TRANSCRIPTOMIC, METABOLOMIC AND GENETIC ANALYSES OF AGE-RELATED RESISTANCE OF CUCUMBER TO *PHYTOPHTHORA CAPSICI*

By

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ABSTRACT

TRANSCRIPTOMIC, METABOLOMIC AND GENETIC ANALYSES OF AGE-RELATED RESISTANCE OF CUCUMBER TO *PHYTOPHTHORA CAPSICI*

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The oomycete plant pathogen *Phytophthora capsici* infects several agriculturally important crop species. In cucumber (*Cucumis sativus*), *P. capsici* primarily causes fruit rot which is characterized by tissue collapse and dense mycelial growth. Previous studies in our lab have shown that some cucumber cultivars exhibit an age-related resistance (ARR) wherein young fruit are highly susceptible but develop resistance at approximately 12-16 days post pollination (dpp). Furthermore, the fruit peel has been shown to be important for conferring ARR and methanolic extracts from resistant peels had inhibitory effects on pathogen development. In the research herein, we sought to elucidate the mechanism controlling this ontogenic resistance in cucumber fruit by employing a diverse array of genetic, genomic, metabolomic and microscopic approaches. Using transcriptome analyses of peels from ARR expressing- and non-expressing cultivars, we identified unique upregulation of defense related factors in resistant-aged fruit peels, including resistance genes and transcription factors. An enrichment for genes involved in specialized metabolism in resistant fruit was also observed, and subsequently followed by an untargeted metabolomic analysis. We identified metabolites, specifically terpenoid glycosides, that may act as antimicrobial components in resistant-aged fruit peels. In a second study, we characterized the response to inoculation at resistant (8 dpp) and susceptible (16 dpp) ages via microscopic and transcriptomic analyses. Scanning electron microscopy of resistant peels showed evidence for infection failure as early as 4 hpi, including deflated or lysed spores and hyphae, that were not observed on susceptible fruit. Furthermore, transcriptome

analysis of the first 48 hours post inoculation (hpi) revealed strong transcriptional defense responses at 4 hpi in both ages. At 24 and 48 hpi, susceptible 8 dpp fruit continued to mount defense along with strong downregulation of genes involved in photosynthesis and other biological processes. In contrast, resistant 16 dpp samples largely downregulated defense responses while upregulating photosynthesis. Weighted gene co-expression network analysis was used to further understand the transcriptional dynamics of infection during the first 24 hours. We identified early defense response modules which showed patterns of increased gene expression as early as 2 and 4 hpi, uniquely in resistant fruit. Several candidate genes involved in conferring this rapid response were identified. The early pathogen death and rapid defense response to infection in resistant-aged fruit indicate developmental changes that may include both pre-formed biochemical defenses and developmentally regulated capacity for pathogen recognition.

To genetically map loci linked to ARR we employed a bulk segregant analysis approach. However, as no easy-to-use computational tools were available for these analyses, I developed a software package to use in our own experiments, but also as a tool for the plant breeding and genetics community. Using this tool, we analyzed bulks selected from two segregating populations derived from ARR expressing and non-expressing parents. We identified one locus on chromosome 3, linked to ARR, and using transcriptome data of the parental lines we identified a set of genes potentially associated with ARR. Together, these studies further our understanding of ARR as a biological phenomenon in general, as well as in the cucumber-*P. capsici* pathosystem specifically.

To Carly, with love and admiration

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CHAPTER I

Introduction

Cucumber as a crop in the genomics era

The *Cucurbitaceae* plant family includes approximately 115 genera and several economically significant crops, of which one of the most important is cucumber (*Cucumis sativus* L.). In the U.S., cucumber production is grouped into two main variety types: slicing (fresh market) and pickling (processing), and about half of all cucumbers produced in the U.S. are specifically grown for the pickling industry (USDA, http://quickstats.nass.usda.gov). Michigan is the leading producer of pickling cucumber, producing over 170,000 tons and accounting for more than 30% of national production in 2015. Depending on the region of production, pickling cucumbers can be hand harvested for multiple harvests per season or can be planted at high density for once-over mechanical harvest (Cargill et al., 1975). In contrast to other fruit, cucumber is typically harvested immature, several weeks prior to ripening.

In 2009, a draft genome of the Chinese fresh market type cucumber cv. 'Chinese Long 9930' was sequenced and assembled (Huang et al., 2009). This study compared Sanger and Illumina assemblies and ultimately used a combination of both technologies for *de novo* assembly. Moreover, this assembly's annotation was later improved using RNA sequencing (RNA-seq) (Li et al., 2011). Data from the above studies are deposited on the cucurbit online database (http://www.cucurbitgenomics.org). The genome of the gynoecious, inbred, pickling type cv. 'Gy14' was sequenced and assembled shortly after, but an accompanying manuscript was not published. The north European cucumber cv. 'B10' was also sequenced (Witkowicz et al., 2011). The recently updated, Cucurbit Genomics Database further improves access to these genomes, other genetic and genomic data for cucumber as well as other cucurbits (Zheng et al., 2019). The database provides standard genomics tools such as BLAST and Genome Browser as well as new tools that allow viewing of gene expression and synteny data (Zheng et al., 2019).

Utilizing Next Generation Sequencing (NGS) tools has aided in characterizing genetic diversity of many crops (Kilian and Graner, 2012). A set of 115 cucumber lines, representing four geographic regions of origin, was resequenced, revealing valuable information regarding the domestication and diversity of cucumber (Qi et al., 2013). More recently, the full USDA plant introduction collection (1234 accessions) was genotyped using genotyping-by-sequencing technology, providing an extremely detailed characterization of the diversity and population structure catalogued in this collection (Wang et al., 2018). Together, these data sets help understand the history of domestication of the crop from its origins in India and Asia. Furthermore, these data can aid in identifying markers linked to traits of interest using genome-wide association studies.

Phytophthora capsici **is a plant pathogen that infects cucumber fruit**

A major pathogen affecting cucumber yields in the mid-western U.S. is *Phytophthora capsici*, an oomycete that causes foliar blighting, damping-off, wilting, and root, stem, and fruit rot in many vegetable crops such as bell pepper, tomato, snap bean, cucumber and other cucurbits (Hausbeck and Lamour, 2004; Granke et al., 2012; Sanogo and Ji, 2012). When climate conditions favor the development of the pathogen, it is estimated that approximately 25% of all vegetable crop yields in Michigan are lost due to infection (Hausbeck and Lamour, 2004). Owing to the importance of the disease, there has been research studying the development, morphology and genetics of the pathogen (Hausbeck and Lamour, 2004) and a draft genome of *P. capsici* was sequenced and assembled (Lamour et al., 2012a).

Interestingly, in cucumber, *P. capsici* specifically causes severe fruit rot, yet does not typically infect other plant organs (Hausbeck and Lamour, 2004). Thus, a field may appear to be healthy but fruit, underneath the canopy, will be highly infected and unmarketable. Symptoms of

P. capsici in cucumber fruit include necrosis, water soaking and ultimately, whole tissue collapse (Granke et al., 2012; Colle et al., 2014). In addition, fruit may appear healthy at harvest, but if the pathogen is present, moist post-harvest conditions may facilitate rot, thus leading to complete losses of harvested commercial loads (Hausbeck and Lamour, 2004).

Dispersal and spread of the disease is predominantly by run-off from rain or overhead irrigation (Granke et al., 2009). Furthermore, irrigation water from contaminated sources contributes to inoculum dispersal, by infecting previously un-inoculated fields (Granke et al., 2012). Infection in the field is usually very persistent and the sexually produced overwintering oospores can survive in the soil for many years (Granke et al., 2012). Due to the high survivability of the oospores and the wide variety of host species, it is extremely difficult to disinfest an inoculated field, and even multiple year crop rotations are typically insufficient (Hausbeck and Lamour, 2004).

While this pathogen is increasingly problematic, the worldwide use of chemical pesticides is being increasingly limited due to environmental and health considerations. Furthermore, because of overuse, resistance to mefenoxam, a fungicide that was previously, commonly used to control *Phytophthora*, was reported in several locations throughout the United States (Parra and Ristaino, 2001; Granke et al., 2012; Sanogo and Ji, 2012). Research aimed at identifying and introducing genetic resistance into germplasm will be highly beneficial to growers in need of practical solutions to this pest.

Pathogen life cycle in the cucumber field

Infection of cucumber fruit is by means of motile, bi-flagellate, zoospores that are transferred to the fruit surface via rain or irrigation (Hausbeck and Lamour, 2004). Once on the plant surface, zoospores use electro- and chemotaxis to identify and swim towards potential

infection sites (Hardham, 2007). Zoospores then encyst, that is, become affixed to the host surface and lose their flagella, and subsequently, hyphae germinate from the spore to penetrate the host. Penetration of the fruit surface is predominately through the formation of appressoria-like swellings – turgor pressure building organs that press against the host cell and enable penetration (Hardham, 2007; Lamour et al., 2012b).

After penetration has occurred, hyphae develop haustoria to absorb nutrients from the plant tissue, and once established, growth of the pathogen ensues to colonize the host tissue. *P. capsici* is a hemibiotroph and thus infection can be divided into two stages: parasitic biotrophy in early infection stages and subsequent necrotrophy as cells begin to die (Lamour et al., 2012b). As infection of the fruit progresses, and as early as three days post infection, lemon-shaped sporangia emerge and asexual reproduction of zoospores ensues to advance infection (Gevens et al., 2006; Lamour et al., 2012b). Optimal climate conditions for growth and infection are approximately 28°C with high relative humidity (Erwin and Ribeiro, 1998). Infection on the fruit surface is visible as a dense white, sporulating, mycelium that quickly covers the entire fruit and can propagate infection in the field.

Constitutive resistance to *P. capsici*

The most desirable solution to this disease problem would be to integrate genetic resistance using plant breeding. As there are multiple hosts for this pathogen, there have been attempts to identify sources of genetic resistance in different plant breeding programs. Research has thus far yielded mixed results; some programs have not yet found stable resistance and often resistant varieties of some crops carry other unappealing horticultural traits (Granke et al., 2012). Moreover, it appears that resistance is conferred through different genetic mechanisms in the different crops (Granke et al., 2012).

Some success has been reported in identifying and breeding of *P. capsici* resistant Solanaceous crops. There are some tolerant tomato (*Solanum lycopersicum*) lines that can be used in infected fields (Quesada-Ocampo and Hausbeck, 2010) and research in eggplant (*Solanum melongena* L.) has identified accessions with resistant fruits (Naegele et al., 2014). In pepper (*Capsicum annuum*), several sources of resistance have been studied and implemented in some of resistant cultivars commercially available (Granke et al., 2012). Most of the genes conferring these resistances have yet to be elucidated. A more recent study screened 66 recombinant inbred lines (RIL) of pepper derived from resistant and susceptible parents, against 20 *P. capsici* isolates and utilized a high-density linkage map to identify a quantitative trait locus (QTL) that explained between 29 to 58% of resistance to seven isolates (Rehrig et al., 2014). The researchers proceeded to examine transcriptomic sequence data of this QTL and suggested an ortholog of the Arabidopsis *DOWNY MILDEW RESISTANT 1* (*DMR1*) homoserine kinase, encoded therein, to be a strong candidate for a component of this resistance.

Screening against isolates of *P. capsici* has been performed for several cucurbit crops, however, identifying a stable source of genetic host resistance has not been as successful (Hausbeck and Lamour, 2004). One study evaluated 115 *Cucurbita pepo* (squash, pumpkin and gourd) accessions for their resistance to the pathogen and found eight accessions that could be used as sources for resistance (Padley et al., 2008). Later research of the inheritance of the resistance trait in a *Cucurbita* true breeding line suggested that resistance was conferred by three independent dominant genes (Padley et al., 2009).

Potential, constitutive host resistance in cucumber fruit was only potentially identified recently. In a previous effort, a detached fruit screening method was developed and harvest-stage fruit of over 300 cucumber accessions were examined, yet no complete resistance was

discovered (Gevens et al., 2006). Our lab performed a subsequent screen of close to 1300 cucumber accessions (Colle et al., 2014). This attempt revealed three potential accessions that might be used as a genetic source for resistance to *P. capsici* in cucumber that are now being incorporated into breeding programs (Grumet and Colle, 2017).

Age-related resistance

During the initial screening for host resistance our lab discovered that younger fruit of the cucumber cv. 'Vlaspik' appeared to be more susceptible to infection than older fruit (Gevens et al., 2006). More specifically, the transition to resistance was at approximately 10-12 days post-pollination (dpp) and appeared to be highly correlated with the end of the period of rapid fruit expansion. Further research showed that other cucurbit fruit such as pumpkin and squash also became less susceptible with increasing age (Ando et al., 2009). Such ontogenic, developmental or age-related resistance (ARR) has been described in several different plant-pathogen systems and in crops such as pepper, grape, rice, wheat, and several cucurbit crops (Kim et al., 1989; Gee et al., 2008; Ando et al., 2009; Zhao et al., 2009; Zhang et al., 2012). ARR to *P. capsici* was also previously observed in whole pepper plants (Kim et al., 1989) and chili pepper fruit (Biles et al., 1993). Though many studies have been performed, the molecular mechanisms controlling these resistances are not well understood and appear to be highly variable between pathosystems (Whalen, 2005; Develey-Rivière and Galiana, 2007).

Age-related resistance may be organ specific or affect the whole plant and has been observed in monocots and dicots infected by different types of pathogens including viruses, fungi, oomycetes and bacteria (Develey-Rivière and Galiana, 2007). Over the years, research into mechanism of ARR has suggested several potential factors correlated with the onset of resistance. Early research in tobacco ARR to *Phytophthora parasitica* showed a correlation

between salicylic acid (SA) and Pathogenesis-Related (PR) protein accumulation and leaf age (Hugot et al., 1999). Results of this study showed that transgenic *NahG* plants, deficient in SA accumulation did not develop ARR. In Arabidopsis, ARR to *Pseudomonas syringae* pv. *tomato* (*Pst*) was shown to be dependent on SA accumulation yet independent of SA signaling (Kus et al., 2002; Carella et al., 2015). Though transition to flowering is correlated with ARR to *Pst*, flowering is not required, nor sufficient, for manifestation of this resistance (Wilson et al., 2013; Wilson et al., 2017). These studies suggest that in Arabidopsis SA may function as an intercellularly accumulated antimicrobial compound in ARR in response to *Pst* (Carella et al., 2015).

A study of the *Nicotiana benthamiana – P. infestans* pathosystem revealed that age played a role in resistance to the pathogen (Shibata et al., 2010). Using virus-induced gene silencing, the researchers determined that both SA and ethylene signaling pathways were important in conferring ARR. Additionally, the authors show that production of the phytoalexin capsidiol is controlled by ethylene signaling in *N. benthamiana* and is important in ARR to *P. infestans*.

Fruit specific ARR, such as the kind observed in cucumber fruit, was also previously shown in multiple cases. One such example was described in grapevine (*Vitis* spp.), where young berries are susceptible to black rot (Hoffman et al., 2002) as well as powdery mildew (*Uncinula necator*) (Gadoury et al., 2003). The genetics, inheritance and mode of resistance were not described. The research showed, however, that the previously suggested mechanism of resistance – accumulation of soluble solids – was probably not the casual factor associated with the onset of ARR. Other examinations of fruit cuticle thickness, papillae formation and phenolic compound

accumulation concluded that these factors were not related to grape berry ARR to powdery mildew (Ficke et al., 2002).

Theoretically speaking, ARR could be conferred by means of preformed defenses such as physical barriers or chemical defenses that accumulate thoughout development (Meldau et al., 2012; Barton and Boege, 2017). In maize for example, the herbivory defense compound 2-β-d-glucopyranosyloxy-4,7-dimethoxy-1,4-benzoxazin-3-one accumulates in the leaves with plant age (Cambier et al., 2000). Alternatively, a developmentally regulated inducible defense response could be the mechanism. For example, in rice, a developmental increase in expression of leucine-rich repeat (LRR)-kinase type genes, *Xa3/Xa26* and *Xa21*, that peaks at the maximum-tillering stage of growth confers ARR to bacterial blight (Cao et al., 2007; Zhao et al., 2009). Recently, transcriptional control of the canonical immune receptor FLS2 was also shown to regulate ontogenic resistance in Arabidopsis to *P. syringae* (Zou et al., 2018). It is, of course, possible that a combination of preformed and developmentally induced mechanisms contributes to ARR.

Cucumber fruit transcriptomes transition from growth to defense with age

Previous research in our laboratory investigated the transcriptome of cucumber fruit tissue of different ages from 0 dpp to 16 dpp (Ando et al., 2012). This study showed that late/post exponential fruit growth – which is associated with the onset of ARR – is highly enriched for genes involved in response to abiotic and biotic stresses and extracellular functions. Moreover, 12 and 16 dpp fruit were uniquely and significantly enriched for putative transcription factor (TF) genes, including primarily stress related and development related factors. These observations suggested a programmatic shift from growth to defense which was correlated with the transition to resistance at 12 dpp. Interestingly, there appears to be genetic variability for the

ability of cucumber fruit to develop ARR. Of the 19 cultivars subsequently screened, only three ('Vlaspik', 'Long Green Improved' and 'Poinsett 76') developed ARR; the homozygous, breeding line 'Gy14' for example, remains susceptible to *P. capsici* even beyond 16 dpp.

Cucumber peels are associated with pathogen defense

To further understand the underlying mechanisms controlling ARR in cucumber we observed that the fruit surface was associated with resistance; peeled fruit were highly susceptible, though confounding effects of wounding could not be eliminated (Ando et al., 2009). When excised peels were placed over intact whole fruits and subsequently inoculated with *P. capsici* spore suspension, the peel sections showed the same infection rating as whole fruit, indicating that the peels alone responded equivalently to whole fruit (Ando et al., 2015). Interestingly, peels excised from 15 dpp fruit that were placed over intact 8 dpp fruit conferred resistance to the whole fruit beneath them, while 15 dpp fruit underneath infected 8 dpp peels did not become infected. This suggested that factors in the cucumber fruit peel were responsible for ARR and prompted further research into differences of young and old peels. Developmental changes in chemical or physical properties of the fruit peel could potentially influence resistance.

Transcriptomic analysis comparing fruit peel and pericarp tissues at 8 and 16 dpp showed that the genes that were most highly expressed in peel tissue were enriched for fruit surface-associated functions such as extracellular, endoplasmic reticulum, cell wall and plastid-related genes (Ando et al., 2015). Moreover, peel tissue was enriched for putative TF genes annotated to be involved in biotic and abiotic stress responses, while TF associated with development were excluded from peel and primarily expressed in the pericarp. Interestingly, genes that were specifically highly expressed in peel tissue of 16 dpp fruit were annotated to be associated with

response to stress, response to abiotic or biotic stimulus, signal transduction, and extracellular and transport functions.

Methanolic extracts from cucumber peels inhibit *P. capsici* growth

No research has yet specifically examined the metabolome of cucumber fruit peel; however, it has been reported that cucumber leaves synthesize chemicals such as C-glycosyl flavonoid phytoalexins in response to powdery mildew infection and chemical elicitation (McNally et al., 2003a; McNally et al., 2003b). Utilization of the compounds' own auto-fluorescence in confocal laser microscopy of treated tissue showed that synthesis was site specific in response to infection. An attempt to down-regulate synthesis of flavonoid phytoalexin precursors yielded mixed results, however, an increase in infection rates of powdery mildew was correlated with reduced chalcone synthase activity, a key enzyme upstream in the pathway (Fofana et al., 2005). A comprehensive study of compounds in cucumber whole fruit identified the existence of some of these same inhibitory compounds in fruit extracts, yet their effect on pathogen infection was not studied (Abu-Reidah et al., 2012). Other research of cucumber fruit volatiles found evidence that they had antimicrobial properties (Sotiroudis et al., 2010).

To further our understanding of the potential effects of chemicals within the peel on infection we examined the effects of chemical extracts on pathogen growth. We developed a high-throughput screening method using microtiter plates; wells were filled with clarified V8 media and treated with chemical extracts from fruit peels of different fruit ages and genotypes. The wells were subsequently inoculated with *P. capsici* spores from strains genetically engineered to express red or green fluorescent proteins (RFP and GFP) (kindly provided by C. Smart, Cornell University; Dunn et al., 2013) and read with a fluorescence plate reader over the

course of five days. Results showed that methanolic extracts from 16 dpp fruit peels inhibited *P. capsici* growth more than those extracted from 8 dpp fruit peels (Ando et al., 2015).

Utilizing transcriptomics to study plant-pathogen interactions

The ability to examine the whole transcriptome of an organism, organ or cell can shed light on complex developmental processes such as pathogen infection. A transcriptomic exploration of the interaction of tomato and *P. capsici* used a custom designed microarray to concurrently examine host and pathogen over the course of infection (Jupe et al., 2013). The researchers described transcriptional programs for distinct stages of infection. The pathogen showed expression of genes enriched for Gene Ontology (GO) terms associated with protein metabolism, gene expression and biosynthetic processes during the biotrophic stage. Genes involved with catabolic processes, including peptidases and proteasomal subunits, were highly prevalent in the transition to necrotrophy. In later infection stages, pathogen gene expression was enriched in expression of signal transduction and metabolic process genes. Furthermore, the researchers examined the expression patterns of genes coding for effectors, which inhibit plant defense response, and identified groups that were specific for different stages, including prior to infection.

Examination of the tomato transcriptome revealed that there were large groups of genes that were specifically differentially regulated during the first 8 hours of infection. Furthermore, there were relatively few genes differentially expressed during the transition from 0 to 8 hours post-inoculation (hpi) that were also differentially expressed during the transition from 24 to 48 hpi, suggesting two different, key, transcriptional profiles at those time points. The initial stages of infection, 0 to 8 hpi, showed a shift from general metabolic to catabolic and specific metabolic processes. GO enrichment analysis of the transition from 24 to 48 hpi showed enrichment for

many different ontologies and suggested a distinct change in regulation of several metabolic and biosynthetic processes associated with the beginning of the necrotrophic stage.

The recent advancement, accessibility, and decrease in costs of NGS technologies have highly benefited the scientific community. Due to the developmental nature of ARR and the several, different potential molecular mechanisms that could be employed to confer this resistance, an NGS transcriptome-wide approach should prove useful in identifying the key factors involved. To our knowledge, only one transcriptomic study of an ARR pathosystem was performed (Gusberti et al., 2013). Apple leaves show ARR to *Venturia inaequalis*, and by performing RNAseq on inoculated tissue at 72 and 96 hpi genes potentially contributing to ARR were identified.

Rationale and objectives

Though there have been recent advancements, little is understood about the regulation and molecular mechanisms conferring ARR in general, and specifically in the cucumber-*P. capsici* pathosystem. Using genetic and genomic analyses to elucidate these mechanisms will be valuable in understanding ARR as a biological phenomenon. Furthermore, pinpointing the components regulating ARR could aid in development of young fruit resistance that could benefit agricultural production.

The objectives of the research herein were: 1) utilize the genetic diversity in cucumber, and developmental regulation of this trait to identify genes and metabolites potentially contributing to ARR; 2) Use gene co-expression networks to identify differentially regulated processes in response to infection in susceptible and resistant-aged fruit; 3) Develop a software package to facilitate genetic mapping of loci linked to the trait; and 4) Use the software and

segregating populations derived from ARR expressing and non-expressing parents, to identify such loci.

ARR may arise by means of either preformed or induced defenses, in either case an age-regulated difference in expression of the defense components is required. In objective 1, we identified two cucumber genotypes, one exhibiting ARR and one that remains susceptible throughout development. We sought to understand what genes and specialized metabolites were differentially expressed uniquely in the ARR expression genotype, specifically in the resistant age. We expected that by using both genetic diversity and development to filter the thousands of developmentally regulated factors we could focus on those which are ARR specific.

For the second objective, we were interested in characterizing the infection process in an ARR expressing cucumber, both in susceptible- and resistant-aged fruit. To this end, we examined inoculated samples using a combination of microscopy, high-throughput phenotyping and transcriptomic experiments. The goals were to identify the mechanisms and timing at which ARR manifests in resistant-aged fruit.

The transcriptomic and metabolomic approaches identified several genes that were associated with ARR, however genetic analyses are necessary to identify genomic loci linked to the trait. We wished to use a bulk segregant analysis approach for this purpose, however as no easy-to-use tools were available, I developed a software package to aid in the analysis. Using this tool, we analyzed segregating populations derived from ARR expressing and non-expressing parents to identify loci linked to this trait.

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CHAPTER II

Transcriptomic and metabolomic analyses of cucumber fruit peels reveal a developmental increase in terpenoid glycosides associated with age-related resistance to *Phytophthora capsici*

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Author contributions:

BNM and RG Conceived and designed the experiments. BNM performed the transcriptomic analysis with support from RG. MC performed the fruit disease assay. YK and BNM performed the qRT-PCR analysis. Metabolomic analysis was performed by BNM with support from ADJ. The text was written by BM and RG with comments from MC and ADJ. All authors reviewed and approved the manuscript.

Abstract

The oomycete, *Phytophthora capsici*, infects cucumber (*Cucumis sativus* L.) fruit. An age-related resistance (ARR) to this pathogen was previously observed in fruit of cultivar ‘Vlaspik’ and shown to be associated with the peel. Young fruits are highly susceptible, but develop resistance at ~10–12 days post pollination (dpp). Peels from resistant (16 dpp) versus susceptible (8 dpp) age fruit are enriched with genes associated with defense, and methanolic extracts from resistant age peels inhibit pathogen growth. Here we compared developing fruits from ‘Vlaspik’ with those of ‘Gy14’, a line that does not exhibit ARR. Transcriptomic analysis of peels of the two lines at 8 and 16 dpp identified 80 genes that were developmentally upregulated in resistant ‘Vlaspik’ 16 dpp versus 8 dpp, but not in susceptible ‘Gy14’ at 16 dpp. A large number of these genes are annotated to be associated with defense and/or specialized metabolism, including four putative resistance (R) genes, and numerous genes involved in flavonoid and terpenoid synthesis and decoration. Untargeted metabolomic analysis was performed on extracts from 8 and 16 dpp ‘Vlaspik’ and ‘Gy14’ fruit peels using Ultra-Performance Liquid Chromatography and Quadrupole Time-of-Flight Mass Spectrometry. Multivariate analysis of the metabolomes identified 113 ions uniquely abundant in resistant ‘Vlaspik’ 16 dpp peel extracts. The most abundant compounds in this group had relative mass defects consistent with terpenoid glycosides. Two of the three most abundant ions were annotated as glycosylated nor-terpenoid esters. Together, these analyses reveal potential mechanisms by which ARR to *P. capsici* may be conferred.

CHAPTER III

Developmentally regulated defense rapidly inhibits *Phytophthora capsici* infection in cucumber fruit.

Ben N Mansfeld, Marivi Colle, Chunqiu Zhang, Ying-Chen Lin & Rebecca Grumet

Author contributions:

All work was performed by Ben N Mansfeld except for the fruit disease assays and cultivar screens which were performed by Marivi Colle.

Abstract

Cucumber (*Cucumis sativus*) fruit are susceptible to infection by *Phytophthora capsici.* However, some cucumber cultivars develop a fruit surface-associated age-related resistance (ARR) to *P. capsici*. Young, rapidly growing fruit are highly susceptible, but become resistant as they complete exponential growth [~16 days post-pollination (dpp); 2-3 weeks prior to ripening]. Analyses of peel of ARR expressing and non-expressing uninoculated fruit identified gene expression and metabolomic changes associated with resistance that potentially functions as preformed defenses. Here we performed transcriptomic analyses of inoculated fruit at resistant (16 dpp) and susceptible (8 dpp) ages, providing a unique opportunity to examine compatible and incompatible interactions in the same genotype. Strong transcriptional changes were observed at 4 hours post inoculation (hpi), with approximately 1800 genes differentially expressed in either age, suggesting an early initial response to infection. At 24 and 48 hpi, susceptible 8 dpp fruit continued to mount defense along with strong downregulation of genes involved in photosynthesis and other biological processes. In contrast, resistant 16 dpp samples largely downregulated defense responses while upregulating photosynthesis. Scanning electron microscopy of resistant peels showed evidence for infection failure as early as 4 hpi, including deflated or lysed spores and hyphae, that were not observed on susceptible fruit. Weighted gene co-expression network analysis identified early defense response modules uniquely expressed in resistant fruit as early as 2 and 4 hpi. Several candidate genes involved in conferring this rapid response were identified. The early pathogen death and rapid defense response to infection in resistant-aged fruit indicate developmental changes that may include both pre-formed biochemical defenses and developmentally regulated capacity for pathogen recognition.

Introduction

Ontogenic, developmental, or age-related resistance (ARR), wherein plants or plant organs transition from susceptibility to resistance as a result of developmental changes (Whalen, 2005; Develey-Rivière and Galiana, 2007), has been described in several different plant-pathogen systems and in crops such as pepper, grape, rice, wheat, and several cucurbit crops (Kim et al., 1989; Gee et al., 2008; Ando et al., 2009; Zhao et al., 2009; Zhang et al., 2012). While providing protection in agricultural systems and potentially playing an important role in the evolution and optimization of host resistance (Meldau et al., 2012), the molecular mechanisms controlling these resistances are not well understood.

Evidence from various systems suggests possible physical, chemical, or physiological changes that may result from age-dependent, non-pathogen specific investment in defense such as cell wall modifications, production of anti-microbial phytoanticipins, or altered hormone balance (Develey-Rivière and Galiana, 2007; Meldau et al., 2012). There are also some examples where ARR may result from developmentally regulated expression of a pathogen receptor, allowing for pathogen-specific induced resistance at the resistant age. In rice, a developmental increase in expression of leucine-rich repeat (LRR)-kinase type genes, *Xa3/Xa26* and *Xa21*, that peaks at the maximum-tillering stage of growth, confers ARR to bacterial blight (Cao et al., 2007; Zhao et al., 2009). Recently, in Arabidopsis, transcriptional control of the canonical immune receptor FLS2 was also shown to regulate ontogenic resistance to *Pseudomonas syringae* (Zou et al., 2018). It is, of course, possible that a combination of preformed and developmentally induced mechanisms contributes to ARR.

Utilizing an ARR pathosystem also allows a unique opportunity to examine both compatible and incompatible interactions within the same plant genotype. In this work we sought

to understand the basis for ARR in cucumber fruit to infection by the oomycete pathogen, *Phytophthora capsici* (Gevens et al., 2006). This soilborne hemibiotroph is a pathogen of many agriculturally important crops including numerous solanaceous and cucurbit species (Hausbeck and Lamour, 2004). Infection is initiated by means of biflagellate zoospores which arrive via water from rain or irrigation (Granke et al., 2012). Upon reaching the host target tissue, zoospores encyst, lose their flagella and germinate forming germination tubes (Lamour et al., 2012). The germination tubes penetrate the plant surface using appressoria and continue growing hyphae. During the early, biotrophic stages of infection haustoria are formed and used for direct interaction with the host cells and nutrient acquisition; the pathogen then transitions to necrotrophy at approximately 48 hours post-inoculation (hpi) and can produce sporangia for asexual reproduction as soon as 72 hpi (Lamour et al., 2012; Jupe et al., 2013). The transcriptome of *P. capsici* infection has been described in tomato leaves using microarray technology (Jupe et al., 2013). The authors identified two major transcriptomic responses in both pathogen and host, at initial infection (8 hpi), and at the transition to necrotrophy (48 hpi) (Jupe et al., 2013).

In cucumber, the primary targets of infection of *P. capsici* are the fruit, which display symptoms of rot and tissue collapse followed by appearance of white mycelia and sporangia. In screening for genetic resistance to *P. capsici*, ARR was discovered (Gevens et al., 2006). Young, rapidly growing fruit are extremely susceptible to infection, and then transition to resistance as they near the end of their exponential growth phase, starting at 12-15 days post-pollination (dpp). The fruit peel has been shown to be important for ARR (Ando et al., 2015). Excised peels exhibit equivalent responses to whole fruit and methanolic extracts from resistant-aged peels had inhibitory effects on pathogen growth (Ando et al., 2015). Cucumber peels of resistant-aged fruit

are enriched for genes associated with defense against biotic and abiotic stresses (Ando et al., 2015).

ARR may arise by means of either preformed or induced defenses; in either case a developmentally-regulated difference in expression of the defense components is required. A comparison of uninoculated peel transcriptomes of ARR expressing and non-expressing cucumber fruit revealed the potential for either or both cases (Mansfeld et al., 2017). Of the 80 genes that were uniquely upregulated in ARR expressing fruit at the resistant age, four putative resistance genes (R-genes) as well as resistance related transcription factors were identified. Furthermore, this set of genes was highly enriched for specialized metabolism genes, including terpenoid synthesis and decoration genes, and untargeted metabolomic analyses of the same tissues revealed an increased accumulation of glycosylated terpenoids in the resistant tissue (Mansfeld et al., 2017). The accumulation of these preformed compounds may work in inhibiting infection, while at the same time developmentally regulated expression of *R*-genes may provide the ontogenic capacity to sense and respond to infection.

In this study, we characterized the response of cucumber peel to inoculation with *P. capsici* at resistant (8 dpp) and susceptible (16 dpp) ages. To our knowledge, only one other transcriptomic study of ARR was performed. This study examined apple leaves of different ages inoculated with *Venturia inaequalis*, at 72 and 96 hpi (Gusberti et al., 2013). Thus, no thoroughly resolved comparisons of the transcriptome analyses of early infections in an ARR pathosystems have been performed. A detailed characterization of infection in ARR response could shed light on the mechanism by which this resistance is controlled, revealing if preformed or induced defenses are recruited. Here, we observed a rapid transcriptional defense response at the resistant age coinciding with observed death and collapse of the pathogen by 8 hpi. Two co-expression

modules were uniquely upregulated in resistant-aged fruit by 8 hpi and were enriched for defense response. These modules contained WRKY transcription factors, ethylene synthesis genes, peroxidases and other defense related genes. These findings suggest that this early response may be crucial in conferring ARR to *P. capsici* in cucumber.

Materials and Methods

Plant Material

A set of 22 inbred cucumber cultivars was tested for ARR. Three to ten fruits at 16dpp were collected from each cultivar were grown in the greenhouse as described in Ando et al. (2012). For all further experiments, greenhouse production of cucumber fruit (cv. 'Poinsett 76') except plants were drip fertigated (1 L/day at 1-2% 20-20-20 fertilizer). In transcriptome experiment one, flowers were hand pollinated, while in transcriptome experiment two flowers were tagged at anthesis and bee pollinated. In both cases pollination was staggered, such that 8 and 16 dpp fruit were harvested on the same day. In all experiments only one fruit per plant was grown to limit competition.

Detached fruit inoculations and sample collection

Harvested fruit were briefly washed with distilled water and allowed to air-dry. Fruits were placed in incubation trays lined with wet paper towels, to maintain high humidity and covered with clear plastic tops. Zoospore suspensions were prepared from *P. capsici* isolate OP97 or NY-0644-1 expressing RFP (Dunn et al., 2013) cultured on diluted V8 agar media (V8 juice 200 mL, $CaCO_3$ 3 g, agar 15 g, distilled water 800 mL). After 7 days, the plates were flooded with 10 mL sterile distilled water to release zoospores. Two 10 µL aliquots were

removed for quantitation by a Countess Cell Counter (Invitrogen) and the mean concentration was used for dilution. The suspension was diluted to a concentration of 5 x 10^5 zoospores/mL. Fruits were then inoculated with ~6 (8 dpp fruit) or ~12 (16 dpp fruit), equally spaced, 30 µL droplets of the diluted zoospore suspension. Incubation was performed under constant light at 23 to 25 °C. For ARR screening development of disease symptoms such as water soaking and mycelial growth on each fruit was monitored daily for ten days. Fruits were evaluated using a disease rating in scale of 1-9 (1=no symptom; 9=extensive mycelial growth and sporulation).

Plant material was inoculated and harvested for two transcriptome experiments; the first included fruit sampled at 0, 4, 24, and 48 hours post inoculation (hpi), and the second at 0, 2, 4, 8, 12, 18, and 24 hpi. In both experiments timepoint 0 was collected at 12:30 pm. At each subsequent timepoint, samples were collected from 6-12 inoculation sites per fruit. Samples from a given fruit were pooled to form a biological replicate. Three replicate fruits were samples for each age at each timepoint. In experiment two, at timepoint 0, the three replicate samples were prepared from a single fruit. Fruits were removed from the incubation chamber and punches were made around each inoculation site using a No. 4 cork borer. Peel discs were subsequently collected by peeling the punched area using a vegetable peeler and immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction. In experiment two, samples taken from uninoculated parts of the fruit were used as the respective control for each time point.

High-throughput RNA extraction

Samples were ground using a mortar and pestle in liquid nitrogen. RNA extraction was performed using the MagMAX Plant RNA Isolation Kit protocol (Thermo Fisher) with slight modifications: 100-150 mg of ground tissue were added to 1000 µL of lysis buffer. High-throughput RNA extraction was performed in 96-well format, on a KingFisher Flex Purification

System (Thermo Fisher). Immediately after the run was complete, the 96-well plate was transferred to storage at -80°C. RNA concentration and quality were measured using Qubit 2.0 Fluorometer (Life Technologies, Inc.) and LabChip GX (Perkin Elmer) respectively. All samples had a minimum RNA quality score of 8.

TruSeq Library preparation and sequencing

Libraries were prepared at Michigan State University's Research Technology Support Facility, using the Illumina TruSeq Stranded mRNA Library Preparation Kit on a Sciclone G3 robot following manufacturer's recommendations. An additional cleanup with 0.8X AmpureXP magnetic beads was performed after completion of library preparation. Quality control and quantification of completed libraries was performed using a combination of Qubit dsDNA HS and Advanced Analytical Fragment Analyzer High Sensitivity DNA assays. The libraries were divided into two pools of 15 libraries each. Pools were quantified using the Kapa Biosystems Illumina Library Quantification qPCR kit. Each pool was loaded onto one lane of an Illumina HiSeq 4000 flow cell and sequencing was performed in a 1x50 bp single read format using HiSeq 4000 SBS reagents. Base calling was accomplished by Illumina Real Time Analysis (RTA) v2.7.7 and output of RTA was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v2.19.1.

QuantSeq 3'-mRNA library preparation and sequencing

For the second experiment, QuantSeq 3'-mRNA FWD libraries (Lexogen) were prepared by the Cornell University, Institute of Biotechnology, Genomics Facility using the manufacturers guidelines. Quality control and quantification of completed libraries was performed using a combination of Qubit dsDNA HS and Advanced Analytical Fragment Analyzer High Sensitivity

DNA assays. The libraries where then loaded on a single Illumina NextSeq500 lane and sequenced in a 1x86 bp single end format. Base calling was achieved by Illumina RTA v2.4.11 and output of RTA was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v2.18.

Sequencing read preprocessing and quasi-mapping

Experiment one: Reads were cleaned, and adaptor sequences were removed using Trimmomatic v. 0.34 (Bolger et al., 2014) with the following settings: LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:35. Quality control was performed using FastQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc). A cucumber transcriptome fasta file was made from the 'Chinese Long' (v2) (Huang et al., 2009; Li et al., 2011) genome using the *gffread* function from the cufflinks software package (Trapnell et al., 2010) and high-quality reads were then quasi-mapped to the transcriptome using Salmon v. 0.9.1 (Patro et al., 2017) with default settings.

Experiment two: Reads' quality was assessed with FastQC and visualized using multiQC (Ewels et al., 2016). Subsequently, reads were processed using BBMap (https://jgi.doe.gov/data-and-tools/bbtools/) with the following settings: ftl=12; k=13; ktrim=r; useshortkmers=t; mink=5; qtrim=r; trimq=10; minlength=20; int=f, and trimmed of any poly A sequences, adaptors and the first 12 nt (as recommended by the manufacturer of the library kit). To increase the mapping success rate, the 'Chinese Long' (v2) GFF3 file was amended to extend all transcript 3'UTRs by 2000 bases using a custom R script. The extended gene models were then used to extract a transcriptome fasta file as above. Reads were then quasi-mapped to this new transcriptome file using Salmon v 0.12.0 with the --noLengthCorrection option.

Differential expression analysis

Read quantification data was imported into R using the tximport R package (Soneson et al., 2015) and differential expression analysis was performed using DEseq2 (Love et al., 2014) with log-fold-change-shrinkage. Contrasts were analyzed comparing sequential timepoints as well as each timepoint vs. uninoculated samples. Differentially expressed genes were called significant using an adjusted p-value (Benjamini-Hochberg adjustment) and a false discovery rate of less than 5%. A cutoff expression change of above two-fold was used to define biological significance. Alluvial plots were drawn using the ggalluvial R package and venn diagrams were created using the overLapper script from: http://faculty.ucr.edu/~tgirke/Documents/R_BioCond/My_R_Scripts/overLapper.R.

Weighted gene co-expression network analysis

Integer value transcript counts from experiment two, were imported into DESeq2 using the tximport R package (Soneson et al., 2015). Genes with less than 10 reads in greater than 75 of the total 78 samples were considered lowly expressed and excluded from the analysis, and 15202 genes remained. The normalized counts matrix was then transformed using the variance stabilizing transformation (VST) (Anders and Huber, 2010) using DESeq2 and imported into the WGCNA package pipeline (Langfelder and Horvath, 2008).

Two separate signed networks were assigned for the inoculated susceptible- and resistant-aged fruit. For each network, the VST counts were used to calculate adjacency matrices using the biweight midcorrelation and a soft thresholding power of 12 (yielding a scale free topology fit of greater than 0.8). The adjacency matrices were used to calculate two topological overlap dissimilarity matrices which were subsequently used for forming gene clustering trees, using

average distances. The gene trees were used for assigning co-expression modules using the dynamic tree cut algorithm with a minimum module size of 30 genes. Module eigengenes were correlated to each other and modules with similar expression patterns (dissimilarity < 0.25) were merged. Gene expression profiles of module genes from the infected resistant network (16 dpp) were plotted based on VST values and compared to control and 8 dpp expression patterns.

To identify modules with different expression patterns in inoculated tissue, read counts were first extracted and normalized by library size using DESeq2 *counts()* function. For each of the resistant network modules, the $\log_2$ (+ 0.5) of the normalized counts of all genes in that module was the dependent variable in a linear model where a natural cubic spline with 3 internal knots, at 3, 8, 15 hpi (as determined by the 0.25, 0.5 and 0.75 quantiles of time), was applied to the time variable using the *ns()* function from the splines R package:

$$\log_2(Counts_{Module} + 0.5) = age + age{:}\,ns(time, df = 4)$$

An analysis of variance was then performed to identify module with significant age X splined-time interactions. The summary of each of those linear models contains the interaction effects for each segment of the spline. Modules with interaction effects in segment 1 and/or 2 (0 – 3 and 3 – 8 hpi, respectively) where identified as early induced modules.

Gene ontology term enrichment analysis

Gene Ontology (GO) term enrichment analysis was performed using the TopGO R package (Alexa et al., 2006) with the entire set of fruit peel expressed genes set as background. Terms were considered enriched if they passed a *p*-value of 0.05 on the Fisher test with the "*weight01*" algorithm and a minimum node size of 100 genes. The previously updated GO term list for cucumber genes (Mansfeld et al., 2017), was used for analysis. To visualize change of

GO terms over consecutive contrasts heatmaps of -$\log_{10}$(Fisher *weight01 p*-values) were plotted using only terms with $P < 0.01$.

Microscopy

Preliminary fluorescent microscopy of infection was performed using an EVOS FL Auto imaging system (ThermoFisher). Excised cucumber peels were affixed to the lid of a 100 mm petri dish using petroleum jelly and inoculated with 10 µL zoospore suspension (~5 x 10^5 spores/ml) of RFP-expressing isolate NY-0664-1 (Dunn et al., 2013). Petri dishes were then sealed with parafilm and carefully inverted and placed on the microscope table. Samples were observed at 4x magnification and images were captured every 30 minutes for 72 hours.

While collecting samples for transcriptome experiment two, a ~2 mm peel plug from the middle section of each fruit was also excised using a razor blade and fixed in 4% glutaraldehyde in 0.1M phosphate buffer for scanning electron microscopy (SEM). After overnight fixation in glutaraldehyde, samples were soaked in 0.1 M phosphate buffer for 40min. After consecutive dehydration in rising ethanol concentrations (25, 50, 75, 90, 100, 100, 100%; 1 hour each), samples were transferred to a Leica Microsystems EM CPD300 critical point dryer (Leica Microsystems) using liquefied carbon dioxide as the transitional fluid. Samples were then mounted on aluminum stubs using adhesive tabs (M.E. Taylor Engineering) and coated with osmium (~10 nm thickness) in an NEOC-AT osmium coater (Meiwafosis Co., Ltd.). Samples were examined in a JEOL JSM-6610LV scanning electron microscope (JEOL Ltd.).

High-throughput infection phenotyping

High-throughput *in vivo* disease phenotyping was as described in Zhang et al. (2018). Briefly, sixteen 6 mm diameter, 5 mm thick, peel tissue plugs were collected from each of three

8 and 16 dpp fruit using a biopsy punch. Plugs were placed in a 96-well black plate and subsequently inoculated with the constitutively fluorescing *P. capsici* isolate NY-0664-1 (Dunn et al., 2013), or with distilled water (control 4 plugs/fruit). Plates were read using a Tecan Spark Plate Reader (Tecan). Fluorescent measurements were taken in each well every hour, over the course of 24 hours at 28 °C. The excitation and emission settings were 536 and 586 nm, respectively. Gain was calculated from a well containing a mycelial mat, and the Z-position was set at 20000 µm.

Results

The cultivar 'Poinsett 76' displays age-related resistance to P. capsici

Our previous ARR studies (Gevens et al., 2006; Ando et al., 2009; Ando et al., 2015; Mansfeld et al., 2017) examined the commercial cucumber cultivar 'Vlaspik', an F_1 hybrid commonly grown for processing cucumber production in the Midwest USA. For further genetic and transcriptomic analyses, we sought to determine whether ARR was a common trait in cucumber and to identify a homozygous inbred cultivar expressing ARR. Testing of several inbred cultivars (Supplementary Table 1) identified a small number that exhibit ARR including the fresh market cultivar 'Poinsett 76' (Figure 3.1). As described for 'Vlaspik', 'Poinsett 76' fruit are initially extremely susceptible to infection, and fruits then become increasingly resistant as they complete their exponential growth phase (Figure 3.1 A). As fruits reached full size, at ~16 dpp, they primarily exhibited localized necrosis at sites of inoculation, with occasional successful infection at inoculation sites (Figure 3.1B).

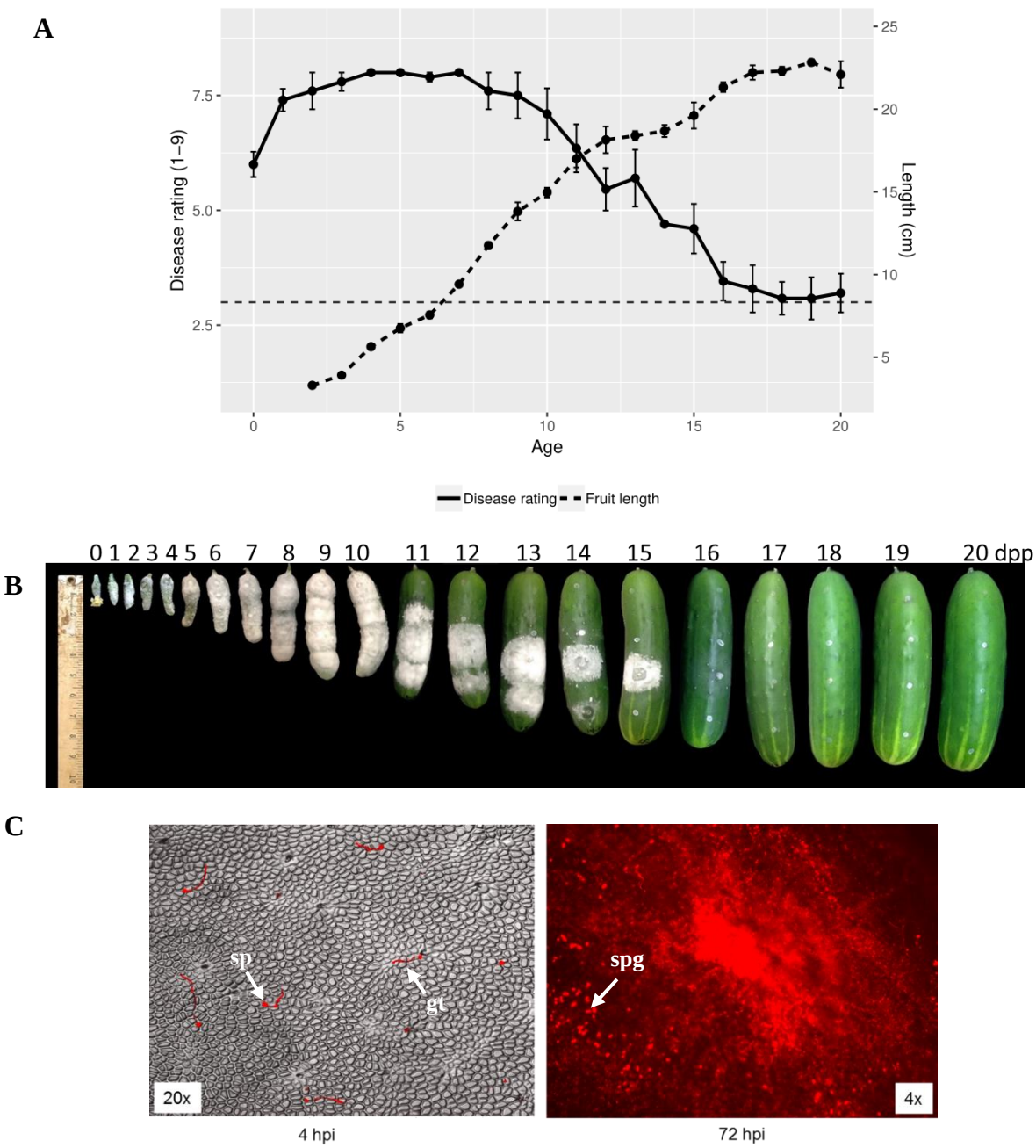

Figure 3.1. Cucumber cultivar 'Poinsett 76' exhibits age-related resistance to *P. capsici*. (**A**) Fruit length and disease rating (DR) as a function of fruit age. Fruit were ranked on a 1-9 DR scale (1=no symptom; 9=extensive mycelial growth and sporulation) at 5 days post-inoculation. The dotted line at DR = 3 represents localized necrosis. Points are means of 5-6 fruit, error bars represent +/- standard error of the mean. (**B**) Representative fruit and disease progression at 5 dpi. (**C**) Fluorescently labeled *P. capsici* on <8 dpp cucumber fruit at 4 and 72 hpi. sp – spore; gt – germ tube; spg – sporangia.

Age-dependent differential transcriptomic responses to infection

As a first step to explore the early stages of infection in susceptible age fruit (8 dpp) we observed germination and growth of a constitutively fluorescent isolate of *P. capsici* NY-0644-1 (Dunn et al., 2013). Consistent with observations of *P. capsici* development on tomato leaves (Jupe et al., 2013), microscopic images taken at 30-minute intervals showed germination and appressoria formation by 4 hpi, extensive growth by 24 hpi, and sporangia formation by 72 hpi (Figure 3.1C).

Based on these results we compared transcriptomic responses of resistant (16 dpp) and susceptible (8 dpp) fruit peels at 0 (uninoculated), 4, 24, and 48 hpi. For each age and timepoint three fruit were inoculated with 10-15 droplets. All inoculation sites for a given fruit were harvested and pooled for sequencing providing ~20M reads per sample. An average of ~82% reads uniquely quasi-mapped to the cucumber transcriptome (Supplementary Figure 1). Pearson's correlations of replicate samples were at least 96% (Supplementary Figure 2) showing high reproducibility among replicates.

Principal component analysis (PCA) confirmed the high within treatment reproducibility (Figure 3.2 A). The first principal component largely reflected time post inoculation, while the second largely reflected fruit age. A similar transcriptional shift in direction and magnitude was observed along the positive direction of PC1 at 4 hpi regardless of age (8 dpp – circles, 16 dpp – triangles) of the tissue, suggesting a somewhat comparable initial response to infection. In contrast, subsequent timepoints (colors) exhibited differential transcriptional responses to infection as evidenced by the PCA. The susceptible 8 dpp samples progressively moved along the positive direction of PC1 with time, while resistant 16 dpp samples largely stayed in same position relative to PC1, suggesting little subsequent change in gene expression.

As was observed by infection phenotyping, successful infection can occasionally occur on resistant 16 dpp fruit. We observed one 16 dpp sample in each of 24 and 48 hpi timepoints to exhibit transcription signatures with similarities to those of infected 8 dpp fruit at the same respective timepoints. These samples had little effect on results (due to treatment of outlier genes in DESeq2), therefore analysis of differential gene expression proceeded including the two samples.

Differential expression analysis showed that approximately 1800 genes were differentially expressed (up or down) at 4 hpi compared to uninoculated tissue, regardless of age, evidence of an extremely rapid and strong response to infection (Figure 3.2 B). As suggested by the PCA, differential expression was markedly different in the susceptible and resistant tissues at subsequent contrasts. While there was an increase in differentially expressed genes in the susceptible 8 dpp fruit peels with time, (4758 and 2505 DEG at 24 and 48 hpi, respectively) the resistant 16 dpp samples had a smaller number of differentially expressed genes at 24 hpi vs 4 hpi (2223) and by 48 hpi only about 500 genes were differentially expressed compared to 24 hpi.

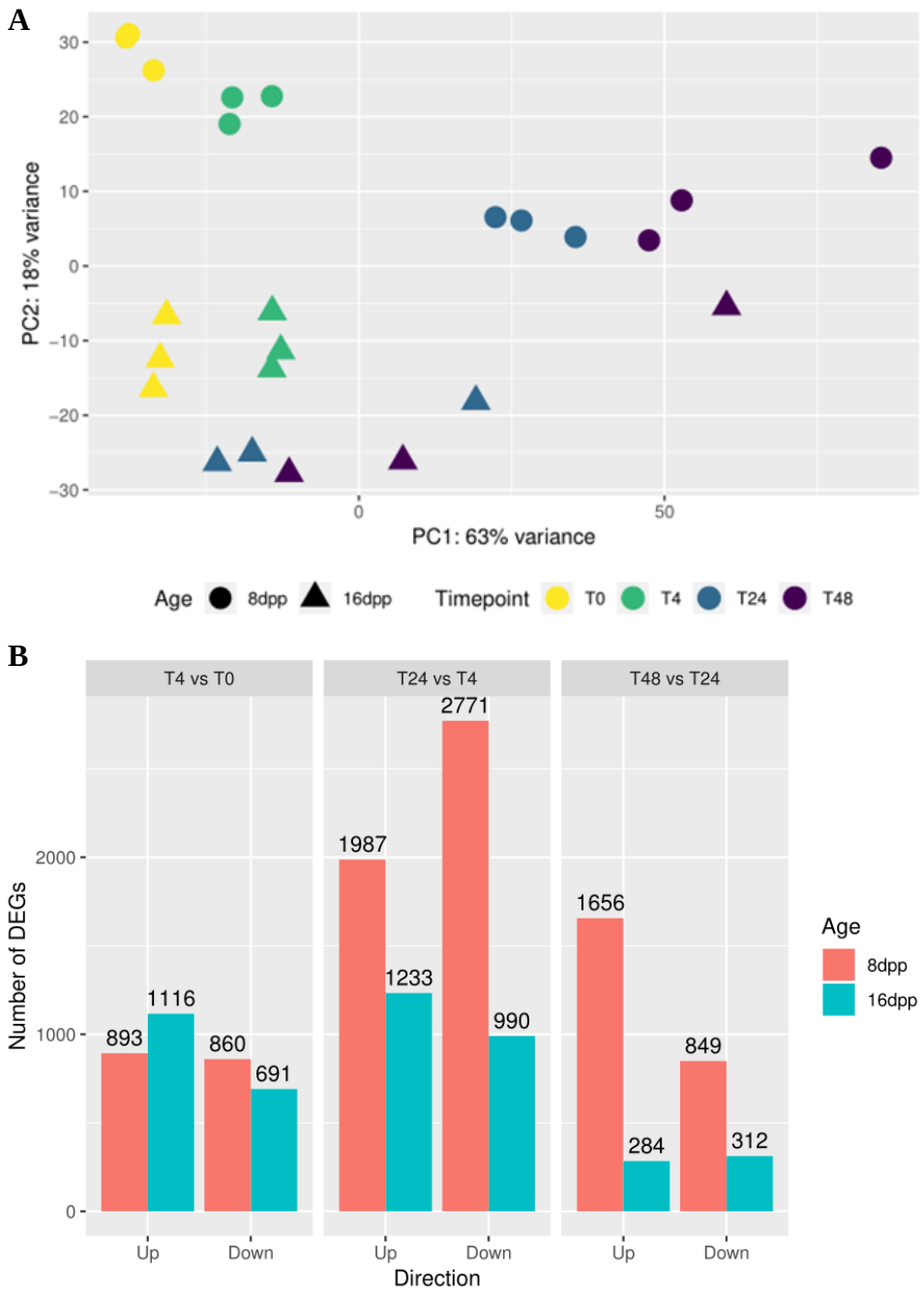

Figure 3.2. Age-dependent differential transcriptomic responses to infection. (A) Principal component analysis (PCA) of 8 and 16 dpp inoculated fruit at 0, 4, 24, and 48 hpi. (B) Number of differentially expressed genes in each of three within-age consecutive contrasts: 4 vs. 0 hpi, 24 vs 4 hpi, and 48 vs 24 hpi.

Resistant-age fruit mount a successful response by 24 hours post inoculation

To further understand the biological processes involved in the two responses, the most significantly enriched GO-terms (Fisher-*weight01* p-value < 0.01) were compared for each consecutive contrast: 4 vs. 0 hpi, 24 vs. 4 hpi, and 48 vs. 24 hpi (Figure 3.3). At 4 hpi, both 8 and 16 dpp fruit upregulated genes in inoculated fruit were strongly enriched for defense related genes. “Response to wounding”, “defense response”, “response to chitin”, “phenypropanoid biosynthetic process, “response to oxidative stress”, “response to karrikin”, “organonitrogen compound catabolic process”, and “drug catabolic process” were of the ten most enriched terms in both ages at this time point. Although the number and GO categories of genes differentially expressed at 4 hpi was comparable between the two ages, fewer than half of the differentially expressed genes in the resistant samples was shared with those differentially expressed in the susceptible samples (Figure 3.4A; blue shading). Analysis of the 613 genes uniquely upregulated in the resistant 16 dpp samples at 4 hpi revealed a potentially unique set of defense related genes involved in an early incompatible interaction.

When comparing 24 hpi to 4 hpi, and 48 hpi to 24 hpi, less than 15% of the thousands of up- and downregulated genes were shared between the ages, respectively (Figure 3.4 B and C). Susceptible 8 dpp fruit continued to upregulate defense (top five enriched terms: “defense response”, “DNA replication”, “translation”, “response to cadmium ion”, and “response to salicylic acid”) while down-regulating photosynthetic processes and other homeostatic processes, such as carbohydrate metabolic processes. In contrast, by 24 hpi, resistant 16 dpp fruit, were upregulating photosynthetic and growth-related genes and downregulating defense (top five downregulated terms: “response to wounding” “ethylene-activated signaling pathway”, “macromolecule localization”, “response to oxidative stress”, “defense response to fungus”)

suggesting a return to normal state (Figure 3.3 B). This is especially evidenced by the large number of inversely regulated genes in the 8 dpp vs. 16 dpp samples at 24 hpi indicating an opposite response (red shading). The set of 251 genes upregulated in 16 dpp and downregulated in 8 dpp was strongly enriched for photosynthesis (*p-value* = 5.2e-13). Collectively these observations suggest that the resistant fruit have successfully mounted a defense by 24 hpi.

Analysis of pathogen growth provides evidence for infection failure in the first 24 hours on resistant fruit

The transcriptomic suggestion of a rapid and potentially successful defense response within 24 hours prompted us to more closely investigate pathogen growth during the first 24 hours of infection using electron microscopy and a high throughput microplate assay. Samples were collected for SEM from 8 dpp and 16 dpp aged cucumber fruit inoculated with droplets of zoospore suspension (5×10^5 spores/ml) at time intervals of 0, 2, 4, 8, 12, 18, and 24 hpi. At each timepoint, three samples were collected, each from a distinct inoculated fruit. The morphological differences between 8 dpp and 16 dpp fruit were readily observed; the younger susceptible fruits had smaller more densely packed cells and trichomes, as well as warts that produced valley regions that in some cases increased spore density due to the surface topography (Figure 3.5 A).

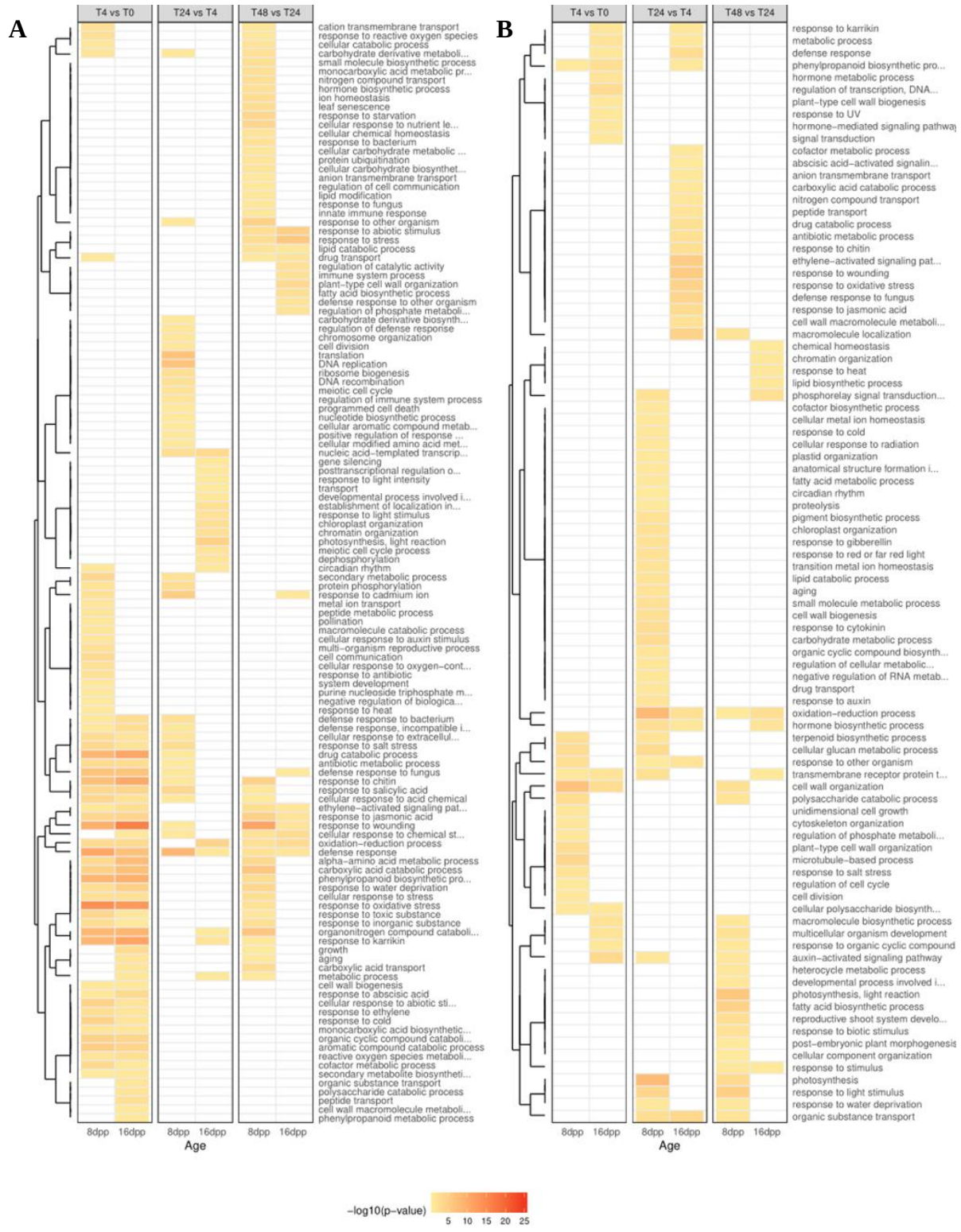

Figure 3.3. Resistant-aged show transient upregulation of defense related genes. Gene Ontology enrichment of up- and down-regulated genes (**A** and **B**, respectively). Each row represents an enriched term at one of the three consecutive contrasts: 4 vs. 0 hpi, 24 vs 4 hpi, and 48 vs 24 hpi. Enrichment p-value threshold of 0.01. Terms clustered by Euclidean distances.

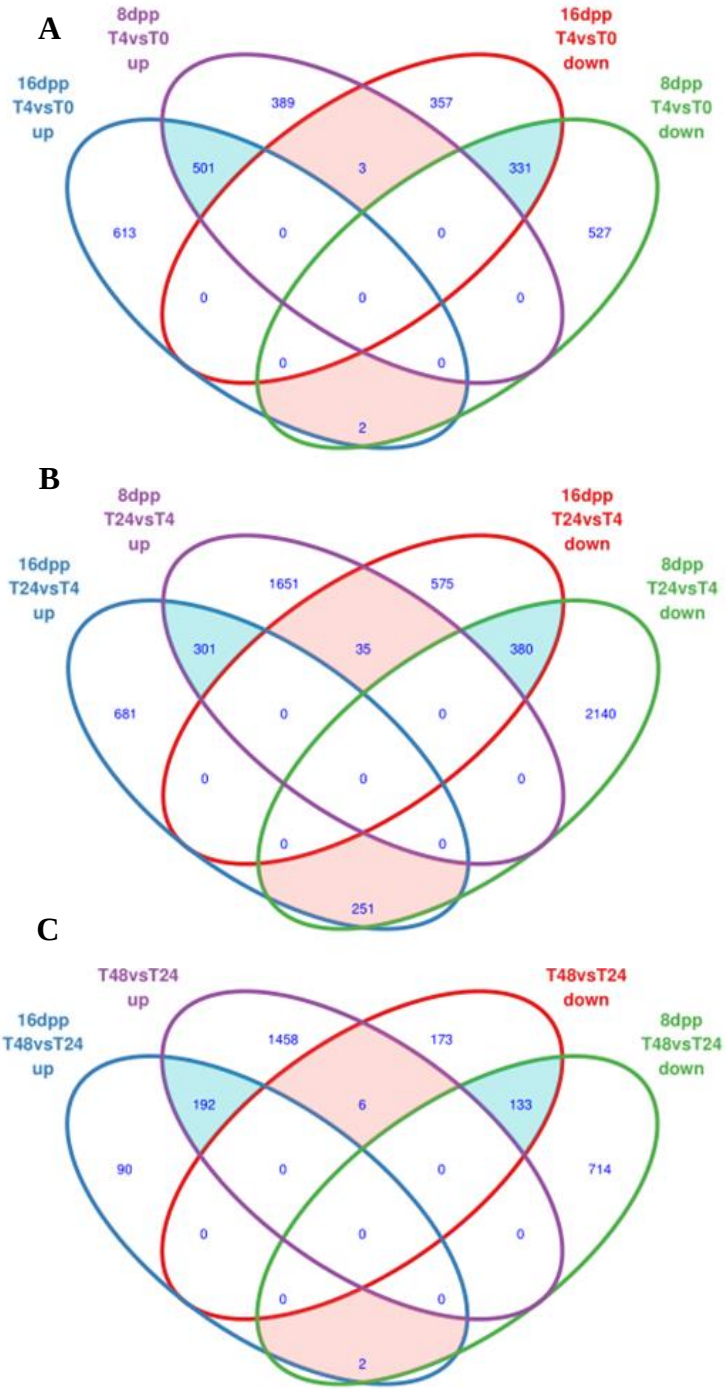

Figure 3.4. Analysis of differentially expressed gene overlap at each timepoint. Venn diagrams comparing all significantly differentially expressed (adjusted P<0.05, fold change ≥ 2) genes in 8 and 16 dpp inoculated fruit at (**A**) 4 vs. 0 hpi, (**B**) 24 vs 4 hpi, and (**C**) 48 vs 24 hpi. Counts in red and blue denote up- and downregulated genes, respectively. Blue and red highlighted areas represent shared- and inverted-differential-expression, respectively.

At 2 hpi, encysted *P. capsici* spores were observed germinating on fruit of both ages. Formation of some appressoria was also observed as early as 2 hpi. By 4 and 8 hpi, some differences were observable between the resistant (16 dpp) and susceptible (8 dpp) fruit. While spores on susceptible fruit continued to germinate and form appressoria, in four out of the six 16 dpp samples, lysed spores and germ tubes were observed, suggesting either preformed antimicrobial compounds or a rapidly induced defense response may inhibit successful infection as early as 4 hpi. As infection progressed, more evidence of failure to infect was observable in the majority of the resistant samples. By 18 and 24 hpi, deflated spores, germ tubes, and hyphae were observed on most of the resistant fruit samples, suggesting that spores that survived an initial defense response may be stopped at a later time, during the first 24 hours. No such histological signs of deflated or burst pathogen structures were observed at any timepoint in samples from susceptible 8 dpp fruit.

Quantitative fluorescence based *in vivo* infection assays provided further evidence of inhibited infection in resistant aged fruit by 24 hpi. After a short lag phase, the signal from fluorescently labeled *P. capsici* on inoculated susceptible 8 dpp fruit grew linearly throughout the 24-hour period (Figure 3.5 B). However, on 16 dpp resistant-aged fruit, intensity of the fluorescent signal plateaued by 8 – 10 hpi, further suggesting early inhibition of pathogen growth. Together, the SEM and bioassay results bolster the transcriptional evidence suggesting that infection may be thwarted by 24 hpi in 16 dpp cucumber fruit.

Transcriptomic investigation of the first 24 hours post inoculation

A second transcriptomic experiment was performed in parallel to the SEM study. Concurrent with the collection of samples for SEM (0, 2, 4, 8, 12, 18 and 24 hpi), inoculated and

uninoculated tissue was harvested for transcriptome analysis using 3′ mRNA sequencing. In total 78 3'-mRNA libraries were sequenced to an average depth of ~5M reads/sample and an average of ~60% reads quasi-mapping to the 3'-extended cucumber transcript sequences (Supplementary Figure 3). Two samples (8dpp_T12_Inoc_1 and 8dpp_T18_Cont_2) had aberrantly low read coverage (<0.5 M reads) and were excluded from analysis. A PCA comparison of timepoints shared between the two transcriptome experiments (0, 4 and 24 hpi) showed tight clustering of samples within their respective timepoints, indicating high reproducibility between the two experiments (Supplementary Figure 4).

The PCA of data from experiment two revealed, modest changes in transcriptomic patterns for uninoculated samples of both ages (open symbols) relative to timepoint 0 (asterisks), likely reflecting a combination of diurnal changes and the effects of fruit detachment from the vine (Figure 3.6 A). In contrast, the inoculated samples (closed symbols) showed strong transcriptional changes, especially for the susceptible 8 dpp fruit. From 4 hpi and beyond, the inoculated 8 dpp samples (circles) exhibited a sequential transcriptomic transition. Conversely, samples collected from the resistant-aged 16dpp fruit (triangles) all clustered together, and relatively closely to uninoculated fruit, from 4 hpi and beyond. Notably, at 2 hpi, samples from uninoculated 8 dpp fruit clustered with uninoculated control, while 2 hpi samples from 16 dpp fruit showed a clear difference from the uninoculated controls, suggesting an earlier response to infection in the resistant-aged fruit.

To further understand the overall trends in gene expression changes in response to infection, all genes that are differentially expressed in at least one timepoint vs. the respective control were displayed as an alluvial plot (Figure 3.6 B). Alluvial plots are comprised of: 1) *Strata* – stacked bars that represent relative counts in each category, in this case upregulated,

downregulated or not differentially expressed (nonDE); and 2) *Alluvia* – curves connecting strata, represent the change in number of observations from one category to another along the x-axis, in this case, the different timepoints post inoculation. Each alluvium can be followed from 2 hpi to 24 hpi and represents groups of genes that share an expression pattern over time (e.g. Up – Down – Up…). The large alluvia from nonDE strata (blue) progressively connecting to either up- or downregulated strata shows a phased response to infection, in which certain genes are involved in early timepoints, while others in later stages of infection.

The figure reveals that response to infection is dramatically different in the two ages. Most evident is that more than double the number of unique genes is differentially expressed at least once in susceptible fruit compared to resistant fruit. Examination of the trends in 8dpp susceptible fruit revealed a pattern of sequential accumulation of differentially expressed genes, as previously suggested by the PCA. The number of differentially expressed genes grew continuously until 18 hpi, and most genes which were differentially expressed at one timepoint continued to be differentially expressed at following timepoints. Of the 679 upregulated genes at 4 hpi, 417 are continuously upregulated in every subsequent timepoint. By 24 hpi, 1411 of the 1585 upregulated genes had been previously upregulated at one and all subsequent timepoints. A similar trend is observable in downregulated genes.

Conversely, by following alluvia of genes initially upregulated at 4 hpi in the 16 dpp resistant-aged samples, it is evident that most are subsequently not differentially expressed at further timepoints. Of the 339 genes upregulated at 4 hpi, 139 genes are not differentially expressed at 8 hpi, and only 40 are continuously upregulated through 24 hpi. Though the number of differentially expressed genes grows until 12 hpi, most genes are timepoint specific, being differentially expressed once or at most at two timepoints. Most striking is the fact that at 24 hpi,

only 255 and 67 genes are up- and down-regulated, respectively, compared to uninoculated tissue, further confirming the culmination of the defense response in resistant-aged fruit.

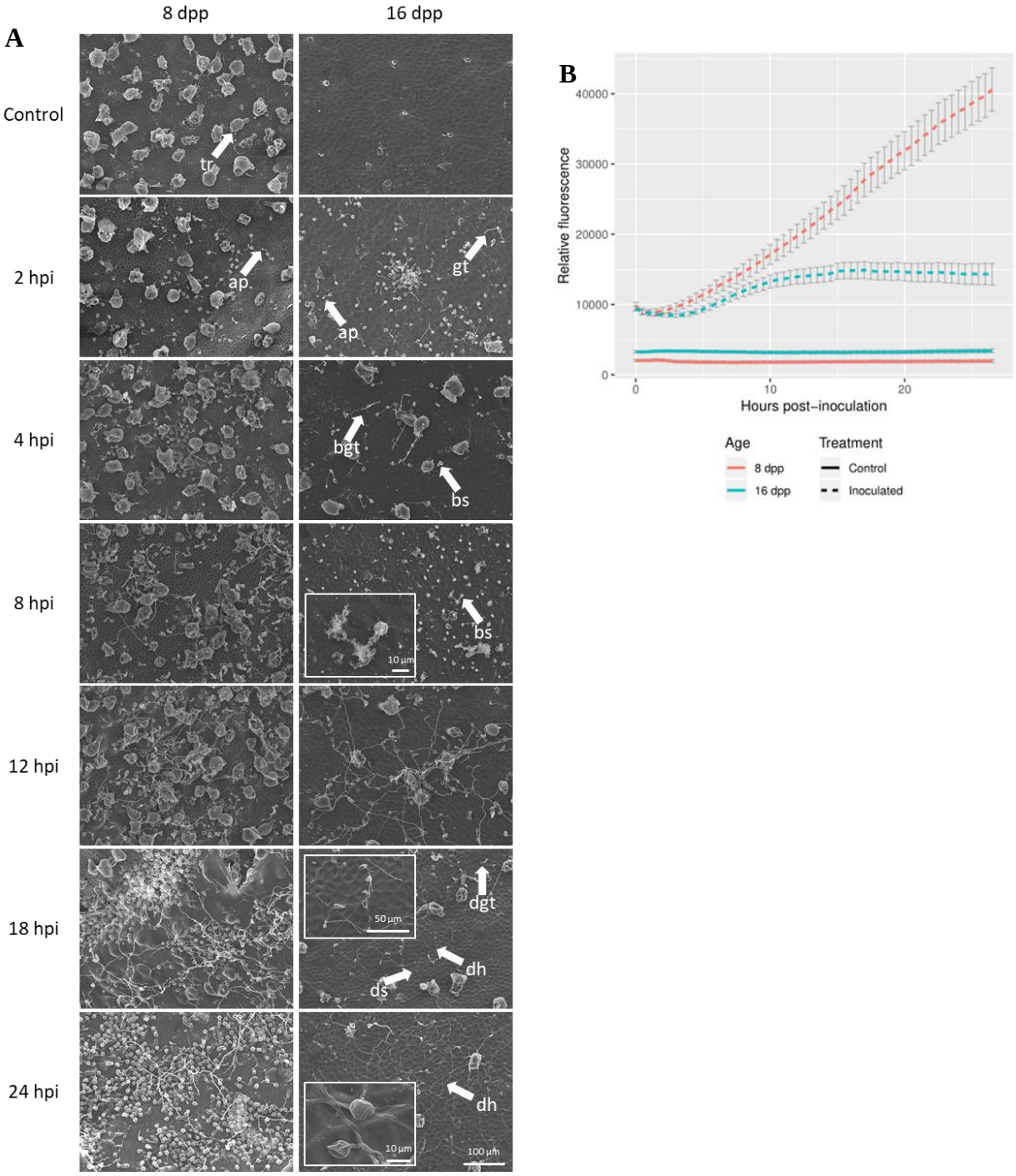

Figure 3.5. Evidence for infection failure in the first 24 hours post inoculation on resistant fruit. (**A**) Scanning electron micrographs of inoculated 8 and 16 dpp fruit. tr – trichrome; ap – appressoria; gt – germ tube; bgt – burst germ tube; bs – burst spore; deflated spores (ds), deflated germ tubes (dgt), and deflated hyphae (dh). Bottom right scale bar for all frames, except for insets. (**B**) High-throughput *in vivo* analysis of pathogen growth on fruit plugs. For inoculated and control treatments, each point is a mean of 36 and 12 replicates, respectively. Error bars are +/- SEM.

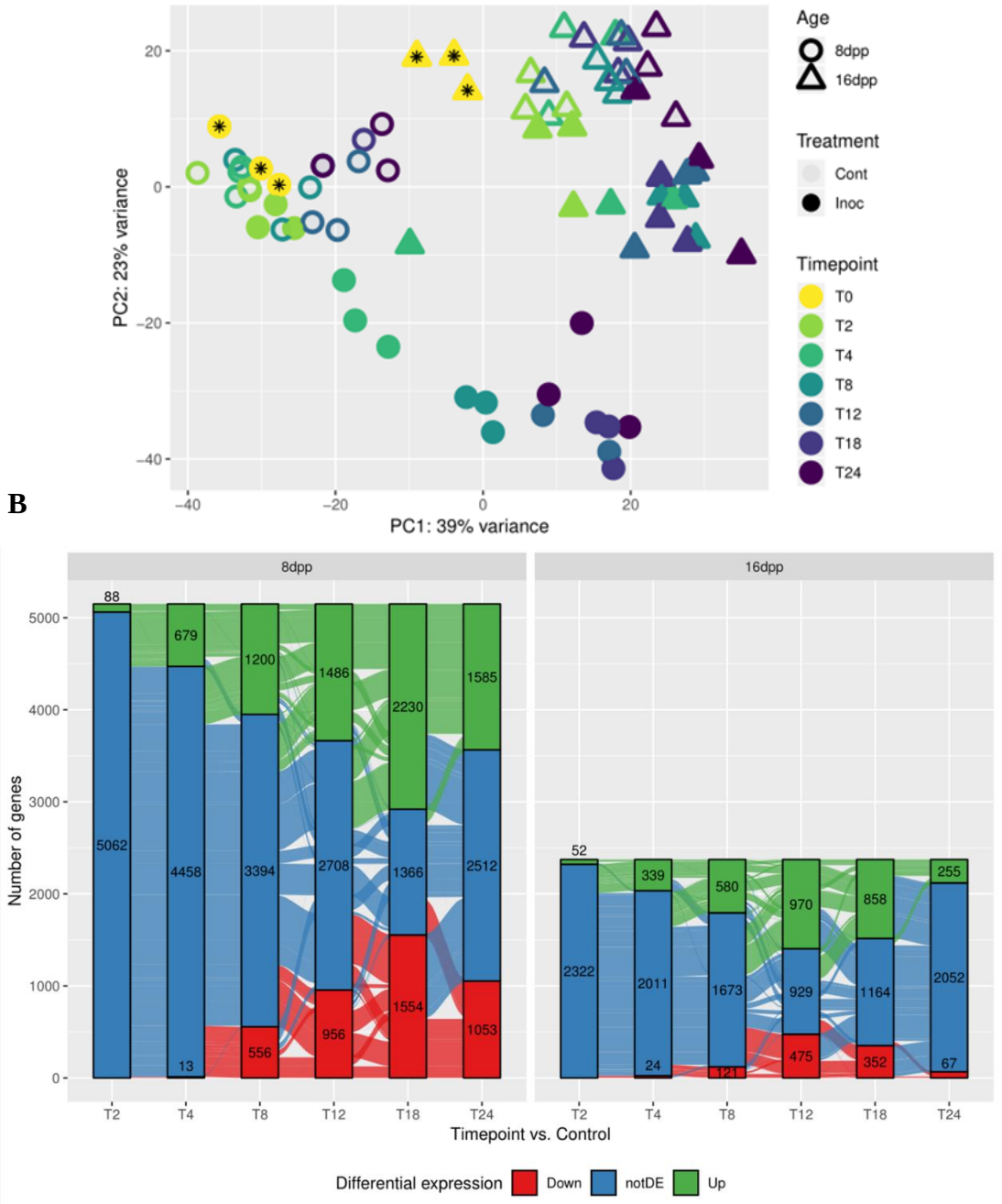

Figure 3.6. Differential transcriptional dynamics within the first 24 hours. (**A**) Principal component analysis of inoculated and control fruit of two ages, 8 and 16 dpp. Samples collected at time 0, which represent both Inoculated and Control treatments, are denoted by an asterisk. (**B**) Alluvial plots showing all genes with significantly changed expression at each timepoint compared to respective controls. Each stacked bar represents the numbers of genes either up- down- or not-differentially (nonDE) regulated at sequential time points. Genes are grouped based on expression patterns throughout time.

Gene co-expression is preserved but not expression patterns over time

To better identify transcriptional co-expression patterns in the data we employed Weighted Gene Co-expression Network Analysis (WGCNA) (Langfelder and Horvath, 2008). Two independent response networks for the susceptible and resistant fruit (Figure 3.7 A, B respectively) were assembled. After module merging (based on eigengene correlation), a total of 15 and 27 modules were defined for the susceptible and resistant inoculated networks, respectively.

Module preservation statistics (Langfelder et al., 2011) showed that gene co-expression was preserved in many modules when comparing the susceptible and resistant networks (Supplementary Figure 5). Module gene overlap (Figure 3.7 C) also showed significant preservation (Fisher's Exact test and FDR < 0.001) of gene co-expression between many modules of the two networks. For example, large subsets of the genes in Susceptible Module 1 were also co-expressed in the resistant network, however these subsets were distributed amongst eight distinct modules, all showing different expression patterns. Thus, while many groups of genes were expressed in concert over time, indicating coordinate regulation regardless of fruit age, these groups also showed diverse module assignment and thus age-specific patterns of expression in response to infection. This suggests a reprogramming of the response network in resistant-aged fruit.

Biological processes identified by weighted gene co-expression network analysis

Using gene module assignment defined by the resistant network, expression patterns of the genes within a given module were compared in control and inoculated fruit of both ages (Figure 3.8). GO-term enrichment analysis of the different modules demonstrated the biological

relevance and function of identified modules (Supplementary File 1). For example, Module R1 (Resistance Module 1) (n genes = 1982) showed patterns of increased expression in response to inoculation in both ages. Uninoculated tissue largely showed unchanging expression levels throughout the entire time course. GO term enrichment showed that this module was strongly associated with translation and ribosome biogenesis suggesting induction of protein synthesis in response to infection.

In Modules R2 and R3 (n genes = 1893 and 1670, respectively) all genes, regardless of age or treatment, showed a similar pattern of change over time. Genes in Module R2, which exhibited gradual decline, were enriched for carbohydrate metabolic process, lipid metabolic process, signal transduction and response to abiotic stimulus. Higher baseline expression of 8 dpp fruit is likely due to the different stage of development, as unharvested fruit at this age are still rapidly growing (Figure 3.1B). Genes in Module R3 showed gradual increase and were enriched for “organonitrogen compound catabolic process”, “aromatic compound catabolic process” as well as” response to water deprivation”. The expression patterns of genes in these modules is probably a result of the fruit being detached during the analysis, i.e. deprived of carbohydrate source and subject to water loss.

Module R13 (n genes = 425) showed a potentially circadian controlled expression pattern, with peak expression at 18 hpi (collected at 6:30 AM) and almost identical patterns of expression in uninoculated fruit of both ages. Inoculated fruit showed a gated expression pattern, wherein the diurnal rhythm is offset in amplitude and period. This module was enriched for processes involved in transcriptional regulation as well as response to light stimulus.

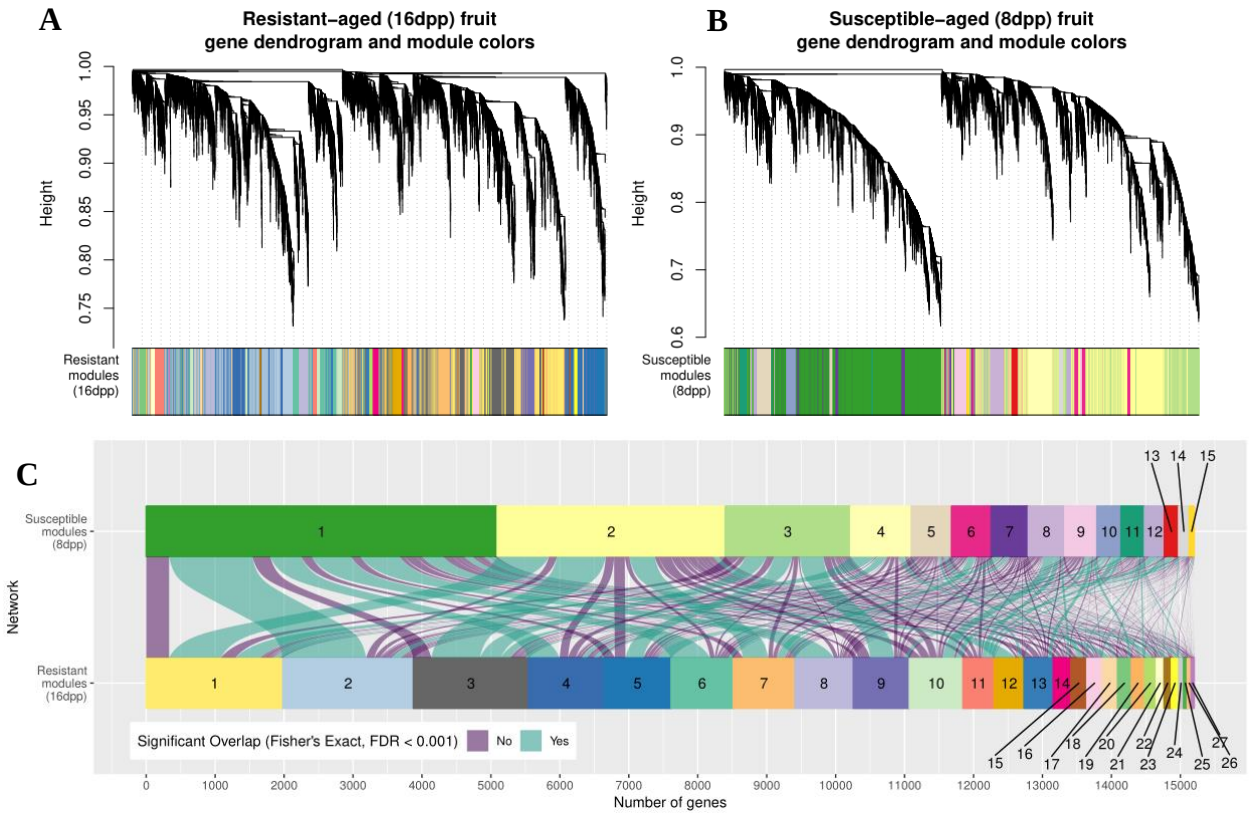

Figure 3.7. Defense response network reprogramming in the two fruit ages. Weighted gene co-expression analysis (WGCNA) was used to analyze all expressed genes in inoculated samples. Separate signed networks were analyzed for resistant (**A**) and susceptible (**B**) fruit. Dendrograms cluster the genes based on their topological dissimilarity. The colored bars underneath the dendrograms show co-expression module assignment. (**C**) Module overlap between the susceptible and resistant networks. Each curve connecting two modules in the different network represents a shared group of genes. Curve width is proportionate to the number of genes. Curve color shows if the overlap between modules is significant based on Fisher's Exact test and an FDR of < 0.001.

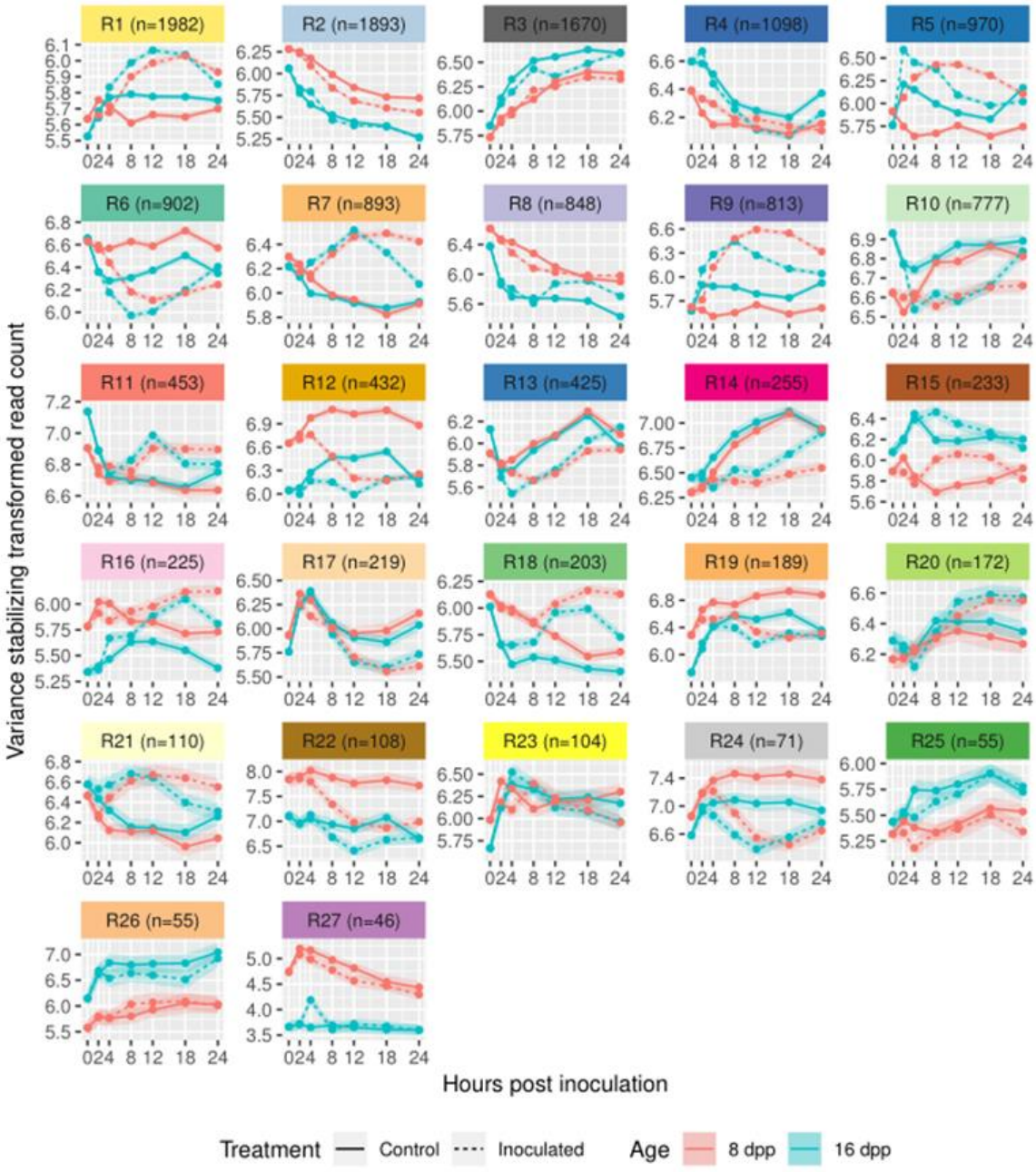

Figure 3.8. Resistant network module gene expression patterns. Each panel represents a module (number of genes indicated in label) from the infection co-expression network of resistant fruit (Figure 3.7A). Comparison of expression patterns of the module-defined genes in the different ages and treatments across time. Data are mean and SEM of variance stabilized normalized read counts for all genes in the module.

Modules induced in early infection of resistant-aged fruit

Several modules indicated differential response to infection between the resistant and susceptible ages. Expression patterns of all genes, in each of the identified resistant network modules, were compared across inoculated samples using regression analysis. A cubic spline basis function was applied to the time variable with three internal knots, splitting the time course into four segments (0-3, 3-8, 8-15, and 15-24 hpi). Most of the modules (22/27) showed significant interaction effects between age and splined modeled time in at least one timepoint, indicating that genes in each module behave differently across time in the two ages, further reflecting the reprogramming of module expression patterns (Supplementary Table 2). Based on the PCA, we were especially interested in modules that showed very early response differences. Six modules had significant interaction effects during the first two spline fractions, 0- 3 hpi and 3-8 hpi (Figure 3.9). Of specific interest were early induced modules that were also associated with defense.

Module R5 (n = 970), is defined by a spike of increased expression in response to inoculation of resistant-aged fruit. Expression levels peak at 2 and 4 hpi followed by decrease in expression starting at 8 hpi. In the inoculated susceptible fruit, the genes identified in this module showed a minimal change in expression prior to 4 hpi. GO term enrichment showed that this module was strongly associated with defense related genes, the five most enriched terms being "response to drug", "response to oxygen−containing compound", "response to organonitrogen compound", "secondary metabolite biosynthetic process" and "response to wounding". Similarly, Module R9 (n = 813) also showed statistically different expression during early infection, with increased expression by 2 and 4 hpi in inoculated resistant-aged fruit. In susceptible fruit these genes show a more gradual increase in expression, which only matches

that of the resistant-aged fruit at 8 hpi. This module was also strongly associated with a resistance response as evidenced by enrichment for “oxidation-reduction process”, “carboxylic acid catabolic process”, “defense response, incompatible interaction” and “aromatic compound biosynthetic process”.

Genes with high module membership are strongly correlated to the module eigengene and thus represent highly connected genes within the module. We used this measure to identify highly connected genes in Modules R5 and R9, as these modules showed patterns of increased expression at early infection timepoints in resistant fruit. The second criterion for selection was those genes that also showed significantly increased expression in resistant inoculated fruit at 2 and/or 4 hpi compared to both the uninoculated control and the inoculated susceptible fruit. We identified 25 genes with a greater than 2-fold expression at 2 and/or 4 hpi in both comparisons as well as a module membership greater than 0.75 (Table 3.1). As expected, the expression patterns of these genes strongly conform to those of the Module eigengene, with a uniquely strong expression at early timepoints in resistant inoculated fruit (Figure 3.10). Many of these genes are annotated to have canonical functions in early defense response, for example associated with reactive oxygen species (ROS) metabolism, gibberellin and ethylene balance, vesicle transport, protein phosphorylation, as well as pathogen perception and response.

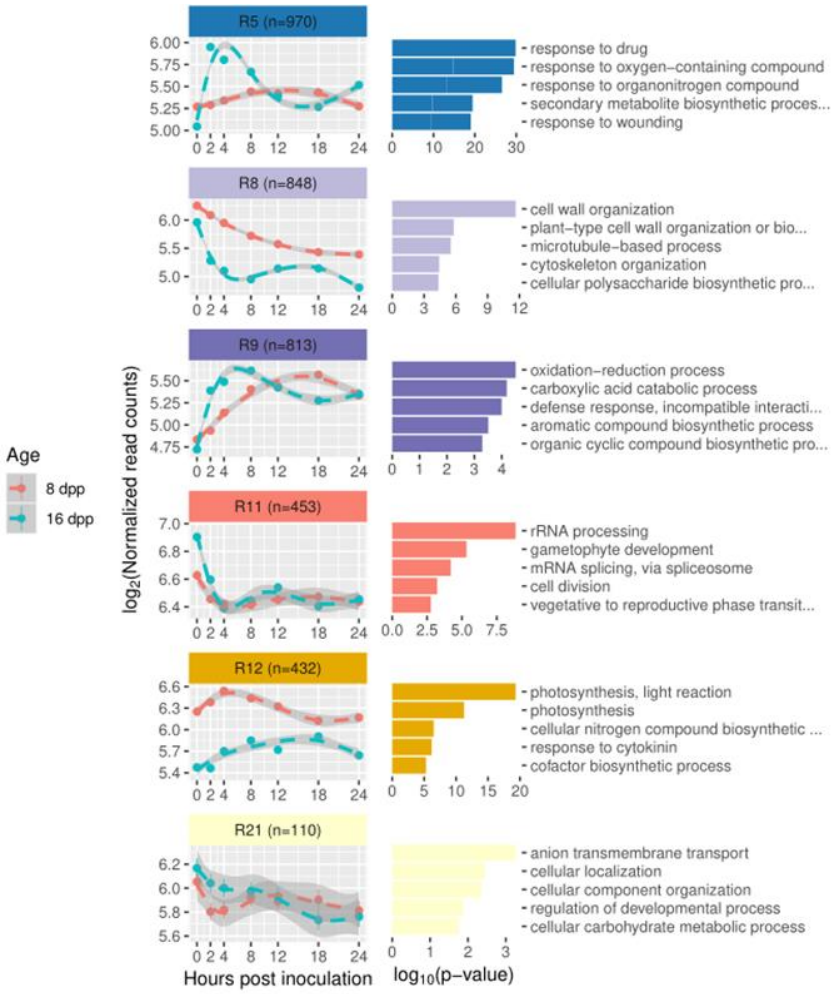

Figure 3.9. Early response modules in the resistant network. Points represent the mean $\log_2$(normalized read count) of inoculated 8 dpp or 16 dpp fruit. Lines and grey ribbons are predicted values (and standard errors) based on regression of $\log_2$(normalized read count) by a natural cubic spline of time with three internal knots (0-3, 3-8, 8-15, and 15-24 hpi). Modules selected are those with a significant interaction effect in one or both first spline fractions (0-3 and 3-8 hpi), i.e. have age-dependent response to infection in early timepoints. The five most significantly enriched gene ontology terms are indicated next to each module.

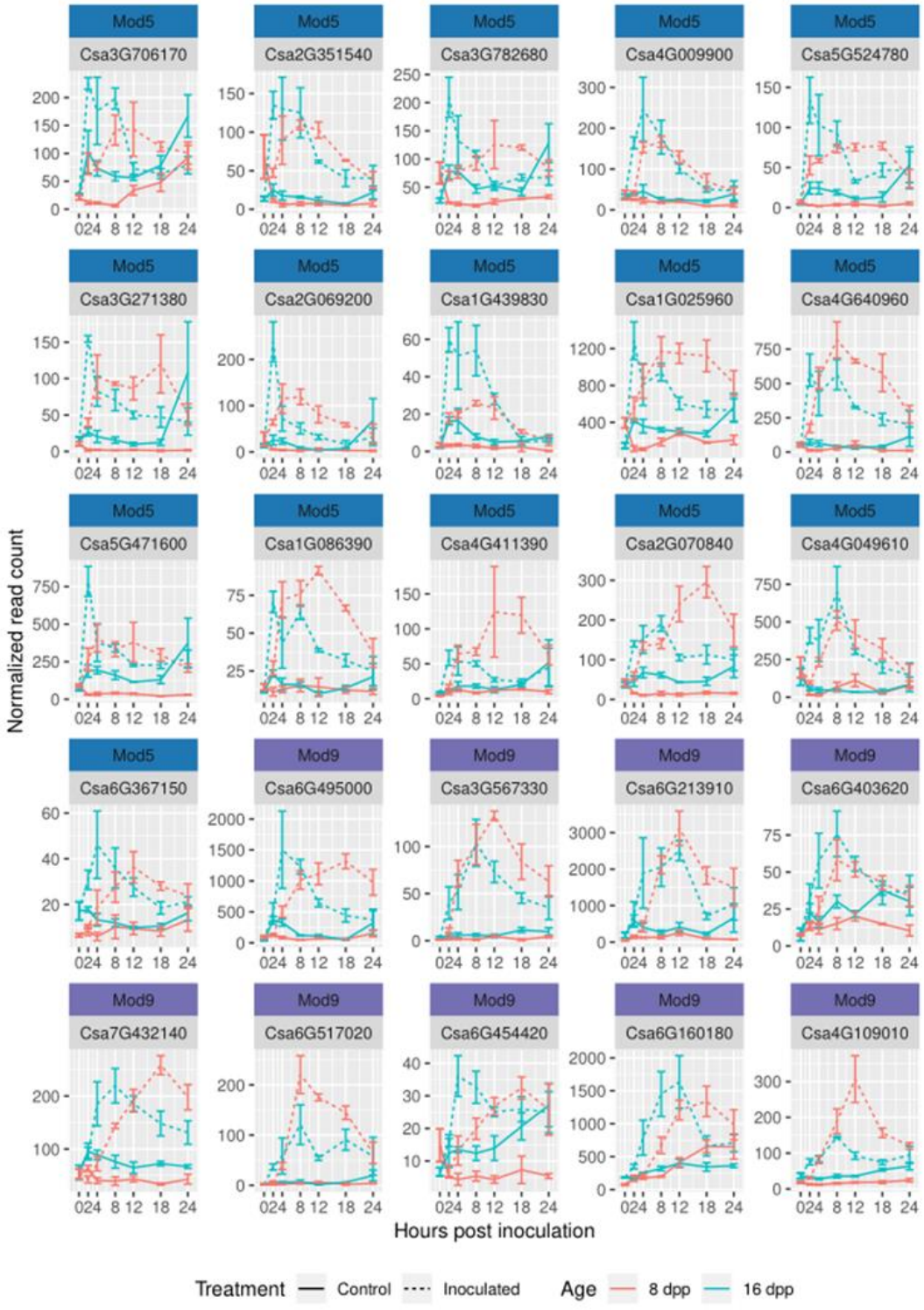

Figure 3.10. Genes induced in early responses to inoculation of resistant fruit. Genes are identified by a module membership > 0.75 and significant fold change of >2 in inoculated resistant fruit (16 dpp) compared to both control resistant fruit and inoculated susceptible fruit (8 dpp). Mean expression for three biological replicates in each age and treatment. Error bars are +/- standard error of the mean.

Table 3.1: Genes with high module membership to Modules R5 and R9 that are differentially expressed in resistant-aged fruit compared to the uninoculated control and inoculated susceptible fruit. Mod. Mem. – Module membership; LFC – log_2(Fold Change); Padj – Benjamini-Hochberg adjusted p-value; R – resistant 16 dpp; S – susceptible 8 dpp; RI – Resistant inoculated; RC – Resistant control.

Module	Gene	Description (Arabidopsis best BLAST hit)	Mod. Mem.	LFC R vs. S	Padj R vs. S	LFC RI vs. RC	Padj RI vs. RC	LFC R vs. S	Padj R vs. S	LFC RI vs. RC	Padj RI vs. RC
			2 hpi					4 hpi			
5	Csa3G706170	unknown protein	0.96	1.39	0.003	1.05	0.394	1.13	0.024	1.22	0.043
	Csa2G351540	chaperone protein dnaJ-related	0.94	1.36	0.032	2.14	0.014	0.49	0.517	2.53	<0.001
	Csa3G782680	3 syntaxin of plants 121	0.92	1.52	<0.001	1.37	0.014	0.80	0.066	0.77	0.176
	Csa4G009900	inositol polyphosphate kinase 2 beta	0.92	1.82	<0.001	1.93	0.006	0.65	0.256	2.29	<0.001
	Csa5G524780	NAD(P)-binding Rossmann-fold superfamily protein	0.91	1.24	0.037	2.21	0.006	0.77	0.249	1.91	0.006
	Csa3G271380	Regulator of Vps4 activity in the MVB pathway	0.91	1.73	0.008	2.22	0.015	-0.26	0.772	1.77	0.032
	Csa2G069200	cinnamate-4-hydroxylase	0.90	1.63	0.031	2.71	0.007	-0.56	0.541	1.38	0.184
	Csa1G439830	gibberellin 2-oxidase	0.89	1.61	0.001	1.76	0.013	1.37	0.008	1.65	0.008
	Csa1G025960	WRKY DNA-binding protein 33	0.89	1.17	0.005	1.54	0.007	-0.05	0.934	1.12	0.035
	Csa4G640960	HXXXD-type acyl-transferase family protein	0.89	1.52	0.045	2.56	0.01	-0.28	0.782	2.31	0.006
	Csa5G471600	EXS (ERD1/XPR1/SYG1) family protein	0.88	1.48	0.001	1.94	0.001	-0.09	0.898	0.92	0.143
	Csa1G086390	receptor-like kinase in in flowers 3	0.87	1.36	0.002	1.50	0.024	-0.58	0.255	1.47	0.013

Table 3.1 (cont'd)

	Csa4G411390	Glycosyltransferase family 61 protein	0.87	1.57	0.009	2.04	0.019	-0.19	0.822	1.47	0.066
5	Csa2G070840	Calcium-dependent phospholipid-binding Copin	0.83	1.19	0.005	1.49	0.015	0.18	0.751	1.13	0.039
	Csa4G049610	1-aminocyclopropane-1-carboxylic acid (acc) synthase 6	0.82	1.72	0.019	2.33	0.023	0.59	0.518	2.43	0.003
	Csa6G367150	ACT domain repeat 4	0.80	1.42	0.006	0.73	0.999	1.33	0.012	1.74	0.006
	Csa6G495000	root hair specific 19	0.95	1.68	0.004	0.41	0.999	1.42	0.022	1.98	0.004
	Csa3G567330	WRKY DNA-binding protein 75	0.94	1.74	0.004	2.51	0.006	-0.38	0.618	2.72	<0.001
	Csa6G213910	Peroxidase superfamily protein	0.93	2.14	<0.001	0.31	0.999	1.88	0.003	1.97	0.007
	Csa6G403620	unknown protein	0.92	1.17	0.037	0.47	0.999	1.17	0.04	1.71	0.009
9	Csa7G432140	HOPW1-1-interacting 1	0.90	1.03	0.005	-0.05	0.999	1.31	<0.001	1.05	0.015
	Csa6G517020	alternative NAD(P)H dehydrogenase 1	0.90	1.96	0.005	2.41	0.022	0.56	0.518	2.70	0.001
	Csa6G454420	unknown protein	0.86	1.23	0.029	0.30	0.999	1.34	0.013	1.36	0.041
	Csa6G160180	ethylene-forming enzyme	0.84	0.74	0.104	1.04	0.263	1.77	<0.001	1.55	0.001
	Csa4G109010	alternative oxidase 2	0.80	1.28	<0.001	1.17	0.015	0.08	0.867	1.57	<0.001

5

Discussion

A rapid infection meets a rapid response

The infection process of *Phytophthora* spp. has been studied extensively on many plants and crops (Erwin and Ribeiro, 1998). However, there are clear differences between the rates and severity of infection in different species and hosts. For example, *P. infestans* infections on potato and tomato are largely symptomless until 3 days post infection (dpi) (Nowicki et al., 2011). As such, many transcriptome studies examine much later time points post inoculation (eg. Gyetvai et al., 2012; Zuluaga et al., 2016). In contrast, *P. capsici* has a rapid infection cycle with visible symptoms, often within 24 hpi, and can reach asexual sporulation by 2-3 dpi (Lamour et al., 2012).

Using both fluorescent and scanning electron microscopy, we observed that *P. capsici* infection of susceptible young cucumber fruit is also extremely rapid. On susceptible-aged fruit, hyphal growth was observed as early as 8 hpi and progressed rapidly throughout the first 24 hours, indicating a successful biotrophic infection. Using *in vivo* high-throughput bioassay, we quantitatively observed a detectable linear growth rate on cucumber fruit peel samples as early as 4-6 hpi, again suggesting quick establishment and rapid progression during this time. As described on other hosts (Jupe et al., 2013), our fluorescent microscopy showed that by 72 hpi asexual reproductive sporangia have been formed in cucumber. It is this rapid infection and reproductive cycle that allows *P. capsici* to be such a devastating pathogen in the field.

As we observed in our previous studies using other cucumber cultivars (Ando et al., 2015; Mansfeld et al., 2017), cucumber fruit of cultivar ‘Poinsett 76’ exhibit ARR at approximately 16 dpp, coinciding with the end of exponential growth. Evidently, inhibition of

zoospore germination is not the mechanism of ARR; regardless of fruit age, within two hours of inoculation most *P. capsici* zoospores have already encysted and germinated (Figure 3.5), and some had formed appressoria. Strikingly however, the SEM of inoculated resistant fruit revealed histological signs, such as burst or lysed spores, as early as 4 hpi, and consistently by 8 hpi.

By 24 hpi, all resistant-aged samples examined either showed no pathogen present or deflated/unviable spores and hyphae. None of these histological signs were present in susceptible samples, on which infection proceeded normally. Similarly, a study of Port-Orford-cedar plants resistant to *P. lateralis* showed a reduction in pathogen tissue as well as deflated hyphae and spores at 24 hpi (Oh, 2004). Though there is limited histopathological evidence of such a severe defense response, similar signs of *Phytophthora* spp. (including *capsici)* inoculum death are observed after exogenous application of phytochemicals such as garlic root exudates or terpenoid-containing essential oils from oregano and other plants (Malajczuk, 1988; Soylu et al., 2006; Khan and Zhihui, 2010; Khan et al., 2011). Evidence in other fungal pathosystems of spore- and hyphal membrane disruption by preformed or induced Defensin proteins also exists (eg. de Beer and Vivier, 2011; Sagaram et al., 2011). Thus, the rapid histological signs of pathogen death might result from preformed potentially antimicrobial compounds, as implicated from peel extract assays and transcriptomic analysis of non-inoculated developing peels (Ando et al., 2015; Mansfeld et al., 2017), or a rapidly induced defense response.

Rapid responses to *P. capsici* have been observed in incompatible reaction on Arabidopsis leaves where failure to penetrate, ROS bursts, callose deposition, and hypersensitive cell death occurred within 24 hpi (Wang et al., 2013). Our *in vivo* bioassay further confirmed that pathogen growth was inhibited by 8-10 hpi on resistant fruit. The findings indicating rapid inhibition of infection were further supported by transcriptional evidence showing that induced

rapid activation of defense related genes at 4 hpi was followed by active downregulation of defense related genes in the peels of resistant age, 16 dpp fruit, by 24 hpi (Figures 3A and B). This downregulation of defense makes intuitive sense if the pathogen has been eradicated or is no longer infecting the host. Conversely, successful infection of tomato by *P. capsici* infection, was accompanied by downregulation of genes associated with primary metabolism processes (Jupe et al., 2013). While we observed similar down-regulation in susceptible cucumber fruit, by 24 hpi resistant-aged fruit showed upregulation of photosynthesis and other metabolic processes, suggesting a return to a "normal" or uninfected state after pathogen defeat.

Together the evidence from microscopy, bioassay and preliminary transcriptome study suggested that biological processes prior to 24 hpi are important in conferring ARR. We thus performed a high-resolution transcriptomic time course analysis within that time frame including inoculated and control tissue at 0, 2, 4, 8, 12, 18 and 24 hpi. Congruent with our first transcriptome experiment, gene expression changes over time were drastically different between resistant and susceptible aged fruit. Double the number of genes were involved in the susceptible response, and susceptible fruit exhibited progressive waves of gene expression changes, peaking at 18 hpi (Figure 3.7 B). A high-resolution transcriptional time series of the compatible response of Arabidopsis to infection by the fungal pathogen *Botrytis cinerea* showed that most differential expression occurred at ~18-30 hpi (Windram et al., 2012). In *Botrytis* infection of Arabidopsis, these timepoints are post-pathogen penetration, and thus the increased gene expression at these times may represent a response which is too late to inhibit infection (Windram et al., 2012). The increased expression at comparable timepoints in our data might suggest a similarly failed defense response in susceptible-aged fruit. In contrast, in the resistant fruit of our study,

relatively few genes were differentially expressed at 24 hpi compared to uninoculated samples, providing an additional indication that the pathogen defense response is largely completed.

A reprogramming of gene co-expression networks of infection at the resistant age

To further understand the effect of fruit age on gene expression patterns in response to *P. capsici* inoculation, gene co-expression networks of both the susceptible and resistant interactions were analyzed using WGCNA (Langfelder and Horvath, 2008). The difference in the number of modules identified and module preservation patterns suggest that the co-expression response in resistant-age fruit was reprogrammed as compared to the network on susceptible fruit (Figure 3.7). While genes within a co-expressed set share similar regulatory mechanisms, these are employed at different times and with different patterns during the infection of resistant and susceptible-aged fruit. Network reprograming in resistant wild type plants and effector-triggered-immunity compromised mutants' responses to infection by *P. syringae* was similarly observed in Arabidopsis (Mine et al., 2018). Specifically, similar genes are activated in compatible and incompatible interactions; however, their timing and expression patterns were altered.

While we identified several modules that exhibit similar expression patterns regardless of infection (i.e. likely a response of fruit detached from the plant), most modules were impacted by infection (Figure 3.8). We focused our analysis on the differences in gene expression patterns of genes identified to be induced by inoculation in the resistant network. In PCA, susceptible samples at 2 hpi clustered with non-inoculated controls (Figure 3.6 A). Conversely, resistant-aged samples at 2 hpi showed a distinct transition along both PC1 and 2 away from the uninoculated controls, suggesting a more rapid transcriptional defense response in these samples. We thus were interested in modules showing differential expression patterns at early timepoints. As optimal timing of defense response has been shown to be crucial for successful resistance

(Tang et al., 2017), the ability to mount a successful defense could be attributed to this early response.

By performing cubic spline regression on module gene expression curves, we identified gene co-expression modules associated with defense that are differentially activated in resistant-aged fruit as early as 2 and 4 hpi (Figure 3.9). Of specific interest were module R5 and R9; in both cases increases in gene expression were delayed in susceptible relative to resistant-aged fruit. Genes of interest were identified using a combination of module membership statistics and differential expression analysis (Figure 3.10). Among the 25 candidate genes of interest showing resistance-specific increase at 2 or 4 hpi, were two WRKY transcription factor homologs *Csa1G025960* (*AtWRKY33,* BLAST E = 2.8e-119) and *Csa3G567330* (*AtWRKY75,* BLAST E = 7.4e-44). While *AtWRKY75* is reported to be associated with phosphate deficiency (Devaiah et al., 2007), its cucumber homologue may function in defense to pathogen infection. *AtWRKY33,* however, has been shown to be important in resistance to *Alternaria brassicicola* and *Botrytis cinerea* in Arabidopsis (Zheng et al., 2006; Birkenbihl et al., 2012). *AtWRKY33* is also rapidly induced by the flg22 epitope as part of microbe-associated molecular pattern immunity, with downstream targets involved in ethylene and camalexin synthesis as well as other transcription factors and pathogen receptors (Birkenbihl et al., 2016). Furthermore, in a proteomic study of *P. capsici* infection, the tomato WRKY33 homolog protein was found to be induced by 8 hpi and localized to the nucleus (Howden et al., 2017).

Among the targets of WRKY33 in Arabidopsis are ethylene biosynthesis genes (Birkenbihl et al., 2016). We further identified that the two ethylene synthesis genes *Csa4G049610* and *Csa6G160180,* encoding 1-aminocyclopropane-1-carboxylate synthase and 1-aminocyclopropane-1-carboxylate oxidase, respectively, are both induced as part of Modules R5

and R9 respectively. Ethylene is generally thought to be important in defense response against fungi and necrotrophic pathogens (Broekgaarden et al., 2015). Specifically, ethylene response, but not SA or JA, was shown to be crucial for inhibition of *P. capsici* growth in habanero pepper, (Núñez-Pastrana et al., 2011). Blocking ethylene perception by means of exogenous application of silver nitrate reduced this inhibition (Núñez-Pastrana et al., 2011). Moreover, silencing of ethylene signal transduction in *Nicotiana benthamiana* resulted in loss of ARR to *P. infestans* (Shibata et al., 2010). The uniquely increased expression of the cucumber *WRKY33* and downstream upregulation of ethylene synthesis observed in resistant fruit could thus be a central component in regulation of the successful defense response in cucumber ARR.

Consistent with a successful hypersensitive response in resistant-age fruit, this group of early induced genes also included two putative peroxidases (*Csa6G213910* and *Csa6G495000*), an NAD(P)H-dehydrogenase (Csa6G517020), as well as an alternative oxidase (Csa4G109010) that could potentially serve in modulating ROS within the first few hpi (Quan et al., 2008). Other genes identified to be potentially involved in defense are genes involved in vesicle transport (*Csa3G782680* and *Csa3G271380*), as well as a signal transduction (*Csa4G009900* and *Csa1G086390*) and specialized metabolism (*Csa2G069200*, *Csa4G640960* and *Csa4G411390*). Finally, *Csa2G070840*, which putatively encodes a calcium-dependent phospholipid-binding copine family protein, might also be important in fine-tuning the response to infection, as its homolog in Arabidopsis functions in stomatal closing during infection and regulation of several resistance receptor genes (Li et al., 2009; Gou et al., 2015). All these genes are canonically involved with response to pathogens, and so their early activation in response to inoculation in resistant fruit could be crucial in conferring ARR by limiting pathogen establishment in early stages of infection.

Conclusion

Many transcriptomic comparisons of plant-pathogen interactions have been performed utilizing both susceptible and resistant genotypes. Yet often the resistant material is of wild or ancestral nature and hence these comparisons may have inherent biases due to any genomic presence/absence variances of resistance or other genes (i.e., Bayer et al., 2016). Even in cases where transgenic plants, mutants or near isogenic lines are compared, pleotropic effects might be present (eg. Krattinger et al., 2016). The ability afforded by an ARR pathosystem to examine both susceptible and resistant responses within the same plant genotype helps us gain valuable insight into the mechanisms that confer plant disease resistance. We have previously shown that developmentally accumulated chemical compounds have an inhibitory effect on pathogen growth (Ando et al., 2015; Mansfeld et al., 2017). Thus, the findings herein, of extremely rapid pathogen defeat could be due to such preformed specialized metabolites.

We also observed an ontogenically manifested capacity to rapidly respond to inoculation, suggesting developmental regulation of an upstream signaling factor. In our previous study we observed developmental upregulation of four pathogen receptors, uniquely in ARR expressing fruit (Mansfeld et al., 2017). Similar in mode to the rice Xa3/Xa26 and Xa21 receptors (Cao et al., 2007; Zhao et al., 2009), a developmental upregulation of one of those receptors in cucumber could allow the perception of *P. capsici* at resistant ages, and thus activation of transcription factors like WRKY33 and its downstream ethylene and other defense response targets. Ultimately, these two developmentally regulated mechanisms, chemical defenses and rapid response (Figure 3.11), could be working in concert to protect the cucumber fruit and seed, and thus the investments made in producing future generations.

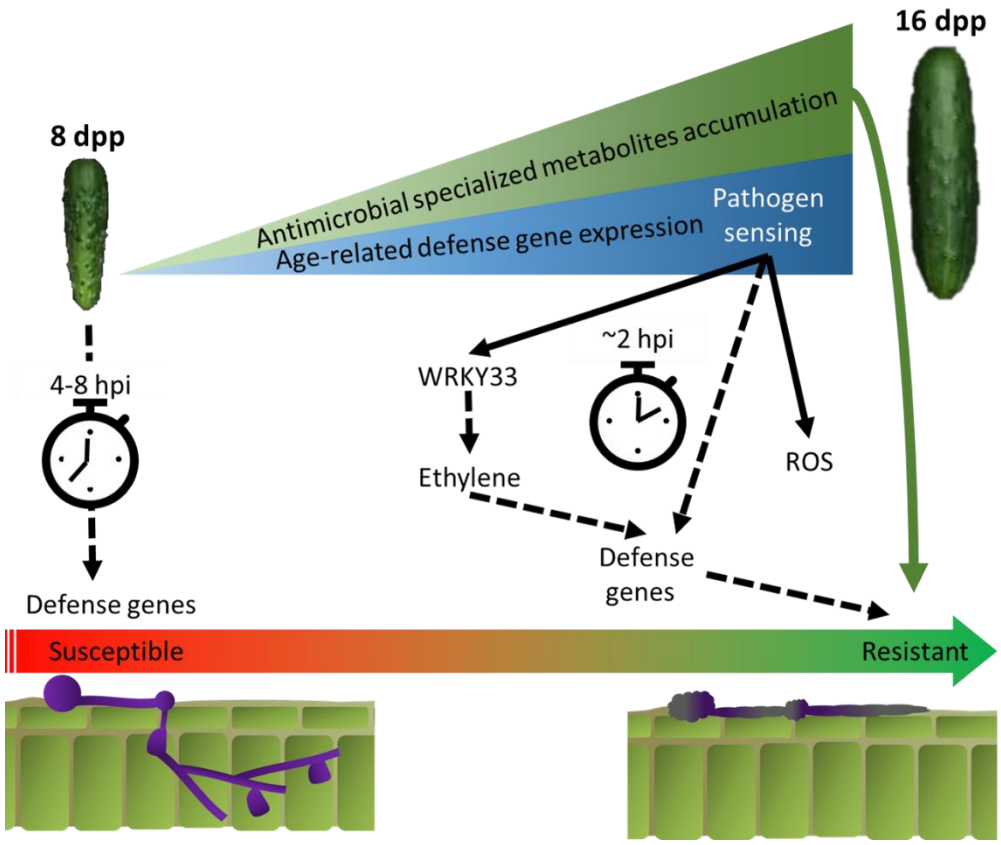

Figure 3.11. Hypothesized model for cucumber age-related resistance to *P. capsici*. In young susceptible fruit there is low accumulation of potentially antimicrobial specialized metabolites. Furthermore, a delayed response (> 8 hpi) to pathogen sensing may be too late to limit pathogen establishment. In resistant-aged fruit, the accumulation of metabolites could directly inhibit pathogen growth. Developmentally regulated expression of receptor-like gene(s) allows the sensing of pathogen-associated molecular patterns or effectors, and thus mediates an early response to infection. Transcription factors such as WRKY33 are expressed and their downstream targets including ethylene synthesis genes and other defense genes are activated. In resistant-aged fruit, metabolism of reactive oxygen species (ROS) is also activated in an early response to infection likely further mediating a strong defense response. By 24 hpi pathogen presence is limited on the fruit surface and mostly non-viable spores and hyphae remain.

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CHAPTER IV

QTLseqr: An R Package for bulk segregant analysis with Next-Generation Sequencing

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Author contributions:

BNM wrote the code for the software with support from RG. The manuscript text was written by BNM and both authors reviewed and approved the manuscript.

Abstract

Next-Generation Sequencing Bulk Segregant Analysis (NGS-BSA) is efficient in detecting quantitative trait loci (QTL). Despite the popularity of NGS-BSA and the R statistical platform, no R packages are currently available for NGS-BSA. We present QTLseqr, an R package for NGS-BSA that identifies QTL using two statistical approaches: QTL-seq and G'. These approaches use a simulation method and a tricube smoothed G statistic, respectively, to identify and assess statistical significance of QTL. QTLseqr can import and filter SNP data, calculate SNP distributions, relative allele frequencies, G' values, and $\log_{10}$(p-values), enabling identification and plotting of QTL. The source code is available at https://github.com/bmansfeld/QTLseqr.

CHAPTER V

Conclusion and future directions

Conclusion

The complexity and appeal of studying ARR stem from the intersection of developmental biology and disease resistance. There are several hypothesized mechanisms by which ARR could be conferred (Whalen, 2005; Develey-Rivière and Galiana, 2007). Accumulated physical or chemical barriers could inhibit pathogen penetration or germination (Meldau et al., 2012; Barton and Boege, 2017), while developmentally regulated components in defense signaling could function to employ the plant's defense programs (Cao et al., 2007; Zhao et al., 2009; Zou et al., 2018). In the research herein, we sought to elucidate the mechanism controlling this ontogenic resistance in cucumber fruit by employing an array of genetic, genomic, metabolomic and microscopic approaches. In any of the cases above, whether physical, chemical, or signaling-associated, a developmentally regulated transcriptional change should be observable.

In experiments described in Chapter II we sought to identify such a change. Though fruit development is a transcriptionally complex process that involves thousands of genes, by coupling developmental transitions with genetic diversity for ARR, we were able to filter out developmental effects and focus on genes potentially contributing to ARR. We identified unique upregulation of defense related factors in resistant-aged fruit peels, including resistance genes and transcription factors. We also identified an enrichment for genes involved in specialized metabolism which prompted an untargeted metabolomic analysis. Though this approach we identified ARR-associated accumulation of metabolites, specifically terpenoid glycosides, that may act as antimicrobial components. We also confirmed previous observations in our lab indicating marked transcriptional transitions to defense at the end of fruit growth (Ando et al., 2012; Ando et al., 2015). Defense related gene ontology terms were of the most enriched in 16 dpp fruit, regardless of genotype suggesting a model in which a developing fruit finalizes its

growth and transitions to defend its metabolic investment before ripening. Such a model is consistent with recent understanding about growth/defense tradeoffs and the optimal defense hypothesis, which posits that defensive resources are allocated to tissues that are most valuable or vulnerable to biotic attack (Meldau et al., 2012; Neilson et al., 2013; Huot et al., 2014). Research in Chapter II was published in the journal *Horticulture Research* (Mansfeld et al., 2017).

We were also interested in the biological processes involved in response to infection by *P. capsici*. Furthermore, we anticipated that comparisons between susceptible fruit and resistant-aged fruit would help elucidate the mechanism of resistance. In Chapter III, we utilized both microscopic and transcriptomic approaches and strikingly observed that pathogen infection on resistant age fruit (or ARR expressing fruit) is largely inhibited by 8-10 hpi. Additionally, using weighted gene co-expression analysis, a strong early response to infection was observed uniquely in resistant-aged fruit. Using a combination of differential expression analyses and module membership we observed an early upregulation of several genes in resistant-age fruit. For example, transcription factors such as *WRKY33* are expressed and their downstream targets including ethylene synthesis genes and other defense genes are activated. Together these results suggested that a developmentally regulated ability to respond to pathogen sensing was crucial for resistance. This induced response, however, could also function alongside the accumulated chemical defenses observed in Chapter II.

While the above experiments identified several genes that are putatively associated with ARR and shed light on a potentially induced mechanism, genetic analyses are necessary to directly link genomic loci linked to the trait. To this end we chose to employ a bulk segregant analysis approach. However, as no easy-to-use computational tools were available for these

analyses, I developed a software package, QTLseqr to use in our own experiments, but also as a tool for the plant breeding and genetics community. A manuscript describing and testing the software was published in the journal *Plant Genome* (Mansfeld and Grumet, 2018). QTLseqr has already aided researchers working in a diversity of crops for agricultural traits as well as in genetic studies of other organisms such as Drosophila and yeast.

In conclusion, the research herein suggests the ARR to *P. capsici* in cucumber is conferred by developmentally acquired induced responses, potentially combined with developmentally accumulated chemical defenses. These induced responses are activated within 2-4 hpi in resistant-aged fruit, suggesting that some mechanism of defense signal transduction must be in place prior to contact by the pathogen. More research is necessary to further elucidate the genes and casual variants that confer ARR.

Future directions

We have utilized several approaches to identify genes and loci associated with ARR, and while several candidate genes have been identified, more work is required to confirm the function of these candidates in conferring resistance. I further suggest using the QTLseqr tool to perform bulk segregant analysis. Segregating populations derived from ARR expressing and non-expressing parents should be screened for resistance to *P. capsici* and resistant and susceptible bulks should be selected for the analysis. We have started in screening such populations and segregation data suggested a major gene component.

Another approach to further narrow the candidate list, would be to test expression of these genes throughout fruit development, in a set of resistant and susceptible doubled haploid (DH) lines. This will further verify if developmental changes in expression of these candidates is directly correlated with resistance. Though several transcriptional analysis methods can serve

this purpose, the nCounter method (NanoString Technologies) could prove to be accurate and affordable for testing this handful of genes in several genotypes and developmental stages. I expect that genes associated with ARR will show consistent differential expression in the resistant DH lines compared to the susceptible lines. Expression patterns of resistant DH lines should mirror those of the resistant parent 'Poinsett 76' and vice versa.

A polymorphism between the two parents could result in a change in expression which is associated with the trait, as described above. An alternate explanation for the phenotypic variance, which doesn't depend on expression differences, would be a non-synonymous mutation in a gene conferring resistance. A regulatory or signaling factor could be altered such that its function has changed, but its expression levels between the two alleles remains the same. In such a case, our current transcriptional comparisons would be insufficient in detecting the true causal gene. A complementary approach would be to compare all detected polymorphisms within an identified locus and scanning for amino acid alterations. We can utilize other genotypes, differing for ARR, to test if these polymorphisms are congruent with the respective ARR phenotype.

Concurrently, I propose fine mapping of any identified loci using larger mapping population developed from backcrossing resistant DH lines with the ARR- parent, 'Gy 14'. Such a population will narrow down the regions of interest and help verify if any of the proposed candidates are still valid. I have also started developing an $F_{2:3}$ population which could also be used for this purpose. Tissue has already been collected from the F_2 lines for further DNA based analyses. NGS based BSA allows us to detect variants between the parents in the population, these can be used as markers (either InDel or KASP (Semagn et al., 2014) type assays, for

example). These segregating populations and markers can be used to identify recombinant lines within that locus to fine map the trait.

Once further narrowing of candidates has been accomplished, several functional genomics approaches should be employed to further understand the function and regulatory mechanisms controlling these genes. While genetic engineering approaches are limited in cucumber, there are a few reports where such methods, as well as genome editing using CRISPR/Cas9, have been used successfully (Sherman et al., 2016; Hu et al., 2017). Another option would be to utilize a TILLING population developed in cucumber, if mutations in this region exist in that population (Fraenkel et al., 2014). Finally, melon (*Cucumis melo*), a close relative of cucumber is more amenable to transformation methods and several transgenic melon lines have been produced in our lab. Many cucumber genomic regions are syntenic with melon, thus several of the identified genes should contain homologues in melon which might be available for genetic manipulation.

Apart from identifying the developmentally regulated factor that confers ARR, further research into the response mechanisms should be pursued. Using our network analyses, we identified several early induced pathways associated with defense in resistant-aged fruit. More research should be pursued in identifying downstream targets of WRKY33 and WRKY75 in cucumber. Chromatin immunoprecipitation followed by sequencing could be used to identify targets of WRKY75, and to confirm if in fact WRKY33 directly activates transcription of ethylene synthesis genes in response to *P. capsici* infection in cucumber. Ethylene has been shown to be important in ARR of *Nicotiana benthamiana* to *P. infestans* (Shibata et al., 2010) and we showed that key ethylene synthesis genes were upregulated in resistant-aged fruit in response to inoculation. Thus, the importance of the ethylene signaling pathway in cucumber

should be examined in early infection. An easy assay would be to chemically block ethylene receptors by exogenous silver nitrate application on the fruit surface, and to subsequently inoculate resistant fruit. This approach was successful in pepper in showing the importance of ethylene signaling in resistance to *P. capsici* (Núñez-Pastrana et al., 2011). Further studies may be performed to understand the downstream targets of this pathway and their importance in resistance.

Ultimately, once the gene(s) involved are accurately identified, these may be used in developing constitutive resistant lines in cucumber as well as other cucurbits, such as watermelon and squash, which are also strongly affected by *P. capsici*. In the future, I envision genome edited cultivars with altered expression of ARR genes that allows for a robust resistance mechanism in the field.

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