FINAL REPORT ON

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PROJECT TITLE: EVALUATION OF RATE-REDUCING RESISTANCE AND DEFENSE RESPONSES IN STRAWBERRY GENOTYPES TO *Colletotrichum gloeosporioides* and *C. acutatum.*

NAME, MAILING AND EMAIL ADDRESS OF PRINCIPAL INVESTIGATOR(S): Tika Adhikari[1] and Frank J. Louws, [1]Department of Entomology and Plant Pathology and [2]Department of Horticultural Science, Campus Box 7609; Shipping: 2721 Founders Dr; 118 Kilgore Hall, NC State University, Raleigh, NC, 27695-7609. E-mail: tbadhika@ncsu.edu and frank_louws@ncsu.edu

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PUBLIC ABSTRACT

Anthracnose fruit rot (AFR) and crown rot (ACR) caused by *Colletotrichum acutatum* (Ca) and *C. gloeosporioides* (Cg), respectively, reduce strawberry yield significantly. Although a few quantitative trait loci (QTL) have been identified, the current understanding of the molecular mechanisms of resistance to Ca and Cg hemibiotrophic infection (HBI) in strawberries is not yet fully investigated. In the absence of complete resistance, strawberry varieties with good fruit quality showing quantitative or partial resistance (i. e., rate-reducing resistance) have frequently been used as a source of resistance to Ca and Cg in strawberry. We used RNA-seq analysis to examine how partially resistant cv. NCS 10.147 and susceptible cv. Chandler strawberry responds following infection by Ca and Cg and to identify molecular events associated with HBI (0 to 48 h post-inoculation [hpi]) development. We found defense responsive genes such as chitinase, peroxidase, glutathione transferase, and leucine-rich repeat (LRR)-receptor-like kinases (RLKs), and transcription factors such as MYB, and NAC, and WRKY are strongly upregulated in leaf tissues of partially resistant NCS 10.147 infected with Ca and Cg, indicating the activation of plant defense mechanisms. Further investigations of key components of plant defense signaling during Ca/Cg HBI and strawberry interactions will help for utilizing these candidate genes in breeding programs and for improving crop resilience to both pathogens.

INTRODUCTION

Colletotrichum acutatum (Ca) and *C. gloeosporioides* (Cg) cause anthracnose fruit rot (AFR) and crown rot (ACR) are considered destructive pathogens of strawberry worldwide including North Carolina (2,6,10,11,12). Planting of resistant varieties is the most cost-effective and environmentally friendly strategy to mitigate disease epidemics. One unique aspect of these pathogens can cause latent infection (symptomless colonization) during which the pathogens interact with the strawberry as a 'hemibiotroph' and can quantify using qPCR (3). Hemibiotrophic pathogens establish a biotrophic interaction with their hosts at an early stage but switch to necrotrophic lifestyle at later infection phases by the successful secretion of effectors into the plant to repress and manipulate host defense and physiology. Both appeared to Ca and Cg pathogens have a hemibiotrophic infection (HBI) strategy so that these pathogens initially colonize host tissues with intimate host contact in a biotrophic phase followed by a necrotrophic phase associated with symptoms (2,10). This unique aspect of these pathogens can cause long latent infections (quiescent) during which the pathogens interact with the strawberry as a 'hemibiotroph'. These pathogens can multiply in a "hidden way" without showing symptoms during the vegetative stages of growth in the nurseries and/ can disease epidemics in fruiting fields. If we can stop or slow down the initial colonization and multiplication of the pathogens, especially on green leaves, then we can substantially reduce the risk associated with these pathogens. The high level of inoculum buildup during the vegetative stage of production has raised two questions: (i) effective management of the HBI phase in leaves and management of early cycles of sporulation limit the epidemic and subsequent anthracnose incidence in plants and on fruit? (ii) can we select resistant strawberry varieties that reduce pathogen multiplication on vegetative tissues? More specifically, there is a lack of understanding on the genetic basis of host resistance to HBI, what degree of host resistance to HBI by Ca and Cg is correlated, and how host genes respond to HBI. In epidemiological terms, *host resistance* can be considered "rate-reducing" when resistance is low to moderate, the pathogen colonizes the host tissues; however, plants are not severely affected and at the same time the rate of disease increase (epidemic development) is reduced. "Rate-reducing resistance" is often governed by several minor genes, but each having additive effects (4,5). Therefore, such resistance usually remains 'durable'. However, our understanding of host - Ca/Cg HBI interactions particularly host responses to biphasic infections (from biotrophic to hemibiotrophic phase) and molecular events underlying rate-reducing resistance remain largely unknown.

RESEARCH OBJECTIVES: The main objectives of this study were to identify differentially expressed genes (DEGs) using RAN-seq that are biologically relevant for the establishment of HBI in strawberry and provide

targets or candidate genes to further investigation of genetic and biochemical factors involved in rate-reducing or quantitative resistance to Ca and Cg in strawberry.

MATERIALS AND METHODS

To investigate strawberry genes induced or suppressed from biotrophic to hemibiotrophic phase, the interactions between strawberry and Ca and Cg were investigated in time-course experiments. To define these pathogenicity phases, 0 h to 48 h after inoculation corresponded to the biotrophic stage and hemibiotrophic stage of infection. The resistant line 'NCS 10-147' line and the susceptible cultivar 'Chandler' were spray-inoculated (7) and tri-foliate leaves were sampled from each treatment at 0, 24, and 48 hours after inoculation (hai). Total RNA was extracted from strawberry leaf tissues using the Qiagen RNeasy Plant Mini kit according to the manufacturer's instructions. RNA was eluted in RNase-free water and checked for integrity and quantity using a NanoDrop1000 spectrophotometer (Thermo Scientific, DE, USA) and on an Agilent 2100 Bioanalyzer using Agilent RNA 6000 Nano Kit according to manufacturer's instructions (Agilent Technologies, Santa Clara, CA, USA). RNA extracted from leaf samples of three independent biological replicates were pooled for each sample per time point. The cDNA libraries were constructed by using a NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA). In brief, mRNA isolation, fragmentation, and priming were performed with the Next Poly(A) mRNA Magnetic Isolation Module (NEB #E7490). Libraries were quantified using the Agilent Bioanalyzer High-Sensitivity chip. Paired-end (2 × 100 bp) sequencing of the cDNA libraries was conducted using the HiSeq™ 2500 platform (Illumina). Sequencing was performed at Genomic Science Laboratory, North Carolina State University. All RNA-seq data were analyzed as described previously (Adhikari et al. 2012). Single-end reads for a length of 100 bp were sequenced. RNA-Seq reads were quality filtered using sickle a windowed adaptive trimming tool. The transcripts from different samples were merged using CD-HIT-EST with an identity % of 95. The transcripts that are differentially expressed were estimated using the Deseq2 package. To estimate differentially expressed genes (DEGs, each cultivar at each time point inoculated Ca and Cg independently as compared with mock (water-inoculated) at 0, 24, and 48 h post-inoculation (hpi). The significance was determined using an FDR < 0.05 and the absolute value of log2FC is greater than 1.

RESULTS

The transcriptional response to Ca and Cg infection on strawberry cultivars prompted us to identify whether biological processes, molecular functions, and cellular components are enriched in the up and down-regulated DEGs at specific time points based on their gene ontology (GO) annotation. Our goal was to examine which

genes were induced or repressed in strawberry cultivars by these pathogens transition from surface to hemibiotrophic colonization (i. e., from 0 to 48 hpi). Analyses of strawberry transcripts in the resistant cultivar inoculated with Ca and Cg compared to mock revealed three GO-terms: biological process, molecular function, and cellular component at to 0, 24 and 48 hpi. Transcript data analysis of enriched biological processes revealed five major groups were primary metabolic process, organic substance metabolic process, cellular metabolic process, nitrogen compound metabolic process, and oxidation-reduction process were upregulated at most time points (Fig. 1A). The second group: molecular function contained heterocyclic compound binding, organic cyclic compound binding, iron-binding, oxidoreductase activity, and intracellular organelle genes were upregulated in the resistant cultivar by both Ca and Cg at 0, 24, and 48 hpi (Fig. 1B). The third group**:** Go-term cellular component consisted of the membrane-bounded organelle, intracellular organelle, intracellular part, and intrinsic component of membrane genes are activated in cellular compartmentations and are indicative of rapid and continued expression to Ca- and Cg-HBIs at 48 hai (Fig. 1C).

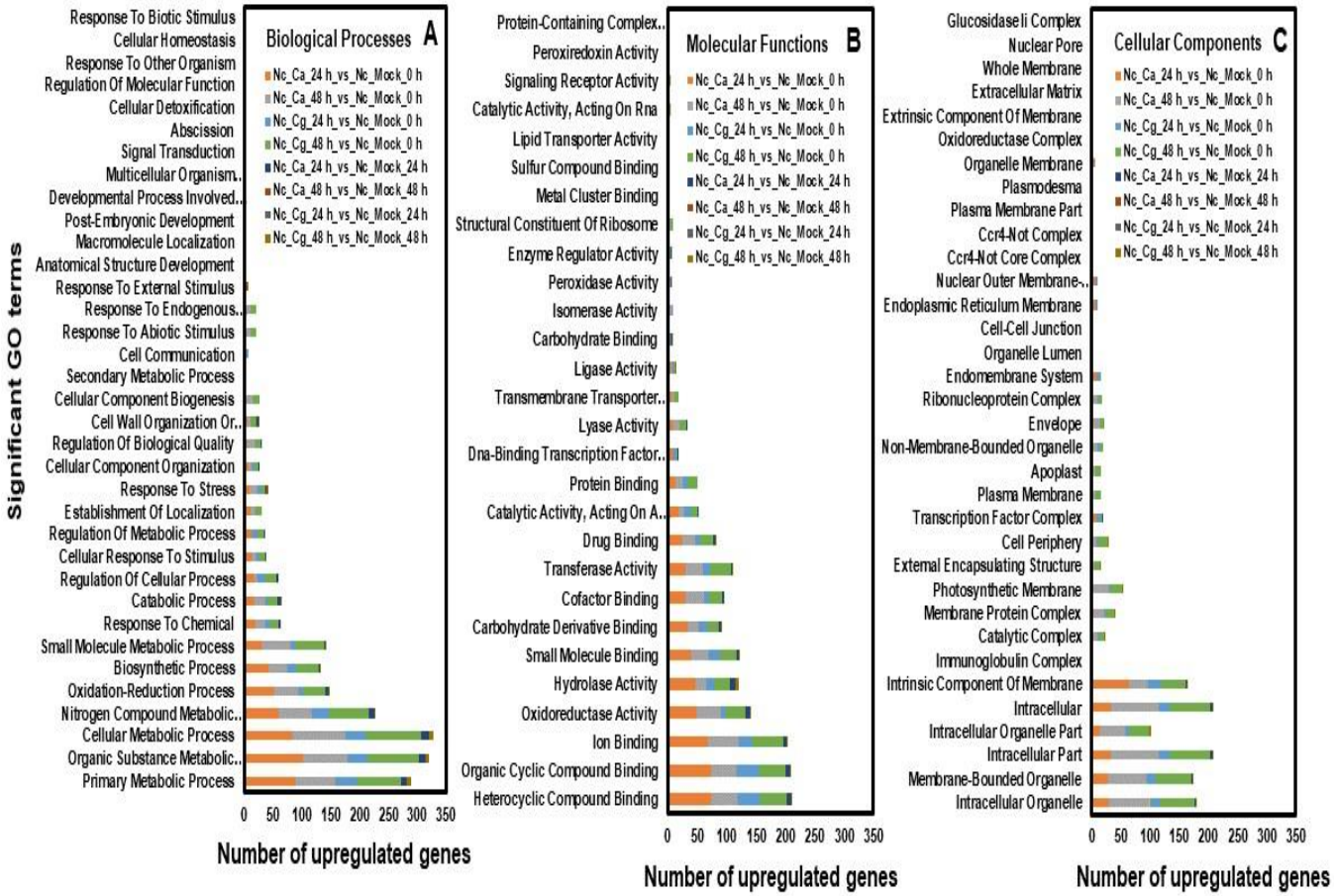

Figure 1. Gene Ontology (GO) categories overrepresented (corrected *P* value < 0.05) in the set of upregulated genes in the resistant cultivar by Ca and Cg at each time point compared to mock.

We also used GO enrichment analyses to understand what genes related to biological processes, molecular functions, and cellular components affected within the leaves of the resistant cultivar. The biological processes related to five genes are organic substance metabolic process, primary metabolic process, cellular metabolic process, nitrogen compound metabolic process, and responses to stress genes were severely reduced after entry of both pathogens at 0, 24, and hpi (Fig. 2D). A significant number of genes under the GO terms associated with molecular functions were downregulated by both pathogens were ion binding, hydrolase activity, heterocyclic compound binding, organic cyclic compound binding, and small molecule binding (more

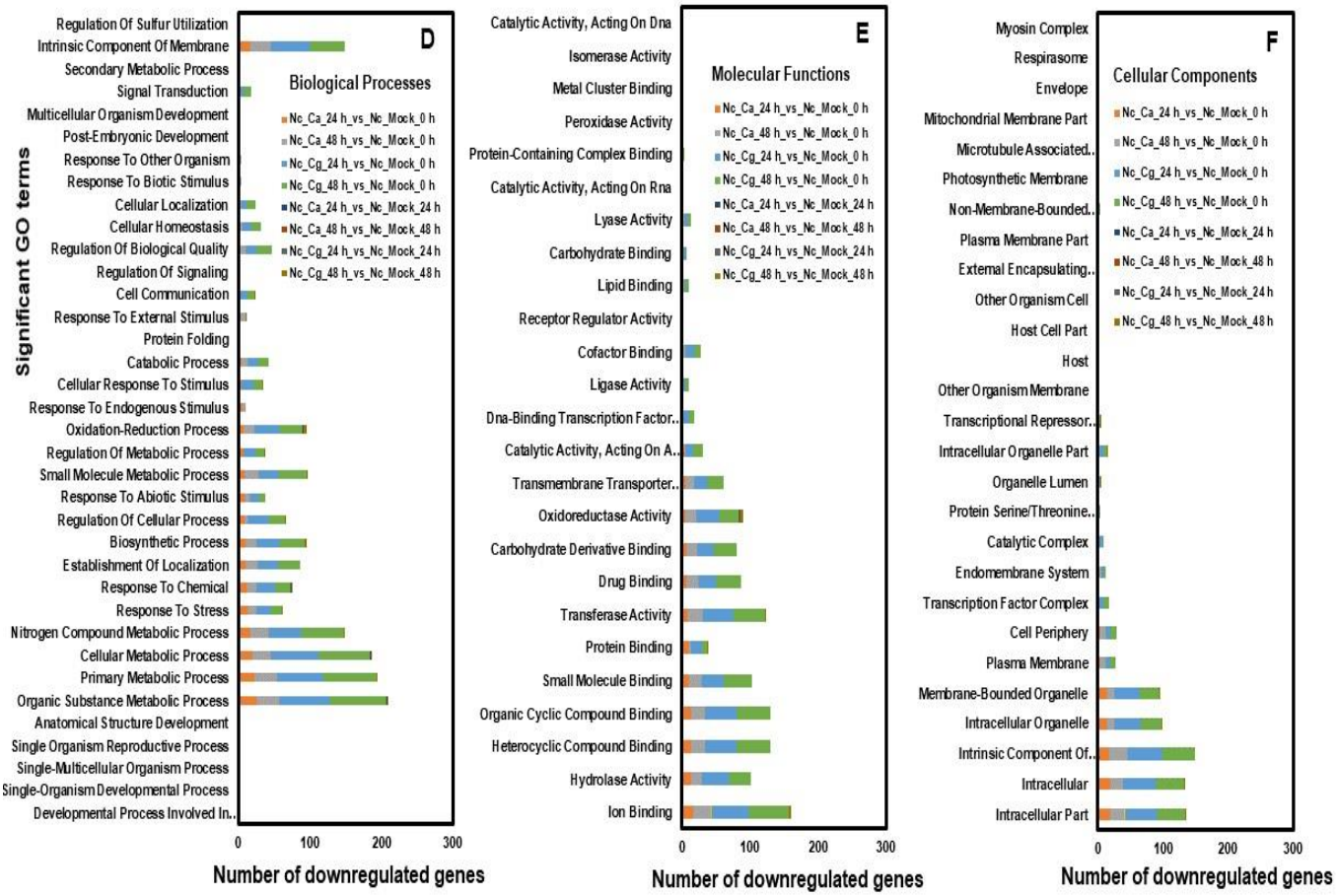

Figure 2. Gene Ontology (GO) categories overrepresented (corrected *P* value < 0.05) in the set of downregulated genes in the resistant cultivar by Ca and Cg at each time point compared to mock.

genes were repressed by Ca than Cg) at each time point (Fig. 2E). Other apparent top five suppressed genes in the cellular components were an intracellular part, intracellular, intrinsic component of membrane, intracellular organelle, and membrane-bound organelle (Fig. 2F). More genes were suppressed by Ca at each time point than by Cg.

We further examined genes that activated (upregulated) or repressed (downregulated) at each time point either Ca or Cg. Some representative upregulated genes are defense responsive (e. g., chitinase, peroxidase), immune receptors or proteins and signal transduction (e. g., mitogen-activated protein kinase kinase, protein kinase, LRR receptor-like serine/threonine-protein kinase) and transcription factors (e. g., NAC, MADS, MYB, WRKY) were activated by Ca and Cg at 24 and 48 hpi (Table 1).

Table 1. Some selected representative defense responsive genes, immune receptors, and transcription factors significantly activated in the resistant cultivar NCS 10.147 during briotrophic and hemibiotrophic development of *Colletotrichum acutatum* (Ca) and *C. gloeosporioides* (Cg). Threshold for differential expression is log2 fold change >1, false discovery rate < 0.05.

Sequence ID	**Gene annotation**	**24 hpi**		**48 hpi**
StrawberryCLC_DN25_c1204_g1204	Chitinase	1.98	1.4	
StrawberryCLC_DN25_c12430_g12430	Chitinase IV			1.93
StrawberryCLC_DN25_c7218_g7218	Chitinase IV	1.73		
StrawberryCLC_DN25_c39268_g39268	Peroxidase	2.99		
StrawberryCLC_DN25_c29676_g29676	Peroxidase	2.52		
StrawberryCLC_DN25_c53035_g53035	Peroxidase 15	2.2		
StrawberryCLC_DN25_c8143_g8143	NAC protein 2	1.5		
StrawberryCLC_DN25_c464_g464	MADS-box protein SOC1	1.47		1.91
StrawberryCLC_DN25_c26676_g26676	MYB-related protein 108	2.5		
StrawberryCLC_DN25_c22396_g22396	WRKY transcription factor 55	2.15		
strawberryCLC_DN25_c2599_g2599	Mitogen-activated protein kinase kinase NPK1	1.94		
strawberryCLC_DN25_c8876_g8876	Protein kinase	1.89		
strawberryCLC_DN25_c12968_g12968	LRR receptor-like serine/threonine-protein kinase			1.7

IMPACT

We profiled plant genes during strawberry - HBI interactions to better understand which genes are associated with rate-reducing or quantitative resistance to Ca and Cg. This study will have a dramatic impact on both major *Colletotrichum* problems in the nursery setting and the fruiting field. In previous work, we documented

that HBI resistance in various genotypes substantially reduced the build-up of Ca in the fruiting fields (12). A major portion of this reduced disease level was because the pathogen did not build-up on the green foliage from the time of planting to fruiting (a form of what is often called rate-reducing resistance). Given the distinct differences in genes expression caused by Ca and Cg in this study, we identified some defense genes (e. g., chitinase, peroxidase), immune receptor genes encoding LRR, protein kinases, and serine-threonine kinases, and transcription factors. We also validated some of these genes by quantitative real-time PCR and gene regulatory network. Single nucleotide polymorphisms (SNP) markers can be developed from the sequences of these genes and used to map the resistance in the mapping populations. Our findings uncover novel genes associated with rate reducing resistance and future studies will allow us a better understanding of HBI interactions, the regulations of host responses, and plant disease development. The long-term goal of this study is to mitigate anthracnose disease epidemics and understand the mechanisms of resistance in the plant that can be exploited through breeding programs to provide durable resistance that contributes to overall yield stability and sustainability of strawberry production in the Southeast US.

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