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Characterizing the Interaction Between Non-Pathogenic Fusarium Oxysporum and Arabidopsis Thaliana to Determine Beneficial Effects Conferred to the Model Plant Host

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Characterizing the interaction between non-pathogenic *Fusarium oxysporum* and *Arabidopsis thaliana* to determine beneficial effects conferred to the model host plant

A Thesis Presented

by

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DEDICATION

To Isabelle and Chester.

I wish you were both here to share in this moment with me.

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ABSTRACT

CHARACTERIZING THE INTERACTION BETWEEN NON-PATHOGENIC *FUSARIUM OXYSPORUM* AND *ARABIDOPSIS THALIANA* TO DETERMINE BENEFICIAL EFFECTS CONFERRED TO THE MODEL HOST PLANT

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Fusarium oxysporum (*Fo*) is a soil-borne fungal pathogen that causes vascular wilt disease on a broad range of plants, including agricultural crops and the model plant *Arabidopsis thaliana*. There are non-pathogenic members of the *Fo* species complex that confer defense benefits against other pathogens to the host plant, however alteration to the host's physiology through interaction with one of these strains, Fo47, have not been described. In this study, we aimed to establish the Fo47-*A. thaliana* interaction and determine if Fo47 reduces disease severity of a pathogenic *Fo* isolate, Fo5176. Additionally, we sought to use bioinformatics to mine transcriptomic data of the infection between Fo47 and *A. thaliana* for putative effectors from the non-pathogenic isolate using a pipeline that is validated by identifying known effectors in the interaction between Fo5176 and *A. thaliana*. Phenotypic characterization of *A. thaliana* plants

inoculated with Fo47 or Fo5176 has revealed a significant increase in rosette biomass of Fo47 inoculated plants when compared to mock (sterile water) inoculated plants. As is observed in other systems, treatment of plants with Fo47 prior to challenging with pathogenic *Fo* significantly reduces the disease severity over time. The results of this study suggest that Fo47 is a possible biocontrol agent against Fo5176, and that inoculation with non-pathogenic *Fo* alters the physiology of *A. thaliana* such that it has a higher rosette biomass without alterations to the water status of the plant. Our pipeline for extracting putative effectors using transcriptomic data as a critical filter generated 13 candidate genes for further experimentation to determine their role in the Fo47-*A. thaliana* interaction. This research reports the first known observation that Fo47 increases the shoot biomass of the host plant it is interacting with, and that the model plant *A. thaliana* can be used as a host to examine the spectrum of interactions capable within the *Fusarium oxysporum* species complex.

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PHENOTYPIC CHARACTERIZATION OF THE NON-PATHOGNEIC INTERACTION BETWEEN *A. THALIANA* AND *F. OXYSPORUM*

INTRODUCTION

The *A. thaliana* and *F. oxysporum* pathosystem

Arabidopsis thaliana is one of the most commonly used model plants by researchers worldwide. The reason for this is that this plant has desirable features like most other model organisms such as a short life cycle, small size, and ample offspring production. However, the extensive community and collection of omics related resources makes *A. thaliana* a unique system to work in. The *A. thaliana* genome was the first plant genome sequenced in 2000, and the information behind this plant has exploded with databases that store genomic, transcriptomic, proteomic, and metabolomic related information along with modeling tools to infer homology and biological meaning from data (Berardini et al., 2015). This research will be utilizing *A. thaliana* as the host for interaction with two different *F. oxysporum* isolates because of the existing knowledge regarding the response to pathogenic *F. oxysporum* in addition to the vast literature base and resources for pathology experiments using this plant.

The fungal isolates used in this research are species complex members of *Fusarium oxysporum*. This asexual fungal species is part of the Fusarium genus, which contains several other notable plant pathogenic fungi like *F. graminearum*, *F. verticilloides*, and *F. solani*. These species share similarities like spore structures however *F. oxysporum* differs from these other

species in several ways. One of the earliest discovered differences is the lack of a sexual stage in *F. oxysporum*, which is seen in other Fusarium species. A more comprehensive understanding of the differences between *F. oxysporum* and its sister species was done through genomics, which revealed accessory chromosomes in *F. oxysporum* that are required for host pathogenicity in addition to features like repeats and transposons that distinguish this species genome structure (Ma et al., 2013, 2010). *Fusarium oxysporum* members fall into mock taxonomic groupings called formae specialis (f. sp.) that are based on pathogenicity on a host. Examples of this obscure classification are that all pea (*Pisum sativum*) infecting *F. oxysporum* isolates are called *F. oxysporum* formae specialis *pisi*, and all isolates that infect tomato are *F. oxysporum* f. sp. *lycopersici* (Baayen et al., 2000).

In terms of plant infections, *Fusarium oxysporum* was listed as the 5th top fungal pathogen for research because of the broad host range of the *F. oxysporum* species complex comprising over 100 crop species, as well as difficulty in managing the disease through generating resistant plant cultivars (Dean et al., 2012). *F. oxysporum* is considered a hemi biotrophic vascular wilt pathogen, but results in a root, bulb, or crown rot in some hosts. This pathogen is classified as a hemibiotroph because it has a biotrophic infection stage where is sustains off of living tissue, and then transitions into a necrotrophic lifestyle of consuming dead tissue. In the soil where this fungus can be isolated from, it is leading a predominately saprophytic lifestyle of degrading organic matter. The term “vascular wilt” is descriptive of the symptoms from plants infected with this type of pathogen like stunting, leaf epinasty, chlorosis, and necrosis as well as vascular bundle discoloration (Michielse & Rep, 2009). Plant responses to vascular wilt fungi are also unique in comparison to responses to foliar or root pathogens because the host response centers on physical containment of the infection by clogging xylem

vessels with gums, waxes, and phenolic compounds, release of antimicrobials, and transcriptional reprogramming of neighboring parenchyma cells (Yadeta & Thomma, 2013). During this thesis work, I will be using two *F. oxysporum* isolates; the pathogenic isolate is Fo5176, and the non-pathogenic isolate Fo47 (Alabouvette, Lemanceau, & Steinberg, 1993). Fo5176 and other brassica infecting strains of *F. oxysporum* are capable of infecting and inducing disease symptoms in *A. thaliana*, and the interaction has been described thoroughly through the lens of microscopy and molecular plant-pathogen interactions. By using a stain that dyes an enzyme specifically secreted by *F. oxysporum*, the growth of *F. oxysporum* f. sp. *conglutinans* within the root was described, and this work highlighted that a known resistance gene in *A. thaliana* to *F. oxysporum*, RFO1, was required to restrict vascular proliferation by the pathogen (A. Diener, 2012). The growth of an *A. thaliana* infecting isolate in root tissue was also characterized using confocal microscopy. This preliminary work found that *F. oxysporum* colonizes more frequently at lateral and apical root tips, grows intercellularly initially prior to intracellular growth in plant cells, and between 5-6 days post inoculation *F. oxysporum* can be observed colonizing the plant vasculature (Czymmek et al., 2007). On a molecular level, *A. thaliana* has been used to dissect genes and pathways involved in the interaction with pathogenic *F. oxysporum*. We know that there are resistance genes to *F. oxysporum* that have a variable distribution among *A. thaliana* ecotypes, and that the introduction of the RESISTANCE TO *FUSARIUM OXYSPORUM* 1 (RFO1) gene contributes to resistance against 3 *A. thaliana* infecting formae specialis (Diener & Ausubel, 2005). Through mutagenesis of key phytohormone signaling components, the role of the cytosolic Jasmonic acid sensor COI1 in promoting disease symptoms to Fo5176 in *A. thaliana* was determined (Thatcher, Manners, & Kazan, 2009). Similarly, 2 subunits of the transcriptional Mediator complex, MEDIATOR18 and

MEDIATOR20 were found to be positive regulators of Fo5176 infection in the *A. thaliana* Col-0 ecotype (Fallath et al., 2017). Akin to Jasmonic Acid, certain auxin signaling mutants display increased resistance to Fo5176 and suggest that Fo5176 utilizes active auxin and Jasmonic acid signaling pathways to colonize the plant root effectively (Kidd et al., 2011). This interaction has also been analyzed on a transcriptomic level, and uncovered broad changes in the expression of transcripts associated with signal perception, Salicylic acid (SA), Jasmonic acid (JA) and Ethylene (Et) signaling, pathogenesis-related (PR) proteins, and stress inducible transcription factors at one and six days post infection (Lyons et al., 2015; Zhu et al., 2013).

Non-Pathogenic *F. oxysporum* interactions with plants

The non-pathogenic isolate to be used in this study has been documented in other plant systems to provide protective functions against several root pathogens through indirect means as well as eliciting plant defense gene expression, but no such characterization has been done in *A. thaliana*. Fo47 was initially isolated from the Châteaubriand region of France where there were low incidences of muskmelon Fusarium wilt symptoms (Alabouvette, 1986; Rouxel & Alabouvette, 1979). Since its isolation out of suppressive soil, this strain has been exposed to numerous plant hosts without causing disease symptoms. Quite the opposite occurs, and typically it is observed that plants inoculated with Fo47 have improved responses to pathogen challenge than plants that are not exposed to Fo47.

The benefits conferred to tomato against pathogenic *F. oxysporum* f. sp. *lycopersici* have been well characterized in terms of Fo47 influencing the defense response to the pathogen. Pre-inoculation of tomato roots primes the plant for defense against the pathogenic formae specialis by increasing the expression of known defense related genes and reduces the amount of pathogenic fungus detected in root tissue (Aimé, Alabouvette, Steinberg, & Olivain, 2013; Aimé,

Cordier, Alabouvette, & Olivain, 2008; Fuchs, Moënne-Loccoz, & Défago, 1999). When tomato roots are exposed to a 1:100 mix of pathogenic *F. oxysporum* and Fo47, there is a delay in the colonization of the vasculature by the pathogen (Nahalkova, Fatehi, Olivain, & Alabouvette, 2008). The xylem sap proteome of tomato inoculated with a 1:1 mixture of Fo47 and the pathogenic formae specialis was dissected and found that it was largely similar to the mock inoculated xylem sap proteome. The major differences between the sap proteomes were that plants exposed to Fo47 alongside the pathogen accumulated a beta-glucanase enzyme and Pathogenesis-Related protein that were correlated with a reduction in disease symptoms, but not reduction in vascular colonization by the pathogen (de Lamo et al., 2018). The overarching observation in the tomato system is that Fo47 primes the plant mostly through inducing defense genes and proteins, and that competition for nutrients and space along the rhizosphere may be important between *F. oxysporum* isolates, these physical interactions are not the major factors in reducing the disease pressure pathogenic *F. oxysporum* exerts on tomato.

In the additional plants that Fo47 has been reported to interact with, similar trends have been reported of a reduction in disease severity due to priming of host defense genes. However, in each system that Fo47 interacts with there is a nuance that demonstrates the potential of this isolate to inhibit pathogenic challengers by other means. In pepper for example, Fo47 primes the induction of Pathogenesis-related genes that promote resistance to *Phytopthora capscisi* and *Verticillium dahliae* as well as stimulates the production of anti-microbial exudates from roots like caffeic acid that inhibit the disease severity of *V. dahliae*, a generalist vascular wilt pathogen (Veloso et al., 2016; Veloso & Díaz, 2012). Cucumber and pea roots have also been inoculated with Fo47, and then examined microscopically to determine the cytological effects Fo47 has on root interactions with pathogens. When cucumber is challenged with Pythium after Fo47

inoculation, Fo47 is found within Pythium hyphal cells inside and outside the root as well as stimulate the production of phenolic and cell wall depositions between the cortical cells and vasculature that the authors hypothesize has a function in impeding Pythium's colonization (Benhamou, Garand, & Goulet, 2002). In pea, there are similar cytological findings of Fo47 stimulating the plant to deposit anti-microbial compounds in the apoplast and cell walls (Benhamou & Garand, 2001). In these cytological reports in addition to fluorescent microscopy of Fo47 in tomato, Fo47 has been observed growing intercellularly along the root epidermis and within the cortical cell layers. A factor that seems to separate Fo47 from pathogenic *F. oxysporum* is the inability of Fo47 to inhabit the vasculature of inoculated hosts. A report of this phenomena being reversed comes from an experiment where an accessory chromosome from the tomato pathogen was introduced into Fo47, and when plants where challenged with that mutant Fo47 vascular bundle discoloration and disease was observed (Ma et al., 2010).

While there is an established history of research into plant interactions with *F. oxysporum*, the goal of this proposed thesis is to contribute novel insights into the infection of *A. thaliana* with *F. oxysporum* by analyzing both the fungus and plant and focusing on the non-pathogenic interaction that has been previously unreported. We aim to determine if Fo47 has a physiological impact on the above ground growth of *A. thaliana*, and that it confers similar defense benefits in *A. thaliana* against root pathogens as it does in other systems.

METHODS

Growth of *Arabidopsis thaliana* for Inoculation

Seeds of *A. thaliana* ecotype Col-0 were sterilized in 1 mL of 70% Ethanol for 3 washes, 5 minutes each, and 1 wash with 50% bleach for 5 minutes. After removing the bleach solution, seeds were rinsed with 1 mL of sterile distilled and deionized water, and stratified for 3-4 days in the dark at 4°C. Seeds were planted into 4" pots filled with an autoclaved mixture of fine grain play sand: MetroMix 360: vermiculite in a 1:2:1 ratio, watered with 800 mL of distilled deionized water, and covered with a clear plastic lid to retain a high humidity for 3 days in the growth chamber with the following settings: 28°C day/24°C night, 14 hour day/10 hour night, bottom watered in the tray daily for 1 hour after removing plastic cover, and light levels ranging from 89-94 μmol·m-2·s-1. After 3 days, plastic lid was removed, and plants were allowed to grow for 11 additional days prior to inoculation with *Fusarium oxysporum* microconidia. Plants are 14 days old at the time of inoculation and have at least 4 fully expanded true leaves.

Preparing Microconidia Suspensions of *Fusarium oxysporum* isolates

Stocks of Fo47 and Fo5176 microconidia suspended in 30% glycerol were thawed from the -80C freezer, and 200 uL of stock was added to approximately 100 mL of 1X Potato Dextrose Broth. Fungal cultures were grown in 50 mL falcon tubes in an incubating shaker that was set to 28°C and 150 rpm. Fo47 is grown for 3-4 days before filtering microconidia from broth, and Fo5176 is grown for 5-6 days to ensure adequate spore quantities. Cultures are filtered through sterilized double layer cheese cloth, centrifuged for 15 minutes at 4,500 rpm, and the supernatant is removed. The spore pellet is rinsed twice with 50 mL of sterile distilled and deionized water prior to quantification with a hemocytometer. An aliquot of the microconidial

spore solution is diluted and viewed under the microscope at 10X to count the number of spores in 5 out of the 9 fields on the hemocytometer. The math to determine the number of spores per mL is as follows:

X Number of Microconidia Counted in 5 Fields x (Dilution Factor/# of Fields Counted=5) x 10,000 spores/mL (constant)= X microconidia/mL

The concentration of the spore solution is used to determine how much of it is needed to make a 50 mL inoculum that contains 1 x 10^6 microconidia/mL. Spore solutions are made and stored in the 4°C fridge until ready to use. The spores are used within 10 hours of filtering from medium.

Root Dip Inoculation Procedure

Arabidopsis thaliana plants with at least 4 expanded leaves are removed from the 4" pots 14 days after planting them, and the roots rinsed briefly in distilled and deionized water twice to remove bulk soil prior to being placed into a petri dish with 50 mL of inoculum for 45-60 seconds. After treatment, plant are re-potted in 4" pots that contain an autoclaved mixture of fine grain play sand: MetroMix 360: vermiculite in a 1:2:1 ratio, and the pots are placed into pipette tip boxes so they may be bottom watered for 1 hour with 75 mL of water daily while preventing cross-contamination between treatments. Mock treated plants are inoculated with 50 mL of sterile distilled and deionized water, and inoculum petri dishes are replaced with fresh inoculum after every 9 plants. Plants are then placed into the growth chamber with the same settings as the plants were grown in. To probe if Fo47 is a candidate for priming *A. thaliana* for defense against Fo5176, plants are inoculated with Fo47 and then 4 days later inoculated with Fo5176.

Additionally, a treatment of simultaneously inoculating *A. thaliana* roots with a 1:1 ratio of 1 x 10^6 microconidia/mL Fo47 and Fo5176 was included to test if Fo47 can reduce disease symptoms through non-priming induced means when it is in equal quantities to the pathogenic isolate. Each 4" pot receives inoculated 3 plants, and per treatment there are 6 pots. The pots are randomly arranged in the growth chamber and re-arranged when removed for photographs.

Disease Score Calculation and Survival Curve Generation

Disease score is on a scale ranging from 0 to 4 with 0 being symptomless, and 4 being necrotic. The stages in between range from wilted/stunted (1) to initial chlorosis (2) and advanced chlorosis where more than 50% of the leaves are chlorotic (3). Per time point and treatment, between 15-18 plants have their disease score assigned. Plants that will be harvested at 6 days post inoculation (DPI) for biomass and RWC quantification have their disease score calculated every 2 days. The plants that are a part of the long-term experiment to determine if Fo47 treatment reduces disease are observed every 4, 7, 10, 14, 21, and 28 DPI. For plants that are inoculated with Fo47 4 days prior to Fo5176 challenge, their disease score was calculated every 3, 7, 11, 18, 25 days after inoculation with Fo5176. Kaplan-Meier plots were constructed to assess plant mortality after challenge with the pathogenic isolate of *F. oxysporum*. GraphPad Prism 8 software was used to compare survival among treatment groups by the Log-rank test.

Biomass Quantification and Determining Relative Water Content of Whole Rosettes

A. thaliana rosette biomass and relative water content (RWC) was determined for 18 plants per treatment at 6 days post inoculation to determine if Fo47 inoculation alters these parameters. To determine the RWC a protocol from (Hummel et al., 2010) was followed that involved quickly measuring the fresh weight of the whole rosette, floating the rosette in a 60 mm petri dish with 10 mL of sterile distilled and deionized water in the dark at 4°C for 24 hours,

weighing again to collect turgid weight, and then drying for 3 days at 50°C in the incubator on a piece of wax paper in the petri dish to generate tissue for dry weight. The RWC is calculated by the following equation:

(Fresh weight-Dry weight)/(Turgid weight-Dry weight) X 100 = RWC %

Statistical Analysis

The GLM procedure and Fisher's Least Significant Difference Test in SAS was used to determine if there were statistically significant differences in the effect treatment has on physiological parameters or disease score. Statistical analysis was performed on each replicate, as well as for 3 out of 5 replicates together for the biomass and co-inoculation data. The 2 replicates that were dropped from each experiment were removed because of their variance from the remaining 3 replicates.

RESULTS AND DISCUSSION

Fo47 Reduces the Disease Severity of Fo5176 on *A. thaliana*

Previous research regarding the protective effect of Fo47 against pathogens has been recapitulated within *A. thaliana* by comparing the average disease score over time of plants inoculated with sterile water as a mock treatment, or 1 x 10^6 spores/mL of either Fo47, Fo5176, and 1:1 combination of Fo5176 and Fo47, or plants inoculated with 1 x 10^6 spores/mL of Fo47 and then challenged with 1 x 10^6 spores/mL of Fo5176 4 days after the inoculation with Fo47. The average disease scores for each treatment and time point that were collected are listed in Table 1, along with standard error in parenthesis and statistically significant differences by Fisher's LSD denoted by letters. The overall average disease score is calculated from replicates 1, 3, and 5. The reduction in disease severity in plants pre-inoculated with Fo47 prior to Fo5176 challenge (Figure 1) is accompanied by a slightly increased survivability in comparison to plants challenged strictly with the pathogen or the mixture of pathogen and non-pathogenic *F. oxysporum* (Figure 2 and 3).

A

Replicate 1

Average Disease Score Days Post Inoculation with Fo5176

Treatment	3	4	7	10	11	14	18	21	25	28
Mock	x	0.00^{A}	0.00^{A}	0.00^{A}	x	0.00^{A}	x	0.00^{A}	x	0.00^{A}
Fo47	x	0.00^{A}	0.00^{A}	0.00^{A}	x	0.00^{A}	x	0.00^{A}	x	0.00^{A}
Fo5176	x	1.00^{B}	$2.5\ (0.15)^{B}$	$3.67\ (0.11)^{B}$	x	4.00^{B}	x	4.00^{B}	x	4.00^{B}
Fo5176:Fo47	x	1.00^{B}	$1.5\ (0.15)^{C}$	$3.28\ (0.11)^{C}$	x	4.00^{B}	x	4.00^{B}	x	4.00^{B}
Mock to Mock	0.00^{A}	x	0.00^{A}	x	0.00^{A}	x	0.00^{A}	x	0.00^{A}	x
Fo47 to Fo5176	$0\ (0.08)^{A}$	x	$0.11\ (0.06)^{D}$	x	$0.94\ (0.18)^{C}$	x	$2\ (0.15)^{C}$	x	3.56^{C}	x

B

Replicate 2

Average Disease Score Days Post Inoculation with Fo5176

Treatment	3	4	7	10	11	14	18	21	25	28
Mock	x	0.00^{A}	0.00^{A}	0.00^{A}	x	0.00^{A}	x	0.00^{A}	x	0.00^{A}
Fo47	x	0.00^{A}	0.00^{A}	0.00^{A}	x	0.00^{A}	x	0.00^{A}	x	0.00^{A}
Fo5176	x	0.56 (0.12)^{B}	1.67 (0.28)^{B}	3.17 (0.31)^{B}	x	3.56 (0.27)^{B}	x	3.67 (0.23)^{B}	x	3.67 (0.23)^{B}
Fo5176:Fo47	x	0.39 (0.12)^{B}	1.11 (0.11)^{C}	2.94 (0.19)^{B}	x	4.00^{B}	x	4.00^{B}	x	4.00^{B}
Mock to Mock	0.00^{A}	x	0.00^{A}	x	0.00^{A}	x	0.00^{A}	x	0.00^{A}	x
Fo47 to Fo5176	0.33 (0.11)^{A}	x	0.89 (0.08)^{A}	x	2 (0.23)^{C}	x	3.67 (0.23)^{B}	x	3.72 (0.19)^{B}	x

C

Replicate 3

Average Disease Score Days Post Inoculation with Fo5176

Treatment	3	4	7	10	11	14	18	21	25	28
Mock	x	0.00^{A}	0.00^{A}	0.00^{A}	x	0.00^{A}	x	0.00^{A}	x	0.00^{A}
Fo47	x	0.00^{A}	0.00^{A}	0.00^{A}	x	0.00^{A}	x	0.00^{A}	x	0.00^{A}
Fo5176	x	0.5 (0.12)^{B}	1.28 (0.18)^{B}	2.33 (0.18)^{B}	x	2.83 (0.22)^{B}	x	4.00^{B}	x	4.00^{B}
Fo5176:Fo47	x	0.11 (0.08)^{A}	1.33 (0.16)^{B}	1.89 (0.18)^{C}	x	2.67 (0.26)^{B}	x	4.00^{B}	x	4.00^{B}
Mock to Mock	0.00^{A}	x	0.00^{A}	x	0.00^{A}	x	0.00^{A}	x	0.00^{A}	x
Fo47 to Fo5176	0.33 (0.11)^{A}	x	1.00^{C}	x	1.11 (0.08)^{C}	x	3.72 (0.16)^{C}	x	3.78 (0.13)^{C}	x

D

Replicate 4

Average Disease Score Days Post Inoculation with Fo5176

Treatment	3	4	7	10	11	14	18	21	25	28
Mock	x	0.00^{A}	0.00^{A}	0.00^{A}	x	0.00^{A}	x	0.00^{A}	x	0.00^{A}
Fo47	x	0.00^{A}	0.00^{A}	0.00^{A}	x	0.00^{A}	x	0.00^{A}	x	0.00^{A}
Fo5176	x	0.72 (0.11)^{B}	1.06 (0.1)^{B}	2.5 (0.23)^{B}	x	3.56 (0.15)^{B}	x	4.00^{B}	x	4.00^{B}
Fo5176:Fo47	x	0.39 (0.12)^{C}	0.67 (0.11)^{C}	1.94 (0.27)^{C}	x	2.83 (0.28)^{C}	x	3.61 (0.22)^{C}	x	3.72 (0.18)^{C}
Mock to Mock	0.00^{A}	x	0.00^{A}	x	0.00^{A}	x	0.00^{A}	x	0.00	x
Fo47 to Fo5176	0.00^{A}	x	0.88 (0.11)^{D}	x	1.41 (0.17)^{B}	x	3.71 (0.14)^{C}	x	3.76 (0.13)^{C}	x

E

Replicate 5

Average Disease Score Days Post Inoculation with Fo5176

Treatment	3	4	7	10	11	14	18	21	25	28
Mock	x	0.00^{A}	0.00^{A}	0.00^{A}	x	0.00^{A}	x	0.00^{A}	x	0.00^{A}
Fo47	x	0.00^{A}	0.00^{A}	0.00^{A}	x	0.00^{A}	x	0.00^{A}	x	0.00^{A}
Fo5176	x	0.39 (0.12)^{B}	1.44 (0.15)^{B}	3.11 (0.16)^{B}	x	3.67 (0.11)^{B}	x	4.00^{B}	x	4.00^{B}
Fo5176:Fo47	x	0.39 (0.12)^{B}	1.28 (0.16)^{B}	2.28 (0.21)^{C}	x	3.28 (0.18)^{C}	x	4.00^{B}	x	4.00^{B}
Mock to Mock	0.00^{A}	x	0.00^{A}	x	0.00^{A}	x	0.00^{A}	x	0.00^{A}	x
Fo47 to Fo5176	0.44 (0.12)^{B}	x	1.33 (0.11)^{B}	x	2.67 (0.16)^{D}	x	3.72 (0.14)^{C}	x	3.72 (0.14)^{C}	x

F

Average Disease Score Days Post Inoculation with Fo5176

Treatment	3	4	7	10	11	14	18	21	25	28
Mock	x	0.00^{A}	0.00^{A}	0.00^{A}	x	0.00^{A}	x	0.00^{A}	x	0.00^{A}
Fo47	x	0.00^{A}	0.00^{A}	0.00^{A}	x	0.00^{A}	x	0.00^{A}	x	0.00^{A}
Fo5176	x	0.63 (0.07)^{B}	1.740 (0.13)^{B}	3.04 (0.12)^{B}	x	3.5 (0.10)^{B}	x	4.00^{B}	x	4.00^{B}
Fo5176:Fo47	x	0.5 (0.07)^{C}	1.37 (0.09)^{C}	2.48 (0.13)^{C}	x	3.31 (0.13)^{S}	x	4.00^{B}	x	4.00^{B}
Mock to Mock	0.00^{A}	x	0.00^{A}	x	0.00^{A}	x	0.00^{A}	x	0.00^{A}	x
Fo47 to Fo5176	0.3 (0.06)^{A}	x	1.09 (0.05)^{D}	x	1.93 (0.12)^{C}	x	3.67 (0.08)^{C}	x	3.83 (0.06)^{C}	x

Table 1: Disease Scores for *A.* thaliana Inoculated with *F. oxysporum.*

Panels A-E are the average disease scores for each treatment and timepoint days post inoculation with Fo5176. These averages are based off of as many as 18 observations per treatment, and standard error for the average is presented in parenthesis. Panel F is the combined result of replicates 1, 3, 5 and contains as many as 18 observations per treatment and time point. Statistical significance was determined with Fisher's LSD Test, and significantly different means are denoted with different letters. Standard error is presented in parenthesis, and averages that lack a parenthesis have a standard error of 0.

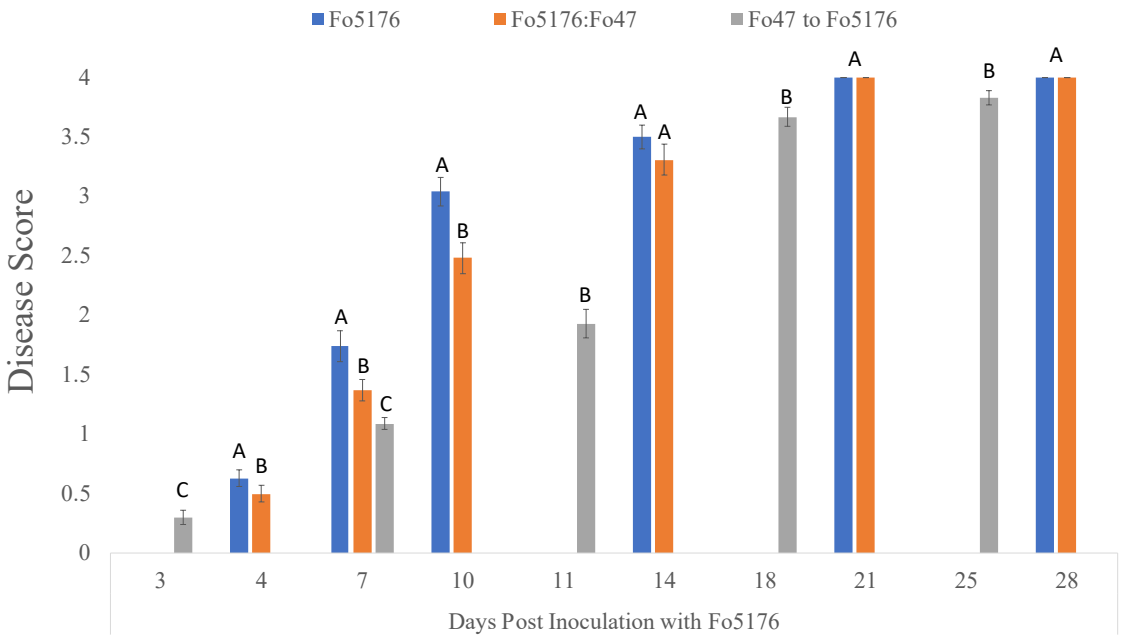

Figure 1: Reduction in Disease Score in Plants Pre-Inoculated with Fo47 Compared to Fo5176 Inoculated plants.

Average disease score of plants in replicates 1, 3, and 5 of the co-inoculation experiments presented with standard error bars. Statistical difference determined by Fisher's LSD Test, and significantly different means are denoted with different letters.

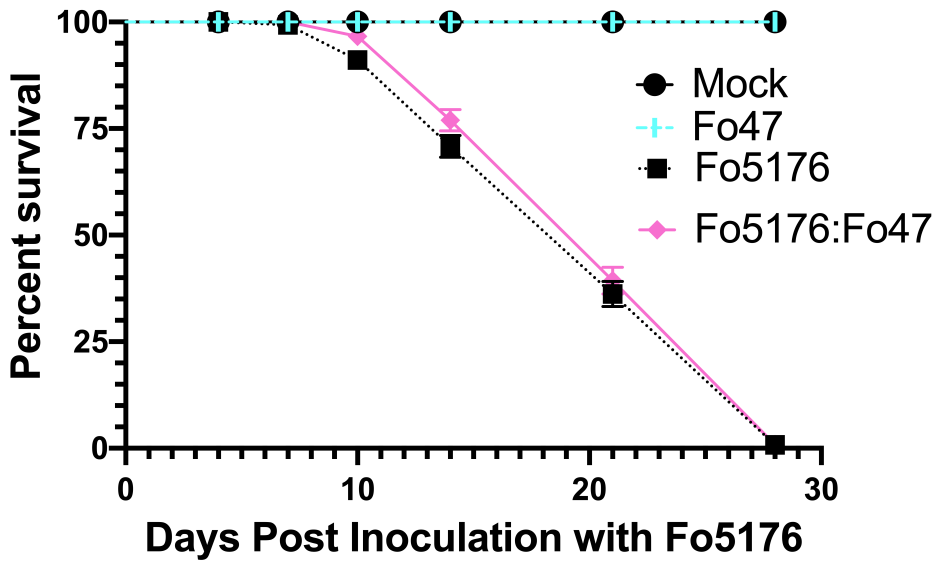

Figure 2: Survivability of *A. thaliana* plants after Inoculation with *F. oxysporum.*

Kaplan-Meier plot of data containing replicates 1, 3, and 5. The Log-Rank test (p-value < 0.005) detected differences between the Mock and Fo47 inoculated plants when compared to the plants that are inoculated with Fo5176, but no significant difference between plants that are simultaneously inoculated with Fo47 and Fo5176 to plants that are exposed to Fo5176.

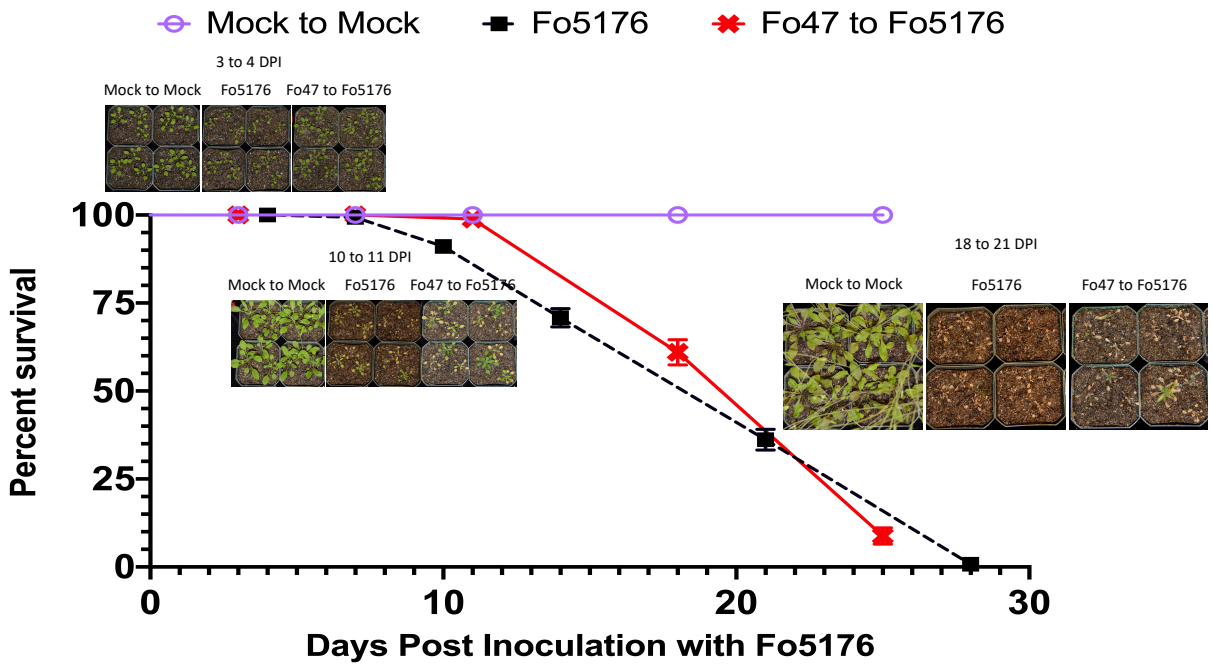

Figure 3: Survivability of *A. thaliana* Inoculated with Fo5176 compared to plants inoculated with Fo47 before pathogen inoculation.

When these two treatments were isolated, we are able to detect statistically significant increase in survivability by the Gehan-Breslow-Wilcoxon test (p-value < 0.01) between plants pre-inoculated with Fo47 prior to Fo5176 challenge and plants inoculated with Fo5176. This graph is composed of all observations in replicates 1, 3, and 5 for the co-inoculation experiment. Pictures included are representative images of plants at indicated days post inoculation with Fo5176.

Taken together, the decreased disease severity and mortality exhibited by Fo47 pre-inoculated plants indicates that this non-pathogenic *F. oxysporum* isolate has the potential to prime the plant for immunity against pathogens as demonstrated in tomato or pepper, or through other means. The plants that are exposed to a 1:1 mixture of pathogenic and non-pathogenic spores do not differ in their survivability or disease score compared to plants exposed to only the pathogen suggesting that Fo47 does not compete successfully against Fo5176 when it is in equal concentrations and, or directly through antibiosis. Future studies should be considerate of time and consider inoculation from the time of pathogen introduction instead of when plants are initially challenged with the non-pathogen. This research would have benefitted from an additional treatment group of mock plants inoculated with the pathogenic isolate at the same time

as the plants that are exposed to Fo5176 4 days after initial inoculation with Fo47. Regardless of experimental design challenges, this work suggests a protective effect of Fo47 on *A. thaliana*.

Rosette Biomass is Greater in *A. thaliana* Inoculated with Fo47

In previous interactions with Fo47, there is a gap in the physiological implications this interaction has on the host plant. When we look outside of Fo47, and Fusaria, there are several examples of fungal interactions resulting in increases in yield in *A. thaliana*. Trichoderma species of fungi are fairly well known for their anti-biotic and anti-fungal effects in soil and the rhizosphere. *Trichoderma virens* reduces disease severity of multiple pathogens in *A. thaliana* in addition to increasing the fresh weight of rosettes and the number of lateral roots. This increase in fresh weight and lateral root proliferation is due to production of auxin-like compounds by *T. virens that* stimulate auxin signaling in the plant (Contreras-Cornejo, Macias-Rodriguez, Cortes-Penagos, & Lopez-Bucio, 2009). *Piriformospora indica* is a root colonizing endophyte capable of interacting with a broad host range and has been reported to improve the biomass of several plants, including *A. thaliana*. Additionally, *P. indica* increases plant height under low nutrient conditions (Banhara et al., 2015). The genus Colletotrichum contains several plant pathogens much like Fusarium, and *C. tofieldiae* is a species that has an endophytic relationship with *A. thaliana*. When in phosphorous limiting conditions, *C. tofieldiae* translocates phosphorous from the medium to the plant roots and improves the fresh weight as well as fitness of *A. thaliana* in this stressful condition (Hiruma et al., 2016). The interaction Fo47 shares with *A. thaliana* mirrors some of these examples in that we know from RNA-seq of the interaction between Fo47 and *A. thaliana* roots involves an induction of key nitrate and phosphate transporter and assimilation genes (Li Guo, unpublished data, Figure 3)

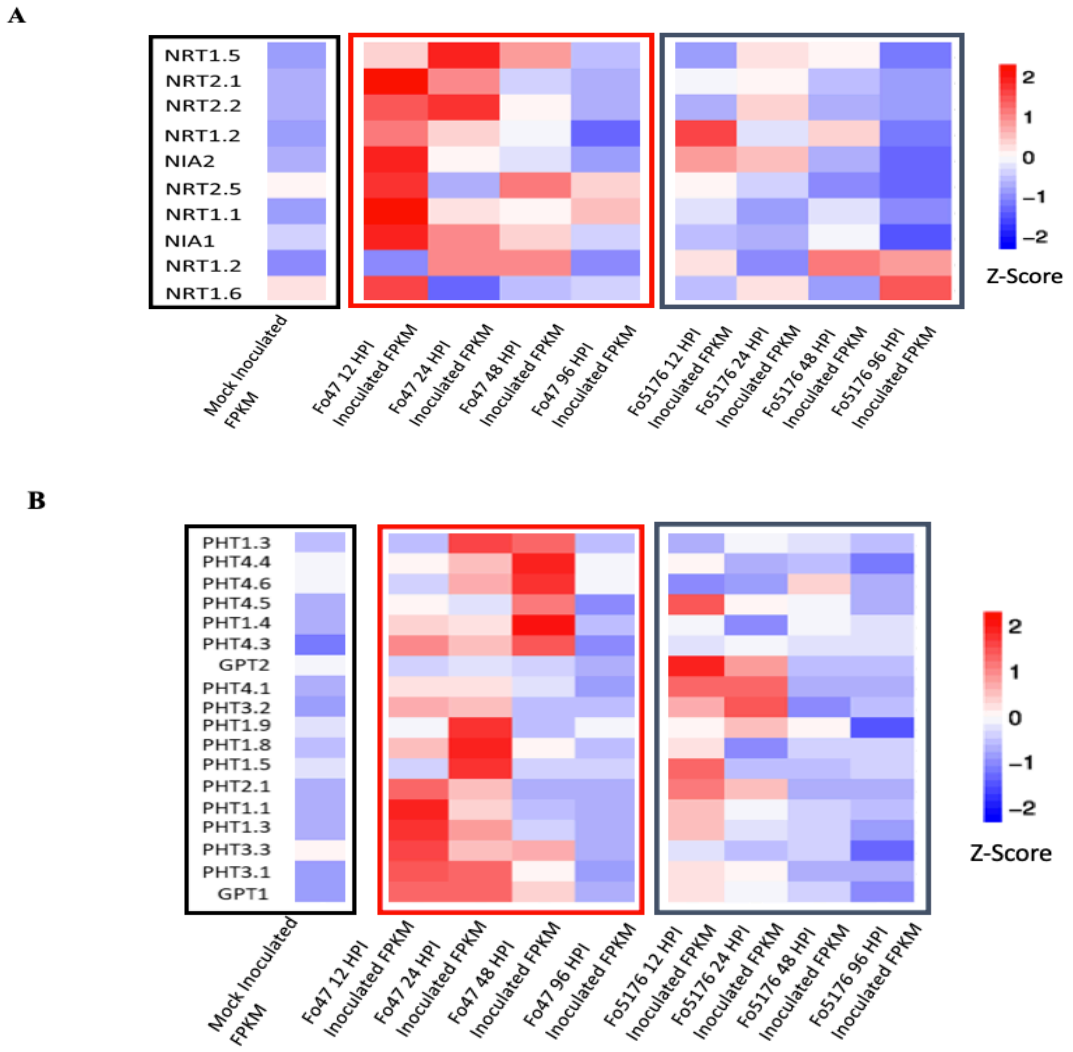

Figure 4: Differentially Expressed Nutrient Related Genes in *A. thaliana* Inoculated with *F. oxysporum*.

Panel A contains genes related to Nitrogen transport and assimilation. Panel B contains genes related to Phosphorous transport and assimilation. Z-scores are composed of the average FPKM values for each timepoint and treatment. Heat Map generated by Dr. Li Guo.

The dry biomass of *A. thaliana* rosettes is greater 6 days after inoculation with Fo47 in comparison to plants mock inoculated with sterile water. Table 2 lists the averages for physiological parameters quantified at 6 DPI for each replicate of this experiment to better define the effect Fo47 has on *A. thaliana* above ground physiology and growth. After reviewing the averages for each replicate of the experiment, it was decided to drop replicates 3 and 4 from the

overall average because of deviation from the other 3 replicates. Figure 5 highlights the increase in rosette biomass of Fo47 inoculated plants in comparison to Mock and Fo5176 inoculated plants.

A

Fresh Weight (mg)

	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Average
Mock	55.94 (2.69)^{A}	49.22 (2.94)^{A}	28.72 (1.74)^{A}	35.44 (1.55)^{A}	55.95 (1.93)^{A}	49.96 (1.57)^{A}
Fo47	54.61 (2.56)^{A}	54.5 (2.46)^{A}	25.72 (1.33)^{A}	41.83 (2.10)^{B}	54.61 (1.11)^{A}	52.33 (1.29)^{A}
Fo5176	11.16 (1.19)^{B}	23.33 (1.47)^{B}	2.56 (0.35)^{B}	13.22(0.92)^{C}	11.17 (0.61)^{B}	14.30 (1.10)^{B}

B

Turgid Weight (mg)

	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Average
Mock	59.72 (2.63)^{A}	52.56 (2.92)^{A}	31.83 (1.77)^{A}	40.78 (1.56)^{A}	59.72 (1.86)^{A}	54.13 (1.51)^{A}
Fo47	58.44 (2.63)^{A}	57.72 (2.42)^{A}	28.72 (1.38)^{A}	46.5 (2.12)^{B}	58.44 (1.20)^{A}	56.52 (1.27)^{A}
Fo5176	19.83 (1.26)^{B}	31.78 (1.65)^{B}	9.5 (0.62)^{B}	21.39 (1.05)^{C}	19.83 (0.69)^{B}	22.31 (1.19)^{B}

C

Dry Weight (mg)

	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Average
Mock	4.5 (0.25)^{A}	4.28 (0.26)^{A}	2.33 (0.11)^{A}	3 (0.16)^{A}	4.5 (0.15)^{A}	4.19 (0.13)^{A}
Fo47	5.28 (0.25)^{B}	5.5 (0.22)^{B}	2.72 (0.14)^{B}	3.83 (0.20)^{B}	5.28 (0.14)^{B}	5.06 (0.14)^{B}
Fo5176	1.27 (0.18)^{C}	2.78 (0.19)^{C}	0.16 (0.09)^{C}	1.22 (0.15)^{C}	1.28 (0.15)^{C}	1.67 (0.15)^{C}

D

Relative Water Content (%)

	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Average
Mock	92.88 (0.60)^{A}	92.53 (0.77)^{A}	89.11 (0.58)^{A}	85.69 (0.72)^{A}	92.88 (0.73)^{A}	91.14 (0.50)^{A}
Fo47	92.66 (0.41)^{A}	93.59 (0.43)^{A}	88.09 (0.73)^{A}	88.74 (0.53)^{A}	92.66 (0.52)^{A}	91.68 (0.39)^{A}
Fo5176	51.01 (2.52)^{B}	69.97 (1.34)^{B}	24.37 (1.91)^{B}	58.57 (1.70)^{B}	51.01 (1.88)^{B}	57.36 (1.66)^{B}

Table 2: Physiological Characteristics Quantified During *A. thaliana* Interaction with *F. oxysporum*.

Panel A is the average fresh weight of rosettes for 18 observations from each replicate. Panel B is the average turgid weight of rosettes, Panel C is the average dry weight of rosettes, and Panel D is the average Relative Water Content that is calculated from the fresh, turgid, and dry weight measurements. The average column in each panel is comprised of the observations in replicate 1, 2, and 5. Statistical significance of the means was determined by Fisher's LSD test, and treatments with significantly different means (p-value < 0.05) are denoted by different letters. Standard error is presented in parenthesis, and averages that lack a parenthesis have a standard error of 0.

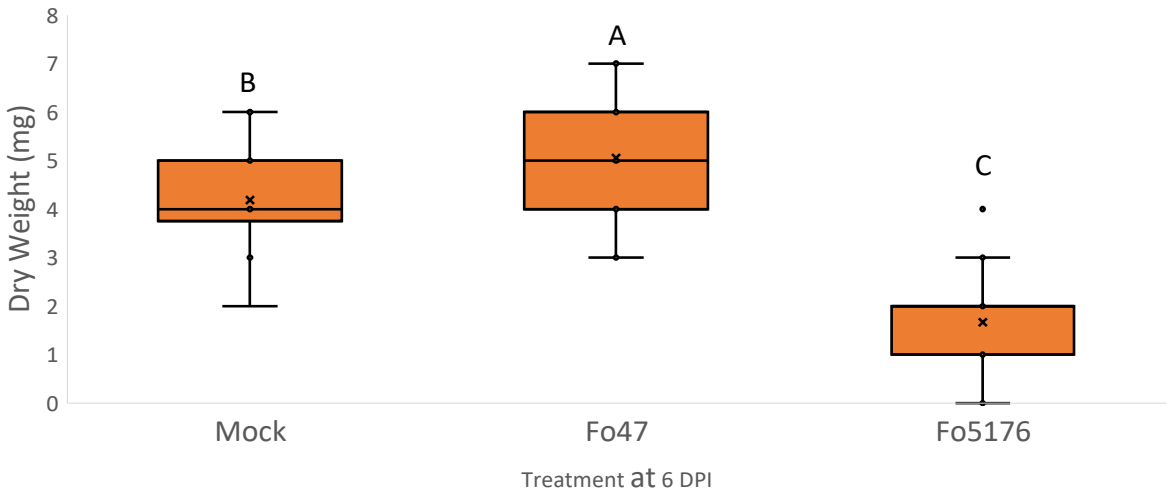

Figure 5: Statistically Significant Increase in Rosette Biomass of Fo47 Inoculated *A. thaliana*.

Box-Whisker Plot of the dry biomass of rosettes 6 days post inoculation. Average dry biomass was calculated for the combined replicates of 1, 2, and 5, and that data is shown here. Within the box, "x" represents the mean, and the line represents the mode. The whiskers extending out of the box depict data that falls 50% outside of the mean with outliers highlighted as dots outside the whiskers.

The means through which this effect occurs could be through promoting *A. thaliana* to increase nutrient absorption and assimilation as RNA-seq data supports. The number of leaves were determined (Figure 6), and there was not significantly more perceivable biomass to Fo47 inoculated plants compared to mock inoculated ones. It is possible the Fo47 inoculation alters carbon partitioning in the rosette and increases the thickness of leaves to result in the significant increase in dry biomass, but with the current physiological data we are unable to quantify leaf thickness (Weraduwage et al., 2015).

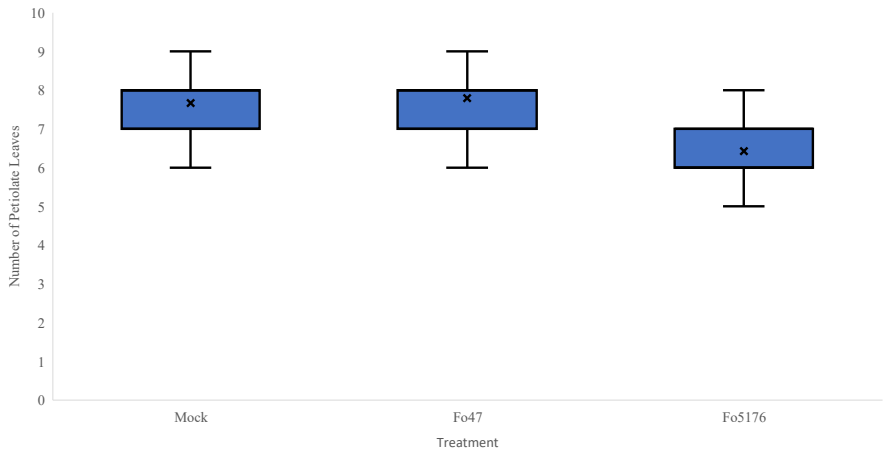

Figure 6: No Significant Difference in the Number of Leaves on *A. thaliana* Rosettes 6 Days After Inoculation with *F. oxysporum*.

Box-Whisker Plot of the number of petiolate leaves illustrates that there is not a difference in the number of leaves between rosettes. Average number of petiolate leaves was calculated for the combined replicates of 1, 2, and 5, and that data is shown here. Within the box, "x" represents the mean, and the bars represent data that falls 50% outside of the mean.

The interaction between Fo47 and *A. thaliana* is markedly different from the interaction that pathogenic *F. oxysporum* have with *A. thaliana*. As it has been observed in other systems, Fo47 displays characteristics of a priming agent that induces the plant immune system and reduces disease severity when the plant is challenged with a pathogen. We choose to challenge plants with Fo5176 4 days after inoculation with Fo47 based on transcriptomic analysis of the interaction between Fo47 and *A. thaliana* roots. Network analysis of differentially expressed transcripts captured an up-regulation in salicylic acid, and plant defense related genes between 2-4 days after inoculation with Fo47 (Figure 7).

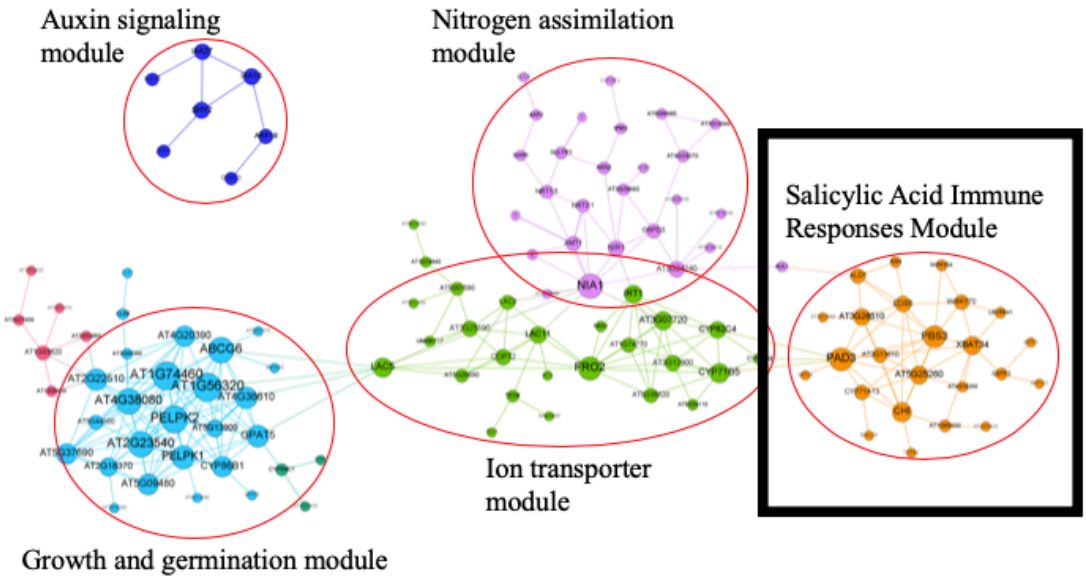

Figure 7: Transcriptional Regulatory Network of *A. thaliana* roots 96 Hours Post Inoculation with Fo47.

Network constructed using STRING protein interaction database and edited in Gephi. This transcriptional network is based off of the RNA-seq data and genes that are differentially expressed between Fo47 inoculation and Fo5176 inoculation. Module highlighted in the black box is enriched for genes with roles in Salicylic Acid defense mediated responses.

It is possible that through priming the molecular arsenal of the plant against a pathogen, Fo47 reduces disease pressure to Fo5176 in *A. thaliana*. Testing this hypothesis further can be done through several means and based on known benefits it would be worthwhile to follow up with qRT-PCR of candidate defense genes, callose staining of the roots, ROS burst quantification, and advanced microscopy of the *in planta* interaction between Fo47, and the pathogenic Fo5176. Using the baseline of the interaction established through this body of work, any perturbations in the observed phenotypes of mutants could be used to identify positive and negative regulators of the reduction in disease severity and/or increase in biomass. The interaction between Fo47 and *A. thaliana* is potentially unique in that the biomass of rosettes that have had their roots inoculated with Fo47 is significantly greater than mock inoculated plants

suggesting a physiological effect on the above ground growth of *A. thaliana* by Fo47. Research with this isolate thus far has not reported alterations to the host plants physiology, and this opens up new research avenues regarding the potential of Fo47 to increase biomass and potentially yield of the commercial crops that it has been reported to interact with.

CHAPTER 2

EFFECTOR PREDICTION IN THE NON-PATHOGENIC *F. OXYSPORUM* ISOLATE UTILIZING IN PLANTA RNA-SEQ DATA

INTRODUCTION

Role of Effectors in Plant Immunology

Plant-microbe interactions exist on a continuum ranging from beneficial to pathogenic. The molecular communication that occurs between microbes and a plant host is a predictor of how the interaction will play out, and proteins are major signals that plants and microbes use to facilitate a parasitic or symbiotic interaction. In the case of parasitic or pathogenic relationships, plants have developed an elaborate system of receptors connected to signaling cascades that regulate multiple aspects of the plant defense response upon recognition of microbe. Jones and Dangl developed a simplified model called the "ZigZag Model" for the plant immune system in 2006 that describes the stages of the plant immune response to a microbe (Jones & Dangl, 2006). In this model, recognition of pathogen associated molecular patterns (PAMPS, more commonly called microbe associated molecular patterns now (MAMPS)), by plant receptor kinases in the cell membrane induces an innate immune response called PAMP Triggered Immunity (PTI) that is characterized by secretion of broad-spectrum antimicrobial enzymes and compounds (Spoel & Dong, 2012). MAMPS are molecules like chitin, beta-glucans, flagellin, and peptidoglycan that are ubiquitous and conserved components of fungi, oomycetes, and bacteria. PTI is sufficient to prevent disease in most cases, and microbes or pathogens that elicit PTI do not result in disease

symptoms. This case is called an incompatible interaction because the microbe or pest is not able to overcome the basal immune responses (Surico, 2013). The next step in the ZigZag Model illustrates an interaction between a plant and a microbe where the microbe has effectors that prevent downstream signal transduction events that trigger PTI or overcome the PTI response. This represents Effector Triggered Susceptibility (ETS), and a compatible interaction because disease symptoms become evident due to microbial infection. Effectors are proteins secreted into the apoplastic space or into the host cytosol by a microbe that can be essential for virulence on a host plant. The last part of this model is Effector Triggered Immunity (ETI), which is a return back to an incompatible interaction between the plant and microbe, due to the plant containing Resistance (R) proteins that recognize effectors or the perturbation the effector causes, and initiate a strong immune response specific to the threat (Jones & Dangl, 2006). Plants use phytohormone regulated signaling cascades that cross-talk with one another to fine-tune responses to microbes ranging from symbioants to pathogens. These same phytohormone signaling networks that are controlled mostly by Salicylic Acid, Jasmonic Acid, and Ethylene gas are the subjects of manipulation by plant pathogens to shift the host response away from the necessary defense (Pieterse et al., 2012).

Effectors within *F. oxysporum*

In the early 2000's the hunt for effectors within *F. oxysporum* had begun with the initial report of the SIX1 (Secreted in Xylem 1) protein from *F. oxysporum* f. sp. *lycopersici* contributing to the virulence of this pathogen on certain tomato cultivars (Takken & Rep, 2010). Since then, numerous SIX and other classes of effectors have been identified in *F. oxysporum* that contribute to the virulence of formae specialis on host plants (de Sain & Rep, 2015). The pathogenic isolate used in this study (Fo5176) contains homologous SIX genes, some of which

function in pathogenicity in *A. thaliana*. Through homology-based comparison between *F. oxysporum* f. sp. lycopersici and Fo5176, 4 homologs to SIX effectors from the tomato infecting strain were identified in this *A. thaliana* infecting strain. Of these 4, SIX4 was 99% similar on the amino acid level, induced during infection of host roots, and deletion of the SIX4 gene resulted in a decrease of disease symptoms and in increase in survival of inoculated plants compared to inoculation with wild type Fo5176 (Thatcher et al., 2012).

Effectors are typically discussed in the context of plant-pathogen interactions, however the function of an effector to modulate the interaction a microbe has with a plant host is also shared by non-pathogenic microbes. *Trichoderma virens* again presents itself as an interesting example to explore pathology concepts in a beneficial interaction. Through bioinformatic analysis of the *T. virens* proteome and transcriptome when interacting with plant hosts, a class II hydrophobin protein was identified that was demonstrated to be a contributor to plant biomass increases, and colonization of the root by *T. virens*. This protein also has roles in the mycoparatisitic effect *T. virens* exerts over *Rhizoctonia solani*, and when over-expressed reduces the colony size of *R. solani* in *in vitro* assays (Guzmán-Guzmán et al., 2017). Arbuscular mycorrhizae (AM) are commonly found interacting with a wide variety of plants, and form intracellular structures called arbuscules that resemble pathogenic haustoria but are sites of nutrient exchange between the fungal symbioant and plant. In the AM fungus *Rhizophagus irregularis*, an effector that shares conserved domains with the crinkler (CRN) effector family is necessary for the formation of arbuscules in *Medicago truncatula* (Voß et al,. 2018). While the effector repertoire of Fo47 has not been defined, a different non-pathogenic *F. oxysporum* isolate CS-20 has had a small secreted protein characterized that reduces disease severity of *F. oxysporum* f. sp. *lycopersici* when plants are exposed to the putative effector prior to pathogen

challenge. This small protein named CS20EP was isolated by fractionating secreted proteins and reduces pathogen symptoms by increasing the expression of the Pathogenesis-Related 1(PR-1) gene and chitinase activity in tomato roots and leaves (Shcherbakova et al., 2016).

In this chapter, we sought to shed some light into the effector potential of Fo47 when it is interacting with *A. thaliana*. A pipeline for putative effector identification was designed, tested using the interaction between the pathogenic isolate Fo5176 and *A. thaliana*, and successfully pulled out the known SIX4 effector as well as other candidates with homology to effectors in other pathosystems. A crucial filter in our pipeline is the use of transcriptomic data of the early interaction between *F. oxysporum* and *A. thaliana* roots between 12 and 96 hours post inoculation. Unlike purely genomic or in silico-based research into detecting effectors, the use of RNA-seq data allowed for us to operate under the principal that effector proteins are specifically induced when the fungus interacts with the plant and select genes that are expressed in planta. In Fo47, we were able to identify 13 putative effectors, and 3 of our candidates share homology with known pathogen effectors. This chapter highlights the use of transcriptomic data to refine the search for putative effectors in a non-pathogenic interaction, and it will be exciting to further elucidate the role of these candidate genes in the Fo47-*A. thaliana* interaction.

METHODS

Transcriptome Analysis of the Interaction between Fo47 and *A. thaliana* roots

Infected plants at 12, 24, 48, and 96 HPI were harvested and roots collected from 5 plants per treatment per time point were subject to total RNA isolation. Meanwhile, the same number of control plants at 12 HPI, Fo5176 and Fo47 fungal mycelia from 5 day-old liquid cultures were also collected for RNA extraction. Three biological replicates were produced for each treatment. Total RNA extractions were conducted using the ZR Soil/Fecal RNA Microprep Kit (Zymo Research, CA, Cat. R2040) following manufacturer's protocol, and the RNA quantity and quality were assessed by NanoDrop 2000 and Agilent 2100 Bioanalyzer. Illumina TruSeq Stranded mRNA libraries were prepared and sequenced by Illumina HiSeq™ 2000 Sequencing System at the Broad Institute in Massachusetts.

Pair-end RNA-seq reads were first assessed for quality by FastQC 0.10.1 and then trimmed by Trimmomatic 0.32 to remove sequence adapters and low-quality reads that had a quality score threshold of 30 every sliding window of 4 nucleotides (Andrews, 2010; Bolger, Lohse, & Usadel, 2014). RNAseq data analysis was conducted using Tuxedo suite pipeline. Briefly, trimmed reads were mapped to reference genomes of Fo5176 and Fo47 (Broad Institute, MA) using Tophat 2.0.9. Mapped reads were assembled into transcripts and FPKM (fragments per kilobase per million mapped reads) were calculated using Cufflinks/2.2.1. Differential gene expression was conducted using Cuffdiff for infected samples per time point versus fungal control to discover differentially expressed fungal genes using a maximum false discovery rate of 0.05 and a minimal two-fold change as thresholds (Trapnell et al., 2012). RNA and transcriptomic resources and data made available by Dr. Li Guo.

Filtering for Putative Effectors in Non-Pathogenic *F. oxysporum*

The reference genomes of Fo47 and Fo5176 were analyzed by SignalP 4.1 to determine which genes has a predicted signal peptide domain with a D value that is greater than 0.45 to reduce the incidence of false positives (Nielsen, 2017). The list of genes that contained a signal peptide domain were then compared to the RNA-seq data set we have of differentially expressed fungal genes when interacting with *A. thaliana* roots, and genes with a signal peptide were selected that were specifically induced at least 2 fold more in planta than in culture conditions, and had an average FPKM of at least 10.

This short list was further reduced by considering protein size, and since effectors are usually small proteins, the cut off was for genes with a product that is 500 amino acids or less. We also examined the sequences of these candidate proteins, and determined if they were enriched for cysteine residues, as well as lacked transmembrane domains. Cysteine residues are common in effectors, and they are thought to contribute to the stability and tertiary structure of these secreted proteins (de Jonge & Thomma, 2009). The known effector from Fo5176, SIX4, was used as a check for the filters to ensure that it was not filtering out known *F. oxysporum* effectors. Further characterization of all candidate proteins by homology-based approaches was performed using HMMER to define the architecture of known domains within these sequences utilizing the UniProtKB proteome reference database (Finn, Clements, & Eddy, 2011). The BLAST tool available through the Pathogen-Host Interface Database (PHI-Base) was used to determine if the candidate Fo47 proteins have significant homology to proteins that have a known role in plant infection processes (Urban et al., 2017).

RESULTS AND DISCUSSION

Effector Screening Pipeline Proof of Concept with Fo5176

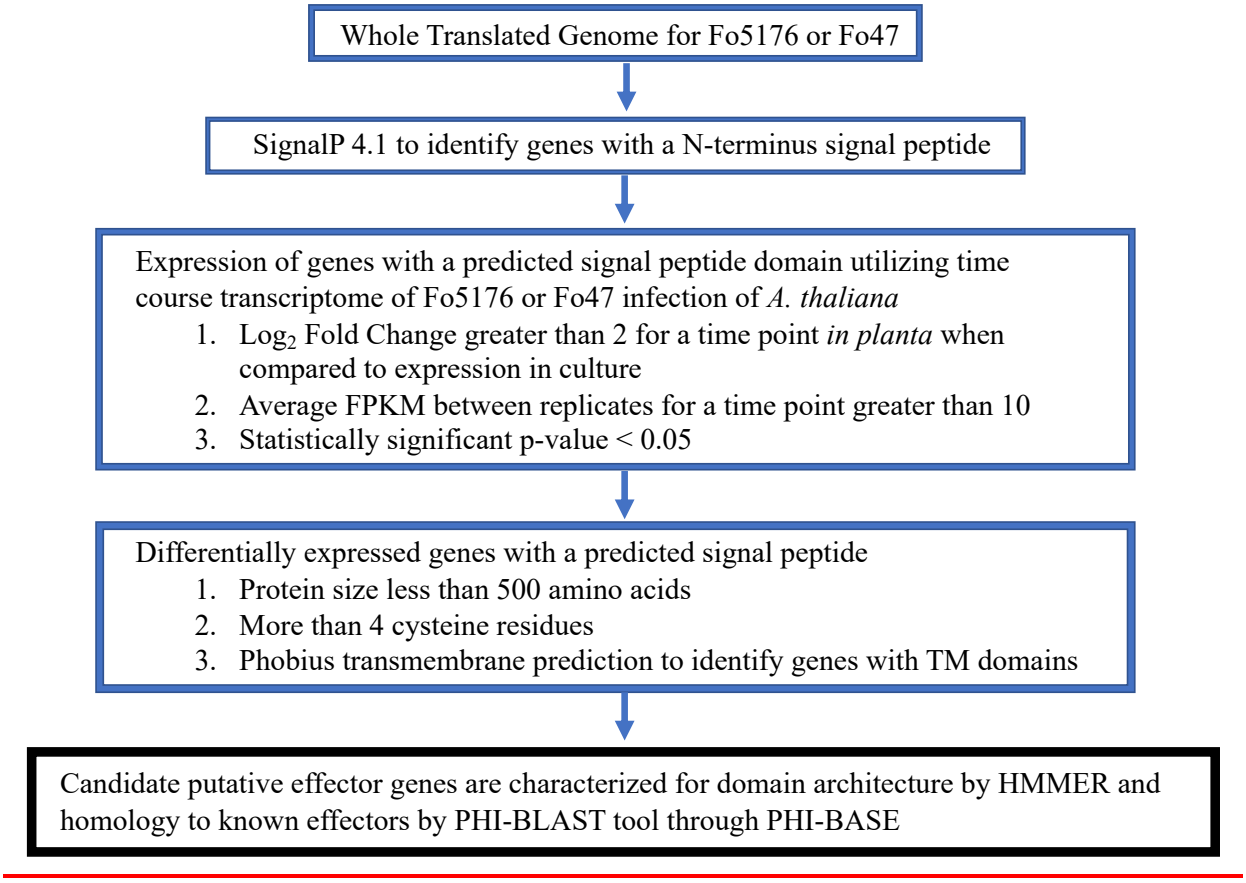

Figure 8: Pipeline for Identifying Putative Effectors from *F. oxysporum*

Figure 8 is a breakdown of the filters applied to the whole translated genome of the Fo5176 isolate to home in on potential effectors. Table 3 describes the candidate effectors identified from Fo5176 and highlighted by a red box within this table is the SIX4 gene. We know from previous research that SIX4 is expressed by Fo5176 when it interacts with *A. thaliana*, and we wanted to use the identification of this effector from our pipeline as a validation measure that we are not excluding known effectors (Thatcher et al., 2012).

Gene ID	12 HPI FC	24 HPI FC	48 HPI FC	96 HPI FC	Protein Size	# of Cysteine Residues	HMMER Predicted Domains	Homology to Known Effectors
FOXB_04098	2.51	4.36	5.34	5.63	433	13	Glycosyl hydrolase family 18 domain	*T. virens* chitinase *CHT42*, reduced virulence on *R. solani*
FOXB_09542	4.47	6.30	5.08	4.95	241	12	Pectate lyase Domain	*F. solani* subspecies *pisi* pectate lysase A *pelA*, reduced virulence on pea
FOXB_11108	5.59	6.58	5.21	5.81	241	12	No Significant Domains	No Significant Hits
FOXB_17950	6.79	5.13	7.19	5.20	96	10	CFEM Domain	No Significant Hits
FOXB_04592	-0.99	3.07	1.16	1.41	401	10	Glycosyl hydrolase family 61 domain	No Significant Hits
FOXB_00712	4.46	6.36	6.14	4.45	236	9	CHAP Domain	No Significant Hits
FOXB_04181	5.26	5.28	4.56	4.58	196	8	CFEM Domain	*F. graminearum* FGSG_02077, reduced virulence on wheat
FOXB_18619	3.95	6.03	7.59	7.73	139	6	No Significant Domains	*Leptosphaeria maculans* AvrLm6, avirulence determinant on *Brassica napus*
FOXB_04959	1.59	3.17	3.60	3.83	221	6	No Significant Domains	No Significant Hits
FOXB_04901	4.04	8.62	10.72	11.33	243	6	No Significant Domains	*F. oxysporum* f. sp. l*ycopersici* SIX4, avirulence determinant on tomato
FOXB_05922	5.42	4.85	1.35	2.27	315	6	CFEM Domain	No Significant Hits
FOXB_06937	5.93	5.02	5.88	5.73	211	5	No Significant Domains	No Significant Hits
FOXB_19213	5.99	8.65	8.72	9.03	131	4	No Significant Domains	No Significant Hits
FOXB_14988	1.55	1.28	1.52	2.81	146	4	No Significant Domains	No Significant Hits

Table 3: Properties of Candidate Effectors from Fo5176 that were Isolated by our Pipeline.

The SIX4 gene, FOXB_04901, was used as a control as previous work determined it is expressed and an effector on *A. thaliana*. SIX4 was pulled out of our pipeline, and is highlighted in red. The gene highlighted in black is homologous to a gene in Table 4 from Fo47. Expression of candidate genes is reported as the Log2 fold change of the *in planta* expression compared to the expression in liquid culture.

Gene ID	12 HPI FC	24 HPI FC	48 HPI FC	96 HPI FC	Protein Size	# of Cysteine Residues	HMMER Predicted Domains	Homology to Known Effectors
FOZG_18295	7.70	9.16	8.92	9.35	241	12	No Domains	No Significant Hits
FOZG_13798	4.28	7.46	7.84	7.27	96	10	CFEM Domain	No Significant Hits
FOZG_17490	7.95	9.73	7.03	7.55	95	8	No Domains	No Significant Hits
FOZG_17267	4.71	4.49	6.17	4.62	135	8	No Domains	No Significant Hits
FOZG_02644	2.44	1.54	1.71	2.37	196	8	CFEM Domain	*F. graminearum* FGSG_02077, reduced virulence on wheat
FOZG_18219	4.46	4.48	4.91	5.29	228	8	No Domains	No Significant Hits
FOZG_16010	3.55	5.98	5.77	4.80	276	7	MEROPS peptidase domain	*Coccidioides posadasii* MEP1, reduced virulence on mouse
FOZG_02301	5.85	4.97	4.69	5.44	326	7	No Domains	No Significant Hits
FOZG_03790	3.31	3.41	1.93	2.30	315	6	CFEM Domain	No Significant Hits
FOZG_15044	4.20	2.44	2.99	2.63	211	5	No Domains	No Significant Hits
FOZG_16552	7.12	9.81	7.86	4.13	247	5	No Domains	No Significant Hits
FOZG_07196	2.01	1.82	4.15	4.62	404	5	Glycosyl hydrolase family 17 domain	*Blumeria graminis* f. sp. *Hordei* BEC1005, reduced virulence on barley
FOZG_04373	5.15	6.01	6.24	5.23	284	4	No Domains	No Significant Hits

Table 4: Characteristics of Candidate Effectors Identified in Fo47 using our Pipeline.

The gene highlighted in a black box is homologous to a gene in Table 3. Expression of candidate genes is reported as the Log2 fold change of the *in planta* expression compared to the expression in liquid culture.

Characteristics of Putative Fo47 Effectors Involved in the *A. thaliana* Interaction

Our pipeline using publicly available genomic data and bioinformatic packages combined with experimental RNA-seq data of the interaction between the non-pathogenic *F. oxysporum* isolate Fo47 and *A. thaliana* was able to identify 13 candidate effector genes that could be important for the beneficial interaction between the two (Table 4). Three of these candidates are particularly unique as they share homology with known effectors from other systems.

The gene highlighted with a black box in tables 3 and 4 are homologous to each other with 100% similarity between Fo5176 and Fo47 on the protein level. These genes, FOXB_04181 and FOZG_02644, share 59% amino acid similarity with a gene from *F. graminearum*, FGSG_02077, that when reduced in expression by transposon insertion results in lower pathogenicity on wheat (Dufresne et al., 2008). In *F. graminearum*, the role or function of this

hypothetical protein is not understood, but like the homologs in Fo5176 and Fo47 it contains a CFEM domain. CFEM domains are unique to fungi and tend to be found in extracellular membrane proteins with functions in pathogenesis (Zhang et al., 2015). The first CFEM domain containing protein was described in *Magnaportha grisea*, MAC1, and this protein was found to be a necessary protein for successful appressorium formation on rice (Choi & Dean, 1997). CFEM domain containing proteins are also found in non-pathogenic fungi, and in *Saccharomyces cerevisiae* the protein CCW14 supports fungal cell wall stability by contributing to its biogenesis (Mrsa et al., 1999). There are two additional genes with CFEM domains in tables 3 and 4, and in both cases the genes are homologous between Fo5176 and Fo47 with 100% amino acid similarity. In the case of CFEM domain containing genes that are homologous between Fo5176 and Fo47, it is possible that these genes function in the broader interaction with *A. thaliana* that is shared between Fo5176 and Fo47 and are not effectors specific for pathogenic or non-pathogenic interactions.

The 2 additional putative effectors identified from Fo47 with structural similarities to known effectors are FOZG_16010 and FOZG_07196. FOZG_16010 is 51% identical on the amino acid level to a metalloprotease effector (MEP1) from *Coccidioides posadasii* that contributes to virulence on mouse by enzymatically breaking down a cell surface antigen that recognizes *C. posadasii* spores (Hung et al., 2005). A similar metalloprotease from *F. oxysporum* f. sp. *lycopersici* (MEP1) also contributes to virulence of this pathogen on tomato by working synergistically with a serine protease to cleave tomato chitinase enzymes and reduce their efficacy in digesting fungal cell walls (Jashni et al., 2015). It is plausible to hypothesize that this MEP1 like gene from Fo47 is involved in suppressing plant enzymatic activity that would prevent the establishment of Fo47 in the host epidermis and cortex.

The final effector with homology data behind it is FOZG_07196, and this gene is 30% similar on the protein level to the BEC1005 gene from *Blumeria graminis* f. sp. *hordei*. In *B. graminis*, this protein functions in cell wall remodeling prior to haustoria formation, and the authors state that it is a protein with a morphogenic role related to pathogenicity but argue that it is not an effector in the traditional sense (Pliego et al., 2013). In the interaction shared between Fo47 and pea roots, microscopic observations have revealed that Fo47 hyphae undergo alterations as they colonize the plant tissue. The authors report that the majority of Fo47 hyphae have a distorted and retracted cytoplasm with an accumulation of inclusions in the space between the membrane and cell wall (Benhamou & Garand, 2001). It is possible that in Fo47 this candidate effector functions in a way that is more of a morphological rather than secreted pathological factor to remodel cell wall components when interacting with *A. thaliana* roots. The effectors that do not have any recognizable domains or architecture are not discounted. In table 3, the SIX4 effector does not have any additional information to support its role as an effector, and it has been reported by other groups to lack recognizable domains (Thatcher et al., 2012). It is a property of effectors to have a high degree of diversity in sequences and resulting structures, and structural similarity does not mean that these sequences share functional roles (Białas et al., 2017). This is why our filters focus on non-structure or homology-based identification methods early on and include them later.

We report a conservative list of candidates that warrant further investigation for their expression in planta and predicted protein sequence and structure that suggests that they have a role in interacting with *A. thaliana*. Without functional characterization and localization, it is challenging to deduce the role these candidate effectors play in the interaction between Fo47 and *A. thaliana*. Our pipeline was validated using expression data from the pathogenic interaction

between Fo5176 and *A. thaliana* to identify the known SIX4 effector gene, and then applied to the non-pathogenic isolate Fo47 that has not had its effector potential described to our knowledge.

Our analysis identified candidates that are shared between the pathogen and non-pathogen and likely represent a core group of CFEM domain containing genes that *F. oxysporum* expresses to facilitate intercellular colonization of the host. We also isolated genes unique to the non-pathogen that could have metalloprotease or cell wall remodeling activity and be a component of Fo47's ability to exist within the plant root without resulting in disease symptoms. The use of RNA-seq data as a filter to remove genes that have the critical signal peptide domain that is necessary for the effector to leave the fungal cell, but are not induced when the fungus is *in planta* in comparison to culture conditions was a major asset in this analysis to lend some experimental basis to an otherwise *in silico* predictive search. It would be interesting to apply this filter to other Fo47-host interactions to determine if there is a core set of genes expressed that have effector characteristic as well as putative candidates that are host-specific, and to determine if Fo47 secreted proteins are a component of the disease reduction and biomass increase in the *A. thaliana* interaction.

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