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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 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 strawberry is not yet fully investigated. Identification of genetic factors associated with resistance could help develop improved strawberry varieties with enhanced resistance. We performed genome-wide transcriptional profiles of a resistant (NCS 10-147) and susceptible (Chandler) genotype inoculated with CA and CG. We identified a large number of differentially expressed genes (DEGs) during the transition from the surface (0 h to 48 h) to hemibiotrophic infection (HBI) and that many were closely related to up- and downregulation of defense, stresses, signal transduction, and hormone signaling. Selected candidate genes will further validate and develop single nucleotide polymorphism (SNP) markers and will be used for mapping populations. The DEGs identified in this study will have important implications for further characterization of mechanisms of resistance to CA and CG in strawberry.

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 have the ability to 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 has the ability to 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? There are practical knowledge gaps that exist in relation to the detection of HBI, their epidemiological role in fruit rot versus crown rot, and whether these two diseases can be managed by host plant resistance to HBI. 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'. Our main goal is to advance our understanding of host-pathogen interactions particularly host genetic variation in response to active phases of the disease. It is unclear what genes are differentially expressed in resistant genotype which may lead to the discovery of mechanisms that confer HBI resistance to CA and CG. The main objective of this study was to identify differentially expressed genes that are biologically relevant for the establishment of HBI in strawberry and lay the foundation for the identified candidate genes in resistance mechanisms.

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 infection. To define these pathogenicity phases, 0 h to 48 h after inoculation corresponded to the biotrophic stage and hemibiotrophic stage of infection. For time-course infection analysis, NCS 10-147' line and the commercial cultivar 'Chandler' exhibited resistant and susceptible to both CA and CG, respectively were spray-inoculated (7) and tri-foliate leaves were sampled from each treatment at 0, 24 and 48 hours after inoculation (hai). Collected samples were immediately placed on ice and kept -80°C until further analysis. 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, comparisons were made for each of the cultivar and at each time point between resistant and susceptible cultivar inoculated with the two pathogen samples (CA and CG independently). The significance is determined using an FDR < 0.05 and the absolute value of log2FC is greater than one.

Results and Discussion

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 downregulated 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 hours after inoculation). Analyses of strawberry transcripts from inoculated resistant and susceptible cultivar samples revealed a response to time between the 0, 24 and 48 h. Transcript data analysis of enriched biological processes revealed that three major groups: cellular metabolic process, organic substance metabolic process, and primary metabolic process were upregulated at most time points (Fig. 1A). The second group contained iron-binding, organic cyclic compound binding, and heterocyclic compound binding genes were upregulated DEGs by both CA and CG (Fig. 1B). The third group consisted of the intracellular organelle; membrane-bounded organelle and intrinsic component of membrane

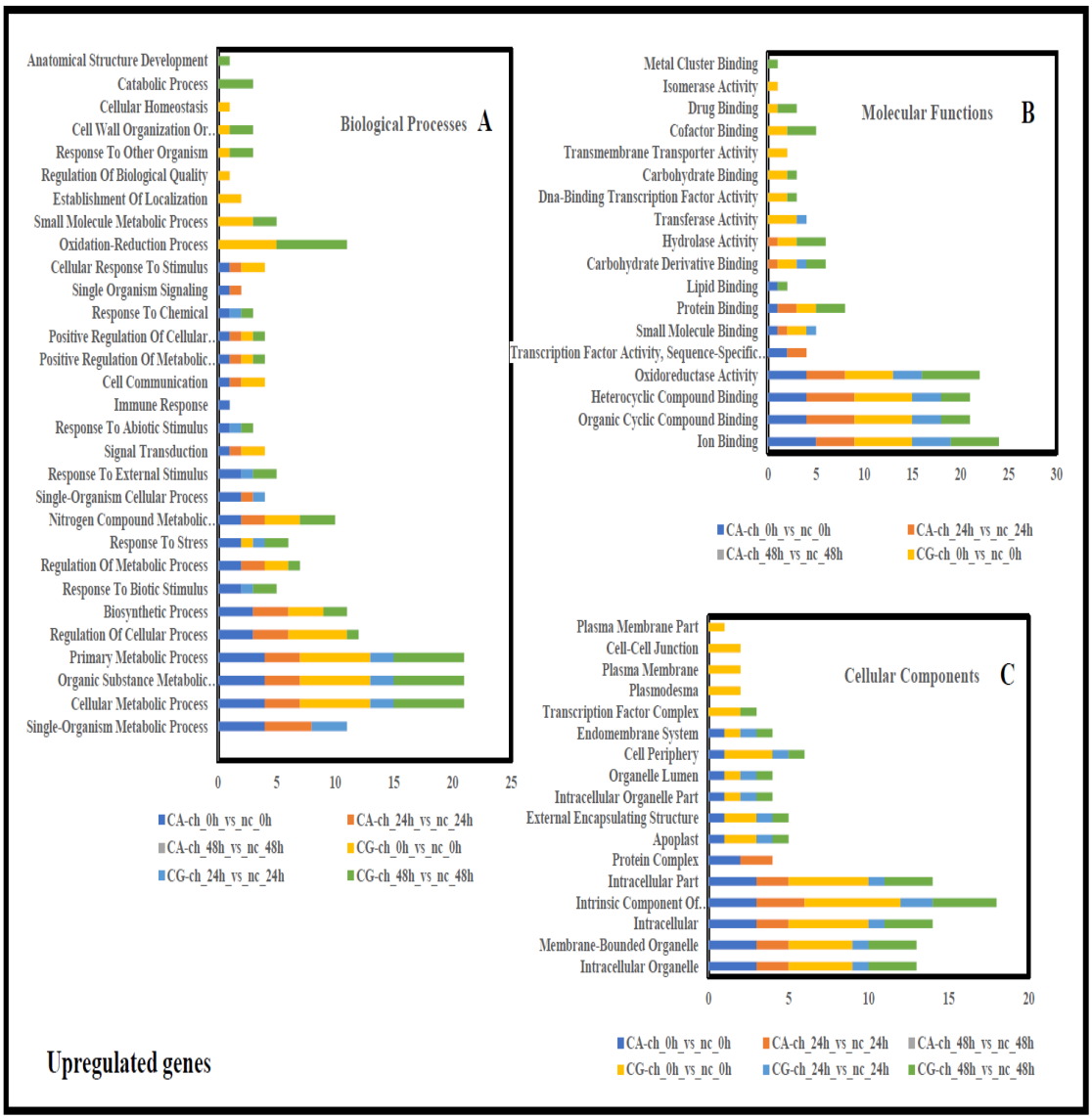

Fig. 1. Enriched functions based on gene ontology (GO) analyses of differentially expressed strawberry genes analyzed at three time points (0, 24, and 48 h). The upregulation patterns were adjusted according to the number of genes present in each GO category. The upregulated genes enrichment to specific biological processes, molecular functions and cellular components presented in A, B, and C groups, respectively.

genes and these genes are likely induced cellular compartmentations and are indicative of rapid and continued expression to CA- and CG-HBIs at 48 hai (Fig. 1C).

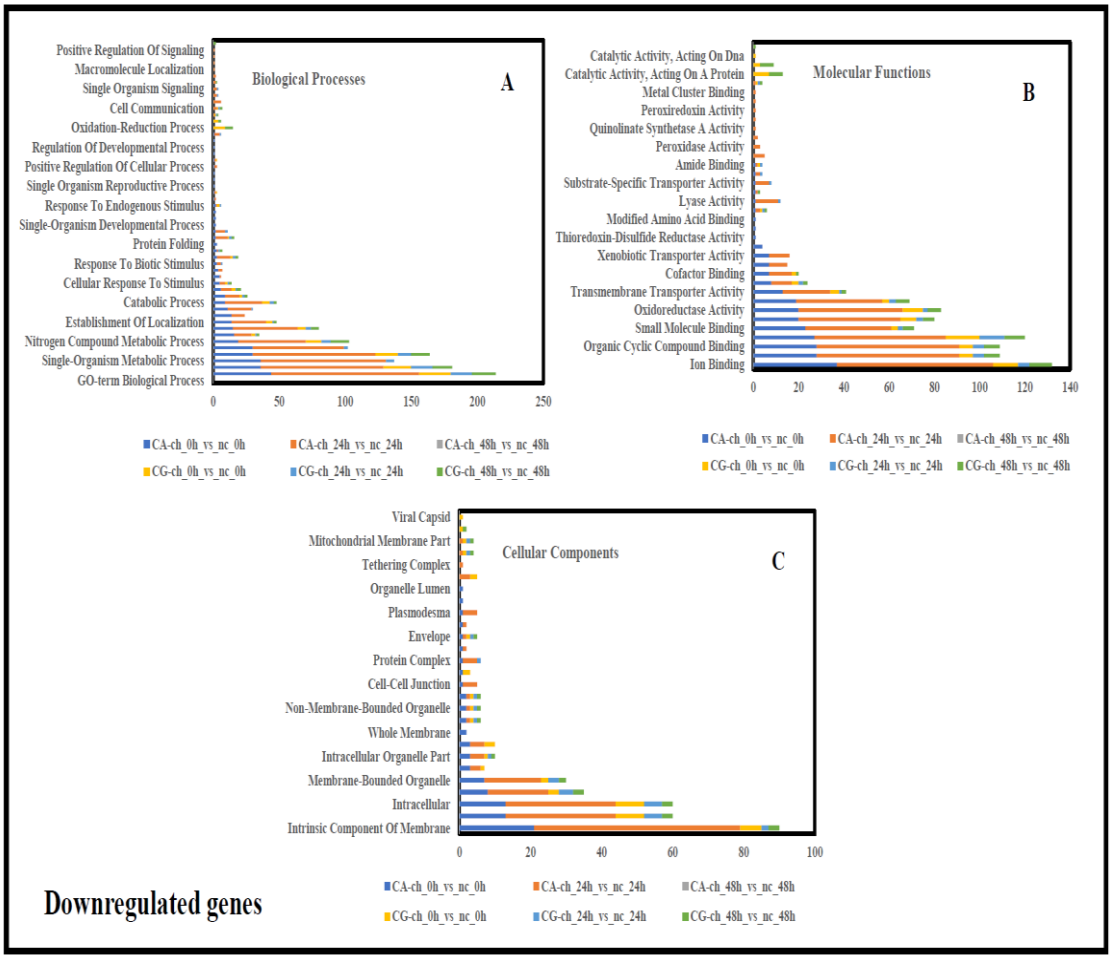

Fig. 2. Enriched functions based on gene ontology (GO) analyses of differentially expressed strawberry genes analyzed at three time points (0, 24, and 48 h). The downregulation patterns were adjusted according to the number of genes present in each GO category. The upregulated genes enrichment to specific biological processes, molecular functions and cellular components presented in A, B, and C groups, respectively.

We also used GO enrichment analyses to understand what genes related to biological processes, molecular functions and cellular components are affected within the leaves of resistant cultivars HBIs. The biological processes related to two genes organic substance metabolic process and primary metabolic process genes were severely affected after entry of both pathogens to hemibiotrophic colonization (Fig. 2A). A significant number of genes under the GO terms associated with molecular functions were downregulated by both pathogens (more genes were repressed by CA than CG) across time points. Based on GO enrichment analysis, 10 downregulated genes related to biological processes were ion binding, heterocyclic compound binding, organic cyclic compound binding, hydrolase activity, small molecule binding, transferase activity, oxidoreductase activity, carbohydrate derivative binding, transmembrane transporter activity, and protein

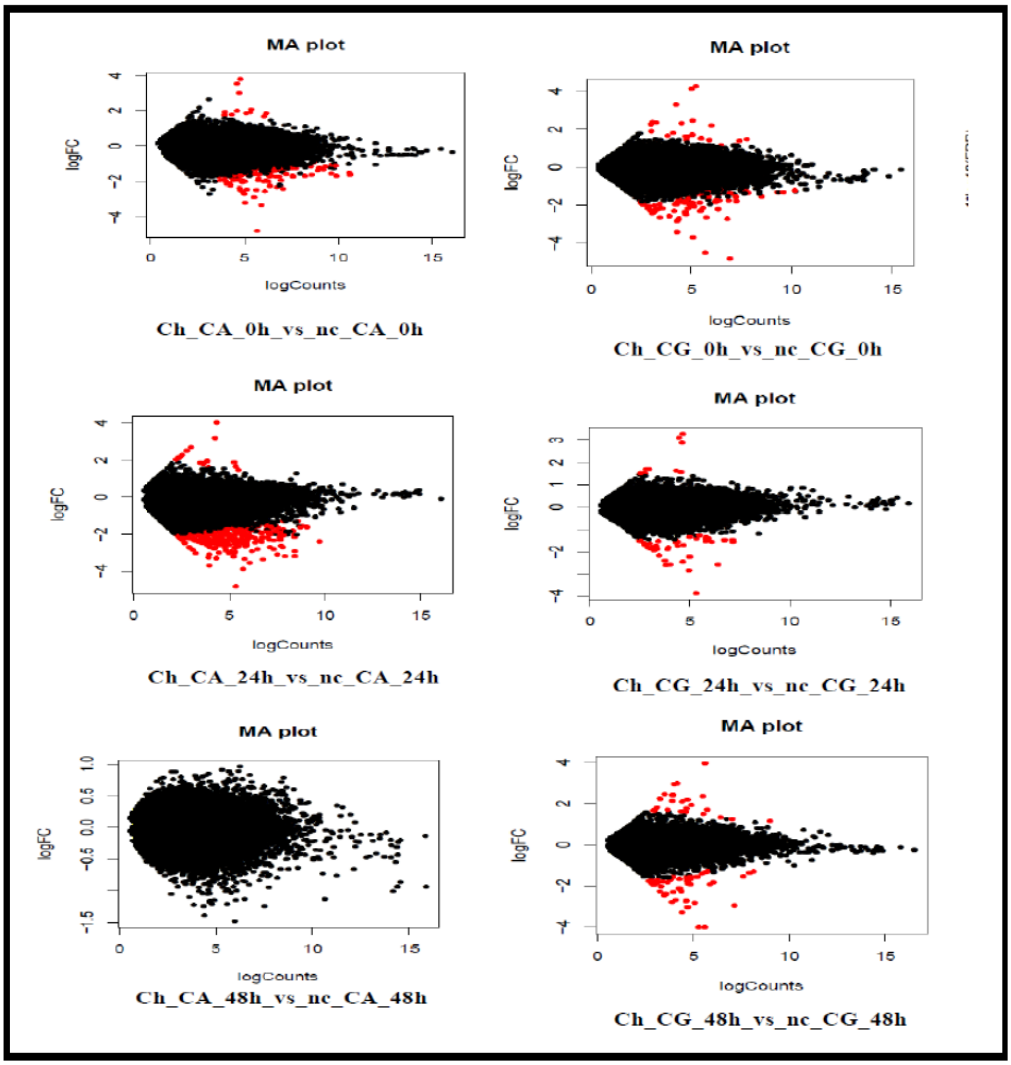

Fig. 3. Transcriptome profiles reveal differentially expressed (DE) strawberry genes during CA- CG HBIs. MA plots showing the log2 fold change in the expression plotted against mean of normalized counts in resistant line NCS 10-147' (NC) when comparing with susceptible strawberry cultivar 'Chandler' (Ch) leave inoculated with CA and CG and samples were collected with 0, 24, and 48 h. Y-axes indicate fold change values ($p < 0.05$) and x-axes indicate mean of normalized counts. Differentially expressed genes (DEGs) are shown in black and red dots indicating no change, up-regulated (above 0-fold change) and down-regulated (below 0-fold change).

binding. Other apparent top five suppression genes in the cellular components were an intrinsic component of membrane, intracellular part, intracellular organelle, and membrane-bound organelle (Fig. 2C). Apparently, more genes were suppressed by CA at each time point than by CG.

Transcriptome dynamics of both inoculated genotypes were compared over time using MA-plots (the log_2 fold change in expression plotted against the mean of normalized counts). A large set of genes was differentially expressed at different time points. For CA inoculated treatment, 12 genes and 101 genes were upregulated

and downregulated, respectively at 0 h after inoculation (hai). There were 266 DEGs between resistant and susceptible cultivars of which 17 genes were upregulated and 249 were downregulated at 24 hai. At 48 hai,

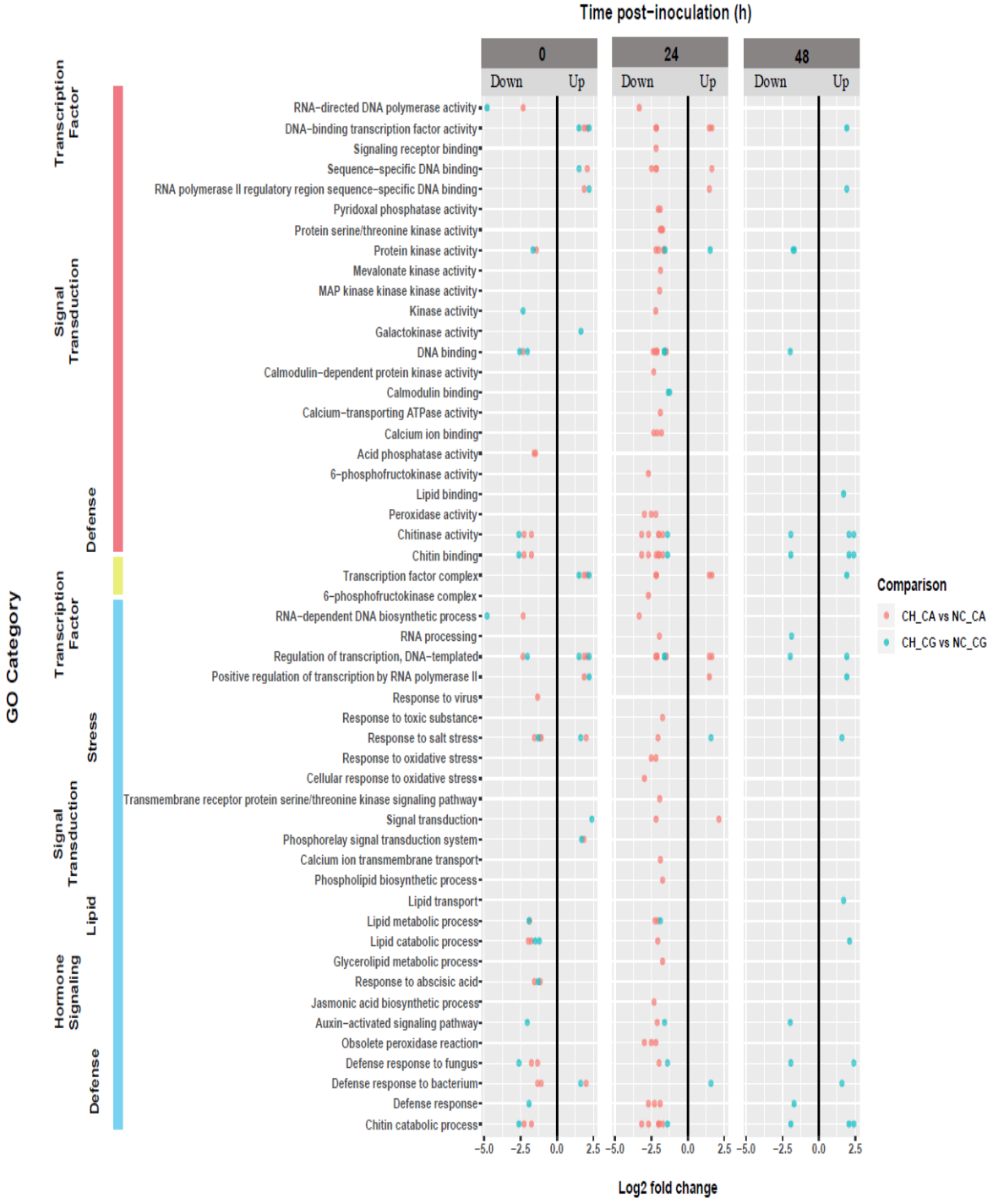

Fig. 4. Gene ontology (GO) categories represented in the set of significantly (*P* value < 0.05) up- and downregulated genes in response to CA- and CG-HBIs. Differentially expressed genes were observed when resistant strawberry cultivars compared with susceptible cultivar. Absolute fold change was plotted on the x-axis and GO terms were plotted on y-axis.

although several genes were induced, no DEGs were detected. For CG inoculated treatments, 22 genes were upregulated, and 72 genes were downregulated at 0 hai; 9 and 42 genes were upregulated and downregulated at 24 hai, respectively (Fig. 3). Likewise, at 48 hai, 25 genes were upregulated, and 51 genes were downregulated. To have a better understanding of the response within leave tissues of each strawberry genotype, we focused on genes that are exclusively responsive in each time point. We examined genes that activated (upregulated) or repressed (downregulated) at each time point either CA or CG. A significant number of genes encoding defense, stress, signal transduction, hormone signaling, were transcription factors were activated or repressed by CA and CG at different time points (Fig. 4).

Impact

If strawberry genotypes confer HBI resistance and this can be a heritable trait with markers, then this will have a dramatic impact on both major *Colletotrichum* problems in the nursery setting and in 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 are interested in further identifying and investigating within our data set of up- and downregulated genes that are related to defense genes (e. g., Chitinase, peroxidase), gene encoding protein kinases and serine-threonine kinases, and calcium- and calmodulin-binding proteins. We are also in the process of validating genes and generating single nucleotide polymorphisms (SNPs) primers from our RNA-seq data that could be useful to the breeder. These SNP markers will be used to map the resistance in the mapping populations. The long-term goal of this project is to prevent 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. This project will help bring assembly of molecular data to closure for a publication on the differential infection and gene expression profiles of the two *Colletotrichum* species on susceptible and resistant strawberry lines.

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