REPORT TO THE NORTH AMERICAN STRAWBERRY GROWERS ASSOCIATION

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PROJECT TITLE: Investigating Defense Responses in Strawberry Genotypes to Colletotrichum gloeosporioides and C. acutatum

Investigators: Tika Adhikari and Frank J. Louws, Center for IPM and Dept. of Plant Pathology, North Carolina State University, Raleigh NC 27695 E-mail:tika_adhikari@ncsu.edu and frank_louws@ncsu.edu

ABSTRACT

Anthracnose fruit rot (AFR) and crown rot (ACR) of strawberry caused by Colletotrichum acutatum (Ca) and C. gloeosporioides (Cg), are devastating diseases on strawberry (Fragaria × ananassa) in the United States. The current understanding of the molecular mechanisms of resistance or susceptibility to the foliar hemibiotrophic infections (HBI) underlying both strawberry defense and fungal attack are not yet fully investigated. We conducted greenhouse experiments to compare genome-wide transcriptional profiles in ‘NCS 10-147’ and commercial variety ‘Chandler’ resistant and susceptible, respectively, to both Ca and Cg. Leaf samples were collected at five time-points (0, 24, 48, and 7 and 14 days after inoculation) and total RNA was extracted and analyzed using IlluminaTruSeq™ at GSL, NCSU, NC. Single end reads for a length of 100 bp were sequenced. Only reads greater than 60 bp were used for analysis. Comparisons were made for each of the cultivars at each time point between the untreated and the pathogen infected samples (Ca and Cg independently). After quality cleaning, 7,763,123,604 reads were detected. Although the expression patterns of the different transcripts were different for each treatment, no samples clustered as a single group, indicating that there is a presence of differential expression among samples. Within each cultivar, the number of genes up-regulated when infected by a pathogen increases up to 48 hrs and then the expression drastically reduces compared to the untreated controls. In the resistant cultivar, there were no significantly differentiated genes except 24 hrs and 48 hrs after inoculation. But in case of the susceptible cultivar differentially expressed genes were present from 7 days after inoculation. As expected, we show a large set of genes that are differentially expressed between the resistant and susceptible cultivars at all time points. In both pathogen infections, the number of genes up-regulated in the resistant line were more than in the susceptible cultivar. This study provides insight into molecular mechanisms of understanding how these two HB fungi infect strawberry and provide a basis to understand and potentially manage these mechanisms for enhanced resistance development in future breeding programs.

INTRODUCTION

Anthracnose fruit rot (AFR) and crown rot (ACR) of strawberry, caused by Colletotrichum acutatum (Ca) and C. gloeosporioides (Cg), are major diseases in strawberry production regions in the southeastern United States. Breeding strawberry for resistance to anthracnose is considered as one of the primary means for managing this disease. Genetic resistance to anthracnose is known but not deployed widely and specific to either pathogen, but not both. We are pursuing the concept of “rate-reducing resistance” or “partial resistance” and seek to understand more about the ecology
and biology of these pathogens (Rahman et al. 2013; 2015). “Rate-reducing resistance” is often governed by several minor genes, but each having additive effects. Recently, we estimated the genetic parameters of crown rot resistance to \( Cg \) using a partial diallel mating design derived from strawberry genotypes from a diverse origin which included NC, FL, CA and OR selections (Jacobs et al, PhD thesis).

Hemibiotrophic (HB) pathogens establish a biotrophic interaction with their hosts at early stages but switch to necrotrophic life style at later infection phases. Thus, hemibiotrophic infections (HBIs) due to \( Ca \) and \( Cg \) are of practical significance in strawberry production. If we can stop or slow down the initial colonization and multiplication of the pathogens, especially on green leaves, then we can substantially reduce risk associated with these pathogens. The pathogens can multiply without showing any typical symptoms during the vegetative stages of growth in the nurseries and can cause heavy damage in fruiting fields. Our main goal is to advance our understanding of host - pathogen interactions particularly host genetic variation in response to active phases of disease and deploy rate-reducing resistance in strawberry breeding programs. The main objective of this project was to determine if strawberry genotypes that confer resistance to HBIs by one pathogen confer equal resistance against the other.

**MATERIALS AND METHODS**

In the previous study funded by NASGA, we identified the selection ‘NCS 10-147’ and commercial variety ‘Chandler’ that exhibited resistant and susceptible responses to both \( Ca \) and \( Cg \). Tips were planted in 50-cell plug trays containing Fafard® 3B potting mix and were rooted under periodic mist (30 second duration, 10 minute interval) in a 21 ± 5°C greenhouse with a 12 h light/dark photoperiod for one week until roots had developed. Plants received overhead supplemental lighting (fluorescent, 11000 lux) for 3 weeks prior to inoculation to increase day length to 14 h and encourage vegetative growth. Three isolates each of \( Ca \) (Ca 34, Ca 40, and Ca 89) and \( Cg \) (Cg 28, Cg 58 and Cg 84) with known pathogenicity to represent natural pathogen diversity found in strawberry plantings in North Carolina were selected and revived on Difco™ potato dextrose agar (PDA, BD Diagnostic Systems, Sparks, MD) plates. The plates were incubated at 25°C for 10-14 days under 12 h fluorescent light. Conidia were harvested by flooding the mycelium with distilled water containing 5 drops of Tween 20 per liter of water and disturbing the mycelium with a glass stirring rod to suspend conidia. Conidial suspension of \( Ca \) and \( Cg \) passed through a doubled layer of cheesecloth to remove cellular debris and measured with a hemacytometer to confirm a final concentration of \( 1.0 \times 10^6 \) conidia/ml, then combining equal volumes of the three isolates of each species. Fungal suspensions were applied to strawberry foliage via handheld sprayer (Solo® model 419) until runoff. Plants inoculated with distilled water served as controls. Inoculation was followed by a 48 h incubation period of intermittent mist (3 second duration, 5 minute interval) and temperatures raised to 25°C to promote pathogen colonization of tissue. After 48 h misting was terminated and temperatures held at 25 ± 1°C for the remainder of the experiment. A total of 45 clonal plants of each genotype were arranged randomly in split split-plot design where fungal pathogen assigned to whole-plots and strawberry genotypes assigned to split-plots. Three leaves were sampled from each plot on each of five time-points: 0, 24, 48, 168 (7 days), and 336 h (14 days) after inoculation. Collected samples were immediately placed on ice and kept -80°C until further analysis. Briefly, mRNA isolation, fragmentation, and priming was performed with the NEBNext Next Poly(A) mRNA Magnetic Isolation Module(NEB #E7490). Libraries were quantified using the Agilent Bioanalyzer High-
Sensitivity chip and then an equimolar pool of 9 samples was made for each lane going on the Hiseq. Sequencing of the cDNA libraries was conducted using IlluminaTruSeq™ sequencing protocols at GSL, NCSU, NC.

**RNA-SEQ DATA ANALYSIS**

Single end reads for a length of 100 bp were sequenced and RNA-Seq reads were quality filtered. Only reads greater than 60 bp were used for further analysis. For each individual sample, we used Velvet assembly with different k-mer sizes. Then transcripts from different samples were merged together using CD-HIT-EST with an identity % of 95. Then a length filter of minimum 200 bp was used to remove the reads that are not assembled into contigs. These contigs were further blasted using USEARCH to a protein database consisting of protein sequences of *Arabidopsis*, *Fragaria vesca* and *Prunus*. Based on the Blast output, if multiple transcripts have the same hit, they were further merged using Blast2CAP3 (Buffalo) (Davidson et al. 2014)). Corset was used to estimate the read count by combining transcripts that have the same reads mapped on them. The principal component analysis (PCA) was further estimated for the 30 treatments using the variance stabilization transformation for the read count data. In addition a Euclidian distance was estimated for the samples and a UPGMA and heatmap were generated using the pheatmaps library in R (Kolde 2013). Comparisons were made for each of the cultivars, at each time point between the untreated and the pathogen infected samples (*Ca* and *Cg* independently). In addition the differential effect of pathogens was determined using a comparison of samples affected with each of the pathogens.

**RESULTS**

Approximately, 7,857,727,784 reads over all the 90 libraries were used. After quality cleaning, 7,763,123,604 reads were used for further analysis. After the first stages of assemblies in Velvet followed by CD-HIT, 1.38 Gbp assembled into 11,588,782 sequences with an N50 of 135bp. Further steps using blast2cap3 and length of 200 bp as minimum resulted in 502,307 contigs. The expression patterns of the different transcripts were different for each of the treatments (Fig. 1). Samples did not cluster as a single group indicating the presence of differential expression among the samples. The samples were clustered into different groups. As expected, the 0 hr treatments merged together, so did the 7 d and 14 d samples. The distance between the samples is observed (Fig. 2). Within each cultivar, the number of genes up-regulated when infected by a pathogen increases up to 48 hrs and then the expression drastically reduced compared to the untreated. Also, in the resistant cultivar, there are no significantly differentiated genes except 24 hrs and 48 hrs after infection. But in the case of the susceptible cultivar differentially expressed genes were present from 7 days. As expected there is a large set of genes that are differentially expressed between the resistant and susceptible cultivars at all time points. In both pathogen infections, the number of genes up-regulated in the resistant line were more than in the susceptible cultivar (Table 1). This data is highly preliminary and further analysis work will be conducted.
Fig. 1. Principal component analysis (PCA) of the 30 samples under study grouped based on sample and treatment.

Fig 2: Heatmap of the Euclidean distance between the 30 samples.
Table 1. Differential genes expression in the resistant line ‘NCS 10-147’ (NCS) and susceptible cultivar ‘Chandler’ (Chan) inoculated with Ca and Cg with samples taken at five time-points.

<table>
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<th></th>
<th>0hr</th>
<th>24hr</th>
<th>48hr</th>
<th>7d</th>
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**Note:** Further RNA-data analysis is in progress.

**LITERATURE CITED**