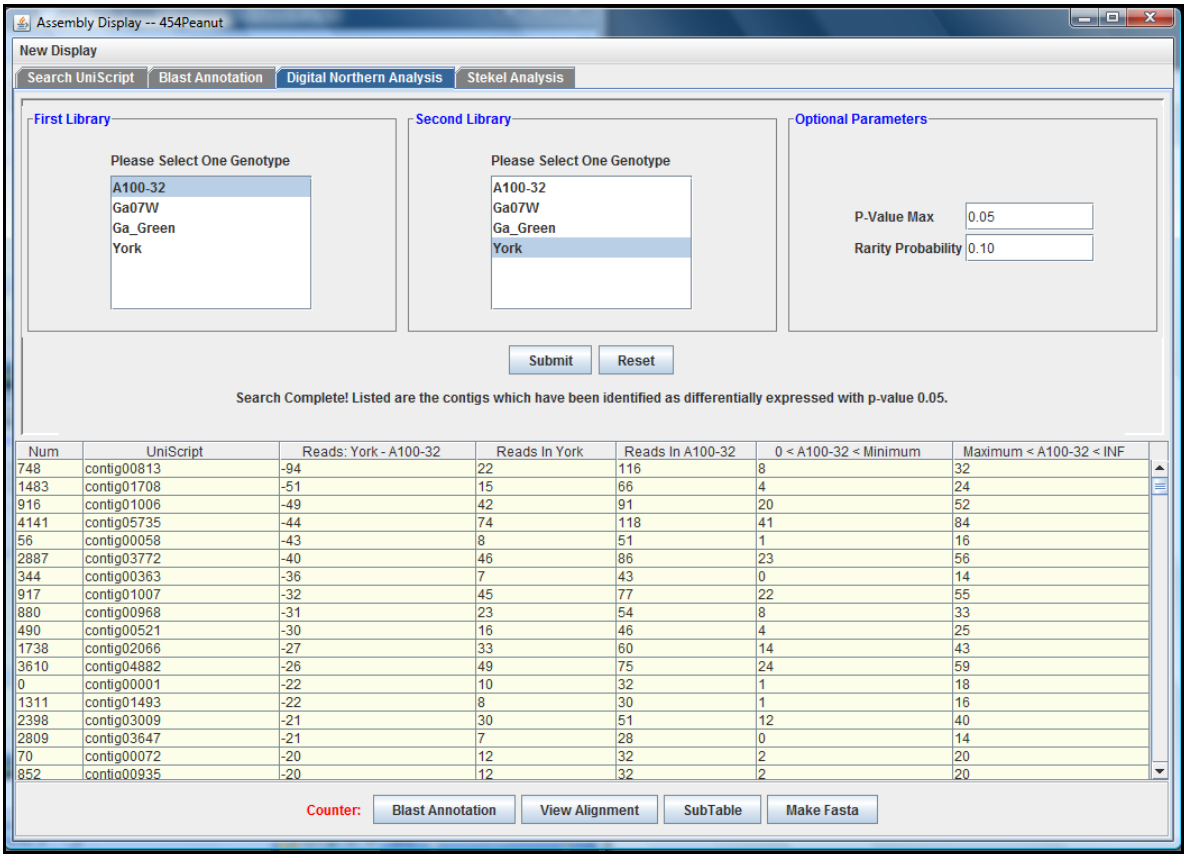


Figure 6. Display of the NG-Magic program output which incorporated the Audic-Claverie statistical method. The A100-32 cultivar was set as the First Library and the York cultivar as the Second Library. The p-value max was designated as 0.05 and the rarity probability as 0.10. The data shows the raw unnormalized read counts. There were 274 contigs assessed to be differentially expressed in the comparison between A100-32 and York.

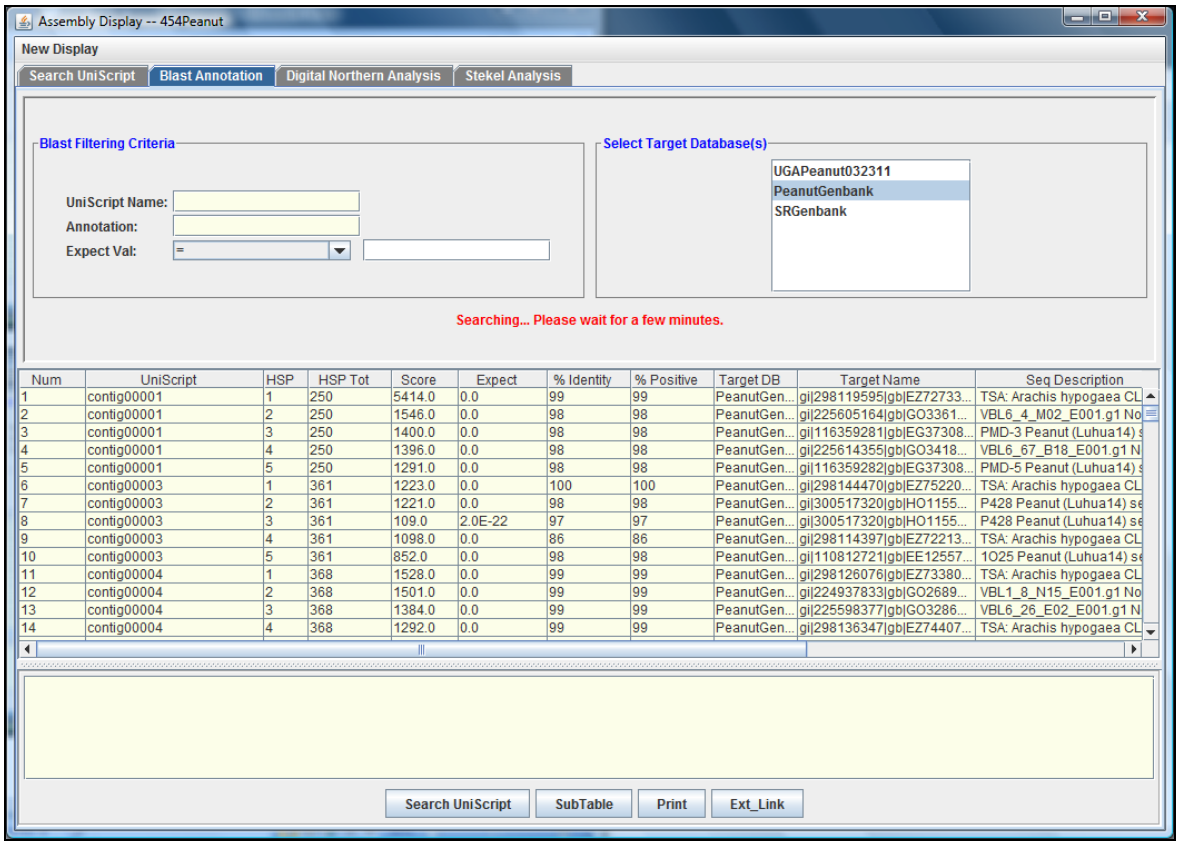
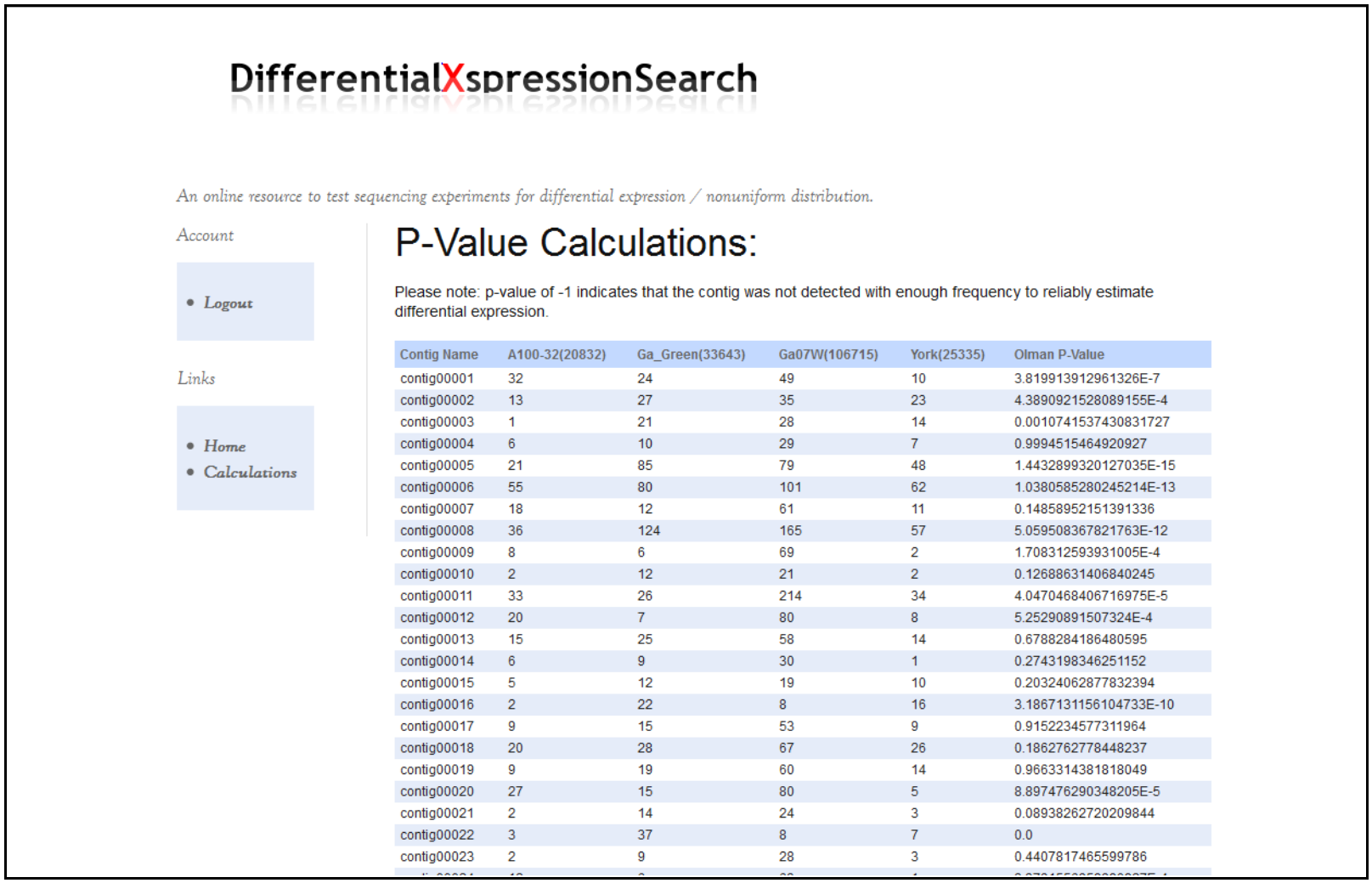


Figure 7. NG-Magic BLAST search output feature. This feature identifies the species of origin of the contig (peanut, *S. rolfsii* or other) and putative gene function. Files downloaded from NCBI containing the nucleotide and EST Blast information for peanut and the EST Blast information for *Sclerotium rolfsii* were loaded into the NG-Magic program. A Blast annotation of the contigs was performed within the program.



Contig Name	A100-32(20832)	Ga_Green(33643)	Ga07W(106715)	York(25335)	Olman P-Value
contig00001	32	24	49	10	3.819913912961326E-7
contig00002	13	27	35	23	4.3890921528089155E-4
contig00003	1	21	28	14	0.0010741537430831727
contig00004	6	10	29	7	0.9994515464920927
contig00005	21	85	79	48	1.4432899320127035E-15
contig00006	55	80	101	62	1.0380585280245214E-13
contig00007	18	12	61	11	0.14858952151391336
contig00008	36	124	165	57	5.059508367821763E-12
contig00009	8	6	69	2	1.708312593931005E-4
contig00010	2	12	21	2	0.12688631406840245
contig00011	33	26	214	34	4.0470468406716975E-5
contig00012	20	7	80	8	5.25290891507324E-4
contig00013	15	25	58	14	0.6788284186480595
contig00014	6	9	30	1	0.2743198346251152
contig00015	5	12	19	10	0.20324062877832394
contig00016	2	22	8	16	3.1867131156104733E-10
contig00017	9	15	53	9	0.9152234577311964
contig00018	20	28	67	26	0.1862762778448237
contig00019	9	19	60	14	0.9663314381818049
contig00020	27	15	80	5	8.897476290348205E-5
contig00021	2	14	24	3	0.08938262720209844
contig00022	3	37	8	7	0.0
contig00023	2	9	28	3	0.4407817465599786

Figure 8. Screenshot of the NG-Magic Web Stat. This website was used for performing the Olman test-statistic. The final column shows the Olman P-value.

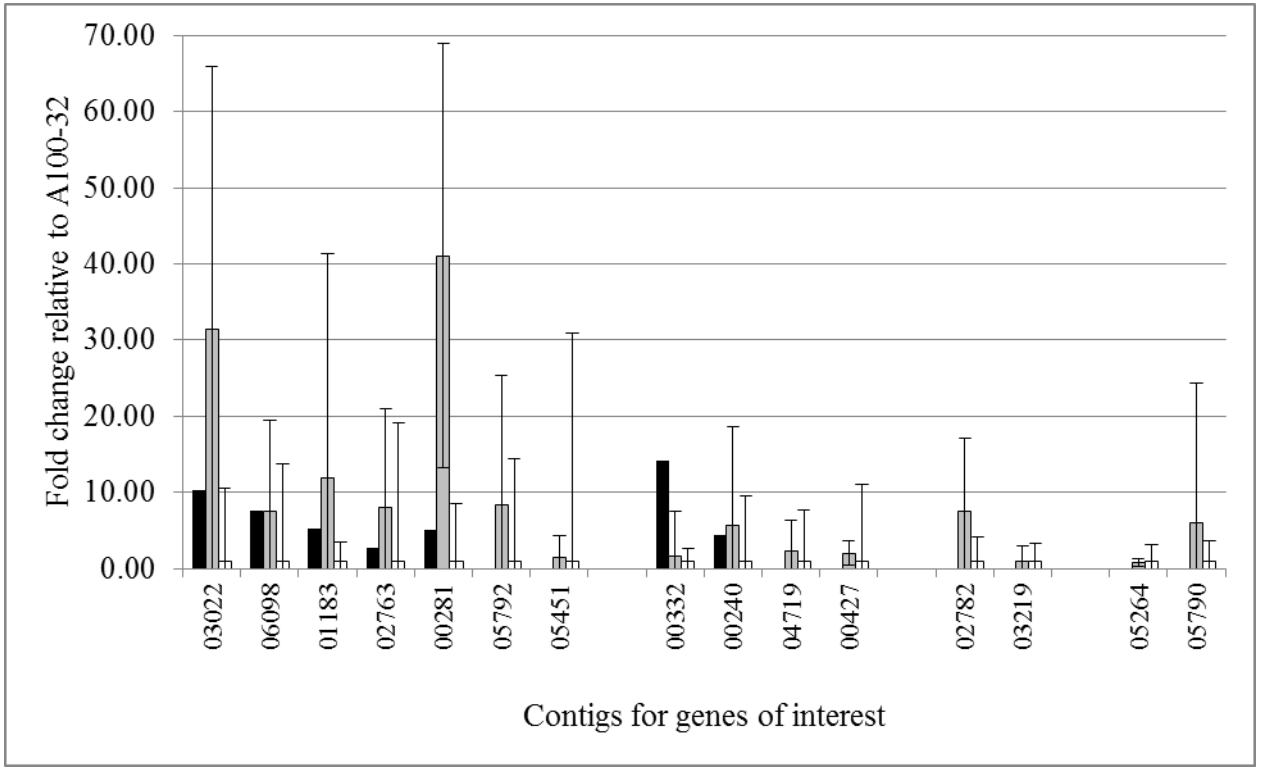


Figure 9. Fold changes of the genes of interest relative to A100-32 using the Livak and Schmittgen normalization method. Standard error bars are shown. The black bars represent the fold changes calculated using the normalized read counts from the 454 data. The grey bars represent the fold change of York relative to A100-32 from real-time PCR data. The white bars represent the fold change of A100-32 normalized to A100-32 for real-time PCR.

CHAPTER 4

CONCLUSIONS

In this thesis I report the findings of my studies on the identification of genes differentially expressed during the early interaction between the stem rot fungus, *S. rolfsii* and peanut. A 454 sequencing experiment was performed on four cultivars of varying resistance. This sequencing generated 225,793 high quality sequences that were assembled into 6742 contigs. The first objective focused on the two peanut cultivars with the extreme variation in resistance levels, A100-32 and York and employed the Audic-Claverie statistical method. Twelve genes of interest, assessed to be differentially expressed and with significant fold changes from the 454 data, were selected for verification using real-time PCR. Although the fold changes from real-time PCR differed from the fold changes calculated using the 454 data, all of the genes selected showed a significant fold-change from real-time PCR. The second objective expanded the focus to all four peanut cultivars and employed the Olman statistical method. Three additional genes, assessed to be differentially expressed and with significant fold changes from the 454 data, were selected for verification using real-time PCR. The contigs assessed to be differentially expressed were also analyzed for trends in gene expression. There were 12 contigs out of 1747contigs assessed to be differentially expressed that showed a pattern of increasing gene expression, with the York cultivar showing the highest gene expression and the A100-32 cultivar showing the lowest gene expression. There were 27 contigs assessed to be differentially expressed that showed a pattern of decreasing gene expression, with the A100-32 cultivar showing the highest gene expression and the York cultivar showing the lowest gene

expression. Statistical analysis of the real-time PCR data revealed that there was too much variation between the biological replicates to reliably conclude that the majority of the genes of interest were significantly differentially expressed. Further experimentation on more biological replicates may reveal outliers in the biological replicates used in this study to perform real-time PCR. Further studies involving characterization of genes that are considered unknown would be useful in assessing the importance of these genes in the initial host-pathogen interaction.