Title
Host resistance and pathogen-derived hormone affect the outcome of a fungal-plant interaction

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Publication Date
2012

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Host resistance and pathogen-derived hormone affect the outcome of a fungal-plant interaction

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy in

Molecular, Cell, and Developmental Biology

By

Stephanie Joy Cole

2012

ABSTRACT OF THE DISSERTATION

Host resistance and pathogen-derived hormone affect the outcome of a fungal-plant interaction

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University of California, Los Angeles, 2012

Professor Andrew Diener, Chair

Like most organisms plants must be able to defend themselves from a variety of pathogens throughout their lifetime. In order to achieve this, plants uses a combination of hormone signaling and defense-related gene responses. However, pathogens also have mechanisms to overcome or manipulate these defense strategies in order to inhabit plant hosts. *Fusarium oxysporum* is a saprophytic filamentous fungus that infects a wide variety of plants and is the causal agent of Fusarium wilt disease. *F. oxysporum* like other soil borne fungi enters through plant roots where it eventually colonizes the xylem ultimately blocking water and nutrients causing wilt symptoms.

Previously, six RESISTANCE TO FUSARIUM OXYSPORUM (RFO) loci were identified in *Arabidopsis thaliana* Columbia 0 (Col0) accession against *F. oxysporum* forma specialis *matthiolae* (garden stock pathogen). *RFO1* and *RFO2* were identified as

a wall-associated kinase-like kinase and receptor-like protein, respectively. Here we discuss the cloning and characterization of another resistance gene, *RFO3,* in Arabidopsis. *RFO3,* like *RFO1,* is a receptor-like kinase (RLK) but belongs to the S Domain 1 (SD1) family. Other members of the SD1 family of RLKs have been shown to be induced by the bacterial pathogen, *Xanthamonas campestris*.

Several pathogens make plant hormones as secondary metabolites. These pathogen-derived hormones can alter plant hormone signaling to make plants more conducive to infection. In plants, jasmonic acid (JA) is considered important for developmental signaling and defense against necrotrophic and insect pathogens. However, we found several strains of *F. oxysporum* produce significant quantities of JA along with JA conjugated to leucine (JA-Leu) and isoluecine (JA-Ile), which is involved in JA signaling in plants. Furthermore, the JA-Ile/Leu produced by *F. oxysporum* is biologically relevant because it can activate the JA-responsive gene *THI2.1* in Arabidopsis seedlings. Interestingly, Arabidopsis *coi1-1* mutants are more resistant to several strains of *F. oxypsorum*. Also, a higher concentration of *F. oxysporum* was detected in COI1 wild-type roots compared to *coi1* mutant roots. Therefore, *F. oxysporum* derived JA may interfere with JA signaling through COI1 and is important for making Arabidopsis more susceptibile to *F. oxysporum*.

The dissertation of Stephanie Joy Cole is approved

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2012

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ACKNOWLEDGMENTS

I would like to thank my advisor, Andrew Diener, for his support and guidance throughout all stages of my dissertation project. Under his mentorship, I have furthered my understanding of genetics and enhanced my critical thinking skills. I appreciate his dedication to me and to my project. I also want to thank my committee members: Drs. Ann Hirsch, Steve Jacobsen, and Jeff F. Miller for their support, advice, and guidance. Additionally, I would like to thank both former and current members of the Diener lab especially, Yunping Shen for their support. I want to express my gratitude to Kym Faull and Alex Yoon for their help with HPLC tandem mass spectrometry. I would finally but most importantly like to thank my parents, brother, sister, and friends for their assistance, love, and support throughout the years.

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Chapter 1

Introduction

***Fusarium oxysporum* is a host-specific vascular wilt fungus.**

Special forms, or *formae speciales*, of the soil-borne filamentous fungus *Fusarium oxysporum* are responsible for devastating vascular wilt and root rot diseases that limit the cultivation of over one hundred crops. However, most *F. oxysporum* is associated with the roots of asymptomatic plants worldwide, and most isolates of *F. oxysporum* are nonpathogenic in infection assays. It is the rare isolate that is pathogenic to specific plants.

The most publicized example of Fusarium crop infection occurs in banana and is known as Panama disease. The causal agent of Panama disease is *F. oxysporum* forma specials (f sp) *cubense*. It was first documented in Australia in 1876 and by the 1960's had affected banana plantations throughout most banana-producing areas. Panama disease slowed after the introduction of a resistant variety, known as Cavendish. But in more recent times Panama disease has reemerged in parts of Southeast Asia and now the Cavendish variety is susceptible to *F. oxysporum* f sp *cubense* race 4, which threatens banana crops globally (Ploetz, 2000). In a recent survey of plant pathologist by Molecular Plant Pathology, *F. oxysporum* was voted one of the top ten fungal pathogens (Dean et al, 2012). *F. oxysporum*, like other soil borne fungal pathogens, causes significant crop loss each year and is difficult to control and treat.

Different classification systems have been applied to *F. oxysporum* pathogens. Classification by morphology places *F. oxysporum* in the kingdom fungi, phylum Ascomycota, class Sordariomycetes, order Hypocreales and family Nectriaceae. No

sexual phase has been observed in *F. oxysporum*, despite the fact that *F. oxysporum* is related to perfect fungi and haploid isolates have either of two idiomorphic mating type alleles that are conserved with its perfect sister species complex *F. verticillioides*. Plant pathologists have classified F. oxysporum in *formae speciales* because the pathogenic isolates have restricted host ranges and typically cause disease in only one or a few closely related plant species. Formae speciales may either be monophyletic, as DNA identity indicates individuals are from the same clonal lineages, or polyphyletic in which case two or more unrelated clonal lineages are pathogens with a similar host range. Different cultivars or varieties of a species may have different susceptibities to isolates from the same formae speciales. Plant pathologists use differences in resistance/susceptibility of cultivars or varieties of a host species to further classify members of a forma specials into races. Genetic exchange can occur between *F. oxysporum* isolates if they belong to complementary vegetative compatibility groups (VCGs). When this occurs, the hyphae from either strain can fuse along with nuclei. Additionally horizontal genetic exchange can occur with supernumerary chromosomes between noncompatible strains of *F. oxysporum*. Supernumerary chromosomes contain unique sequences that carry a large number of genes that code for secreted proteins and other genes specific for plant infection (Kistler & Rep, 2010). These supernumerary chromosomes can be transferred between *F. oxysporum* strains and convert nonpathogenic strains into pathogenic strains (Ma et al, 2010).

Pathogens are categorized as biotrophs, necrotrophs, or hemibiotrophs to describe their lifestyles, or the kind of virulence that is utilized to further infection and

produce disease. However, the lifestyle of a particular pathogen is not definite for all types or phases of infection and virulence of a pathogen may have characteristics that do not exclusively fit into one type. Biotrophic microbes obtain energy from living hosts. Some biotrophs are obligate biotrophs, like *Hyaloperonospora arabidopsidis* (downy mildew), and cannot survive on their own without a host. The second type of pathogen is the necrotroph that acquires energy from dead or decaying tissue such as *Botyis cinerea* (gray mold). These pathogens typically have a wider host range than biotrophs, produce large quantities of degrading enzymes, and generate toxins (de Wit, 2007; Glazebrook, 2005). These life styles are in contrast to *F. oxysporum,* a hemibiotroph capable of colonizing a living plant host as a biotroph but eventually killing the host and living off the dead tissue like a necrotroph. After killing the host, *F. oxysporum* forms chlamydospores, a thick-walled spore structure, and can survive long periods of time associated with organic matter in soil. Once new roots grow in the vicinity providing nutrients, *F. oxysporum* chlamydospores germinate and begin colonizing root tissue (Beckman, 1987).

Though *F. oxysporum* isolates infect many important crop species in nature, *Arabidopsis thaliana* is not an agriculturally significant plant host of *F. oxypsorum*. Nevertheless Arabidopsis has many advantages that are not available with other plants species including TDNA insertion mutants, a fully sequenced genome, and deciphered hormone pathways. Closely related Cruciferous isolates of *F. oxysporum* are capable of infecting Arabidopsis and causing disease symptoms similar to what is seen in their original host. These isolates include: *F. oxysporum* f sp matthiolae (garden stock

pathogen), *F. oxysporum* f sp conglutanins race 1 and 2 (cabbage pathogen), and *F. oxysporum* f sp raphani (radish pathogen) (Diener & Ausubel, 2005). Therefore, Arabidopsis is used a model host for studying *F. oxysporum* pathogenicity and host defense.

Vascular wilt diseases

Vascular wilt disease is routinely caused by Fusarium and Verticillium species but other bacteria and fungi can also cause vascular wilt disease. Wilt disease is characterized first by a stunting of the plant and drooping lower leaves. Also, many vascular wilt pathogens cause lower leaf abscission. This is followed by chlorosis of the leaves starting in the vasculature and migrating throughout the leaves. Finally, the infected plant collapses and dies. It is thought that disease symptoms are caused by two methods. First, the colonization of the xylem vessels by the pathogen blocks water and some nutrients from the root to reach the shoot. This blockage then causes the plant to wilt and die. A second theory is that the toxins and secondary metabolites made by the pathogen cause disease symptoms (Dimond, 1970; Beckman, 1987).

Soil-borne vascular pathogens, like *F. oxysporum*, enter the plant through young root tips where the root is actively growing. Mature roots are comprised of cells that are more rigid than younger root cells that contain cork and a higher lignin concentration making them more difficult colonize. Talboys (1957) describes three phases of infection by vascular wilt pathogens. The first phase, called the primary determinative phase, determines whether the pathogen can invade and inhabit plant roots. During this phase

the pathogen must make it through the cortex and endodermis of the root. Additionally, cell wall degrading enzymes are produced by vascular wilt pathogens. These enzymes help with the initial entry into host. The second phase, the secondary determinative phase, describes the ability of the pathogen to colonize the vascular system. Once the fungus passes through the cortex and endodermis and reaches the xylem, it colonizes the lumen of the vessels. The xylem is a nutrient poor environment with few amino acids and sugars. The xylem also has low oxygen levels making it a difficult environment for a pathogen to thrive in. However, some pathogens can increase nutrient levels of the xylem by breaking down surrounding cells as seen with *Ophiostoma ulmi,* the causal agent of Dutch elm disease (Svaldi & Elgersma, 1982). Mycelia established in the xylem also form conidiospores. These conidiospores can migrate short distances in small plants or great distances in larger plants. The conidiospores can germinate and form more mycelia and begin infecting a new area of the plant. In the final phase, the expressive phase, fungal activity causes disease symptoms in the plant to become apparent. During this time the fungus begins to produce toxins and secondary metabolites that become diluted and distributed though vasculature. The role that some of these toxins play is unknown but have been hypothesized to cause disease along with blockage of vessels. Pathogens also produce polysaccharides and glycoproteins that can cause wilt symptoms by increasing the viscosity of the vasculature and decreasing the vascular flow rate (Figure 1-1).

External factors also have an effect on wilt disease. For *F. oxysporum* infection, elevated temperature reduces the time of disease progression. In tomato it was noted

that increasing growth temperatures increased disease severity and decreasing temperatures decreased disease severity. The optimum temperature of *F. oxysporum* f sp *lycopersici* (FOL) infection in tomato was determined to be 28°C. Temperatures above 34°C or below 20°C did not elicit disease symptoms (Clayton, 1923). Furthermore, the soil itself can affect disease symptoms. There are documented cases of suppressive soils where Fusarium wilt has rarely been seen. Suppressive soils contain some factor(s) including nutrient content, pH, presence of biological control microbes, and clay content that are able to suppress or reduce disease symptoms. Jones and Woltz (1970) found amending soil with lime caused an increase in soil pH which decreased wilt in tomato. However, the reduced symptom development was lost if manganese and zinc or zinc and iron were added with the limestone. This study emphasized the importance of micronutrients on *F. oxypsorum*. Also, nonpathogenic strains of *F. oxysporum* are able to suppress pathogenic strains of *F. oxysporum* (Fravel et al, 2002).

Plant response to vascular wilt pathogens

In plants, resistance to pathogens is controlled by primary and secondary determinants. As described by Talboys (1972), primary determinants affect the initial infection into the root. When the pathogen first enters a plant root, deposition of lignin around the inner surface of cell walls and around hyphae increases, especially in young cells that are less ridged and more vulnerable. Phytoalexins, a class of antimicrobial compounds, are also important primary determinants. One of the most well studied is

camalexin, which can inhibit the growth of the bacterial pathogen *Pseudomonas syringae* (Glazebrook & Ausubel, 1994). Camalexin is also produced after infection by *Alternaria brassicicola, P.syringae,* and *B. cinerea* (Schuhegger et al, 2006*).* Meanwhile, secondary determinants affect colonization of the vascular system. In response to infection, plants produce gels and tyloses. Tyloses are protrusions of the cell wall into the xylem vessels caused by auxin like compounds, wounding and pathogen infection. Tyloses increase resistance to the flow of water through xylem and can even block the vessel completely. Tyloses are helpful for defense in that they block the pathogen from growing further up into the vascular (Elgersma, 1973). Tyloses can also be detrimental, if too many vessels are blocked, water flow is blocked from the root to the shoot (Talboys, 1958). Gels, like tyloses, are made by the plant and secreted into the xylem to block pathogens and are made from compounds such as pectin. Also, plants make aromatic and phenolic compounds as a defense mechanism**.** When infected, hormones such as indole 3 acetic acid (IAA) increase in the plant, which can be from the plant or from pathogens that produce IAA. High levels of IAA can lead to hyperauxiny. Hyperauxiny can cause the collapse of xylem vessels due to hyperplasia of xylem parenchyma and formation of tyloses and gels. IAA and other hormones can both increase defense or susceptibility to a pathogen depending on the level of response (Dimond, 1970) (Figure 1-1).

The rate of response by the plant to a pathogen is crucial to whether or not the plant is resistant or susceptible. If the plant's first primary determinant response to a pathogen is delayed, then the pathogen has overcome the first line of defense making

the plant more vulnerable. If the plant continues to have a delayed defense response the pathogen with continue to colonize the roots and the plant will be susceptible to disease (Talboys, 1972).

Plant immunity

All organisms have some form of defense mechanism in order to protect themselves from predation. In plants, the whole organism contributes to host defense and all tissues are competent to respond to infection and damage by pathogens and pests as there is no separate organ or specialized circulating cells that respond to pathogens. At the cellular level, the response is called innate immunity as it shares analogy to innate immunity in animal cells. Coincidences and analogies of innate immunity in plants and animals are the result of convergent evolution as revealed by molecular characterization. In addition to an immediate cellular response, plants produce mobile signals to activate systemic responses in distant tissues.

MAMP-triggered immunity (MTI)

During infection, plants recognize pathogens by responding to molecular features or activities of invading microbes that are either general or specific. General molecular features, or patterns, of both pathogenic and nonpathogenic microorganisms are referred to as microbe-associated molecular patterns (MAMPs), formerly referred to as pathogen-associated molecular patterns (PAMPs). Also plants respond to molecules that result from pathogen-derived activities that cause damage to the host, or damage-

associated molecular patterns (DAMPs). Recognition of and response to MAMPs (or DAMPs) results in MAMP-triggered immunity (MTI), which is considered the first line of defense against an invading pathogen. MAMPs are molecular components produced by a specific class of pathogen and are important for microbial fitness. For example, flagellin in bacteria and chitin in fungi are considered MAMPs. MAMPs are recognized by the plant through pathogen recognition receptors (PRRs). Because PRRs detect patterns on class of pathogen, MTI is thought of as providing broad spectrum resistance. PRRs are characteristically receptor like proteins (RLPs) or receptor like kinases (RLKs). In Arabidopsis, there are approximately 600 RLKs and 50 RLPs that in addition to defense are involved in development and hormones response (Shiu & Bleeker, 2001; Fritz-Laylin et al, 2005). All known PRRs are cell membrane-localized and respond to transmembrane or secreted proteins. Two of the most well characterized PRRs are Flagellin Sensing 2 (FLS2) and Elongation Factor Tu Receptor (EFR). Both of these PRRs are RLKs with a leucine rich repeat extracellular domain and an intracellular Serine/Threonine kinase domain (Zipfel et al, 2006). FLS2 was discovered in Arabidopsis and interacts with a 22 amino acid segment of flagellin, flg22 (Chinchilla et al, 2006). FLS2 mutants were found to be more susceptible to infection by *P. syringae* DC 3000 (Göhre et al, 2008). Functional orthologs of FLS2 have been found in rice and tomato, which interact with flg15 instead of flg22 (Robatzek et al, 2007; Takai et al, 2008). EFR interacts with elf18 of elongation factor, one of the most abundant proteins produced by bacteria (Kunze et al, 2004). ERF mutants are more susceptible to Agrobacterium transformation (Zipfel et al, 2006). There are also known fungal PRRs,

e.g. CEBiP and LeEIX1 and 2. CEBiP recognizes chitin and is a RLP. RLPs have an extracellular domain and only a short C terminal tail that lacks a signaling domain. However, CEBiP interacts with CERK1, which is a RLK. Interaction between RLPs and RLKs is considered a possible mechanism for downstream signaling for RLPs. LeEIX1 and LeEIX2, both RLPs, were discovered in tomato and they interact with ethylene-induced xylanases. PRRs appear to focus on highly conserved sequenced of MAMPs that are required for function.

PRRs may not act alone in MTI signaling. Other coreceptors like BRI-associated kinase 1(BAK1) have been shown to form complexes with FLS2 and EFR after activation by MAMPs (Chinchilla et al, 2007; Schulze et al, 2010). BAK1 was originally determined to be a coreceptor for brassinosteroid signaling with its partner Brassinostreoid Insensitive 1 (BRI1) (Nam and Li, 2002). In addition to brassinosteroid signaling, BAK1 mutants are more susceptible to pathogens and this phenotype is independent of hormone (Kemmerling et al, 2007). Additionally, BAK1 belongs to the somatic embryogenesis receptor kinase (SERK) family and other family members are predicted to act as coreceptors for RLKs and RLPs as well (Zipfel, 2008).

Quantitative trait loci (QTL) for resistance to *F. oxysporum* have been reported in interactions with melon (*F. oxysporum* f sp *melonis* race 1.2), cotton (*F. oxypsorum* f sp vasinfectum (FOV) race 1), and Arabiodopsis (*F. oxysporum* f sp matthiolae (FOM)) (Ulloa et al 2011; Perchepied et al, 2005; Diener & Ausubel, 2005). In cotton, Ulloa et al (2011) found multiple loci that potentially contribute to resistance to FOV race 1 on nine separate chromosomes. Ulloa et al (2011) also identified a major locus on chromosome

16 previously found to provide resistance as a single gene, *Fov1*, but noted in their study that Mendelian ratios were distorted indicating that it is likely that multiple genes contributed to resistance against FOV race1. In a cross between Arabidopsis accessions Columbia-0 and Taynuilt-0, six *RESISTANT TO FUSARIUM OXYSPORUM (RFO)* loci were identified. From QTL mapping, *RESISTANCE TO FUSARIUM OXYSPORUM 1 (RFO1)* was identified as a major contributor to resistance and enhances resistance to multiple strains of *F.oxysporum* along with fungal pathogen, *Verticillium longisporum* (Diener & Ausubel, 2005; Johansson et al, 2006). Therefore, like most PRRs, *RFO1* provides a broad spectrum of resistance. Additionally, *RFO1* belongs to the wall-associated kinase-like kinase family of RLKs. It is unknown what MAMP *RFO1* recognizes or what is the signaling mechanism for *RFO1*. Finally, *Ve1*, a RLP in tomato, was first discovered to be a resistance gene against *Verticillium dahlia*. *Ve1* recognizes fungal protein Ave1. Homologs of Ave1 were found in other pathogens including FOL. De Jonge et al (2012) found Ve1 also provides resistance to FOL in tomato. Both *Ve1* and *RFO1* seem to provide broad spectrum resistance and are RLK or RLPs characteristic of PRRs in MTI.

Effector triggered immunity (ETI)

The MTI response is thought of as a priming response to the stronger, quicker ETI response. ETI has its own set of receptors called resistance proteins (R proteins); most belong to the nucleotide binding site leucine-rich repeat (NBS-LRR) class of receptors. Unlike MAMPs, pathogen effectors or avirulence (AVR) factors are small

proteins. In rare cases, the host receptor directly interacts with a pathogen effector. For example, in rice Pi-ta is a receptor that recognizes AVR-Pita made by *Magnaportha grisea,* the causal agent of rice blast (Jia et al, 2000). For most R proteins a direct interaction cannot be found with AVR proteins, therefore, new models were proposed. In the decoy model, a host protein that does not function in defense is modified by the pathogen and the modified host protein is recognized by the R protein. In the guard model, the host “guardee” protein that functions in immunity is modified and then in recognized by the R protein (Dangl & Jones, 2001). Once effectors are recognized by host R proteins the effectors must be modified or eliminated for the pathogen to evade recognition by plant. Effectors that are indirectly recognized by R proteins are predicted to be deleted by excision from the pathogen. Pathogen virulence is not affected by this excision because there may be other closely related effectors to replace those that are lost. Effectors that directly interact with resistance proteins are more likely to modify a few amino acids so as to avoid recognition by R proteins. Downstream of ETI response is the activation of MAP kinase pathway that activates WRKY/TGAs transcription factors. These transcription factors activate genes for reactive oxygen species and phytoalexins. ETI is associated with the hypersensitive response (HR), a type of controlled cell death used to kill a portion of the infected plant to reduce the spread of the pathogen. The HR is also associated with salicylic acid (SA) signaling and is used against biotrophic pathogens (Dangl & Jones, 2001).

In the interaction between FOL and tomato, *Immunity (I)* genes were discovered that provide race specific resistance to FOL. Three races of FOL have been identified,

each having a corresponding dominant resistance gene in tomato. The first *I* gene was discovered in Missouri accession 160, described by Bohn and Tucker in 1939, and was integrated into tomato lines and for several decades suppressed FOL infection. Resistance to FOL lasted until FOL race 2 emerged in the 1940's but did not become a problem until the 1960's. A new *I* gene, called *I-2,* was found to provide resistance to FOL race 2 and once again was integrated into commercial varieties of tomato. Finally, FOL race 3 emerged in Australia (1979) and Florida (1982) and a final *I* gene, *I-3*, was found to provide resistance to race 3 (Huang & Lindhout, 1997). So far *I-2* is the only *I* gene that has been cloned. *I*-2 belongs to the NBS-LRR class of receptors, which is typical of R genes. In addition to the *I* genes found in tomato, eleven candidate effectors secreted by FOL into xylem sap known as *Secreted in Xylem* (*SIX*) genes were also identified. SIX4 (AVR1) is important for *I* resistance and can suppress resistance of *I-2* and *I-3*. Recently, SIX4 was shown to have conserved virulence function in Arabidopsis (Thatcher et al, 2012). SIX3 (AVR2) activates *I-2* resistance (Houterman et al, 2009). SIX1 (AVR3), a cysteine-rich protein, is required for full virulence of I-3 line (Rep et al, 2005). Whether the SIX proteins are recognized directly by I receptors or interact indirectly as in the guard or decoy models has yet to be determined. Takken and Rep (2009) describe how the interaction between SIX effectors and I receptors fits into the model of the arms race between pathogen effectors and R proteins (Figure1-2). Tomato plants recognize the SIX effector, SIX4, via the I receptor, and in turn, the FOL SIX4 effector evolved new effectors (SIX3 and SIX1) to overcome resistance. Tomato I receptors, I-2 and I-3, are also evolving to recognize emerging FOL effectors.

The role of hormones in plant pathogen interaction

Hormones are important for all stages of development, growth, biotic and abiotic stress in plants. SA, jasmonic acid (JA), and ethylene (ET) are considered the traditional examples of plant defense hormones. SA is typically associated with biotrophic and hemibiotrophic pathogen defense response. SA mutants are more susceptible to the biotrophic pathogen *Peronospora parasitica.* Meanwhile, JA and ET both contribute to necrotrophic pathogen and insect defense. JA and ET mutants are more susceptible to the necrotrophic pathogens *B. cinerea* and *A. brassicola*. Furthermore, SA is described to antagonize JA and ET signaling (Thomma et al, 1998; Glazebrook, 2005). More recently, other hormones were reported to contribute to the defense response. Auxin, important for growth and development, increases susceptibility to biotrophs and decreases the expression of the SA-responsive gene, *Pathogen Related 1(PR1).* There is also believed to be an antagonistic relationship between SA and auxin because increasing SA decreases plant growth. Gibberellins (GA) increase SA after biotic or abiotic stress. Furthermore, DELLA mutants, negative regulators of GA signaling, are more resistant to *P. syringae* due to elevated SA. Conversely, DELLA mutants are susceptible to *B. cinerea* and *A. brassicicola* because of a decrease in JA signaling. Abscisic acid (ABA) has been shown to both increase susceptibility and increase resistance depending on the pathogen tested. For example, ABA levels can increase after stress, which makes plants more susceptible to virulent *P. syringae*. Furthermore, ABA provides resistance to *A. brassicicola* and *H. arabidopsidis* (Fan et al, 2009)

Many microbes, pathogenic and nonpathogenic, take advantage of plant hormone signaling. One mechanism used by microbes to affect hormones in plants is to synthesize hormones. For example, the necrotrophic fungus *B. cinerea* makes ABA (Seiwers et al, 2006). Meanwhile, *F. oxysporum* produces ABA and JA (Dorffling et al, 1984, Mierch et al, 1998). ABA and cytokinins were detected in cultures of fungus *Ustilago maydis* and in tumors formed on infected maize (Bruce et al, 2011). Several *Pseudomonas* and *Xanthomonas campestis* strains synthesize IAA when grown on media containing tryptophan. *P. syringae pv syringae* was able to produce high concentrations of IAA in culture without the addition of tryptophan (Fett et al, 1987). Occasionally, hormone production by microbes causes hormone imbalance in the plant and leads to symptoms. The pathogenic fungus *Gibberella fujikuroi* causes bakanae disease in rice. Infected rice plants are taller that uninfected plants because *G. fujikuroi* produces GAs, hormones that regulate growth (Ou, 1985). Another example includes *Agrobacterium tumefaciens*, the cause of crown gall disease. The galls are caused by increased levels of auxin and cytokinin produced by the pathogen (Akiyoshi et al, 1983). For pathogens, the production of a hormone is typically correlated with disease but the role the microbe-produced hormones play in disease is not well understood. Another strategy for microbes for taking advantage of plant hormones is to make mimics of them to alter hormone signaling. The classic example is coronatine, which is synthesized by several *P. syringae* species. Coronatine is a mimic of JA conjugated to isoleucine (JA-Ile), the active signaling form of JA (Fonseca et al, 2009). *P. syringae* uses coronatine to take advantage of the antagonism between JA and SA to make plants more

susceptible to infection (Katsir et al, 2008). Additionally, coronatine affects hormone signaling in order to keeping stomata open thereby allowing more bacteria to enter. Another strategy is for the pathogen to induce the plant to produce a particular hormone. Many pathogens produce effectors that can increase hormone concentration. AvrBs3, an effector made by *X. campestris* pv. *campestris*, induces auxin-responsive genes, whichresults in cell hypertrophy (Marois et al, 2002). Furthermore, *Pst* AvrPtoB increases ABA concentration in the plant and suppresses MAMP-induced genes. As a result, growth of the bacteria is enhanced and basal defense responses are reduced (de Torres-Zabala et al, 2007).

F. oxysporum and hormone interactions

For *F. oxysporum*, plant hormone interactions have presented unexpected results. As stated previously, defense against necrotrophic pathogens typically utilizes JA and ET signaling while defense against biotrophic pathogens requires SA. Additionally, most of the experiments done to determine the effect plant hormones have on *F. oxysporum* infection have been done in Arabidopsis due to availability of TDNA insertion mutations in components of biosynthesis and signaling pathways. Berrocal-Lobo and Molina (2004) found the overexpression of Ethylene Response Factor 1 (ERF1), a transcription factor that is JA and ET dependent, enhanced resistance of Arabidopsis seedlings on plates using both FOX f sp conglutinians (FOC) and FOL. Furthermore, ERF1 expression was not induced in either *coi1-1* (JA) or *ein2-5* (ET) mutants but ERF1 expression was still induced in NahG (SA) mutants. Therefore, ERF1 is JA and ET dependent and SA independent. Furthermore, Berrocal-lobo and Molina

showed that infection in Arabidopsis with FOC and FOL increased disease severity in *ein2-5* (ET), *coi1-1* (JA), NahG and *npr1-1*(SA), but not in *pad4* or *eds1* (SA). *pad4* and *eds1* mutants were unchanged compared to WT. NahG, *npr1-1*, *jar1-1*, *ein2-5* showed even greater reduction in fresh weight than WT. Not only does JA and ET affect *F. oxysporum* infection, but SA does as well. The importance of SA in *F. oxysporum* infection was also seen by Edgar et al (2006). Adding SA prior to infection increased resistance to FOC. However, adding methyl jasmonate (MeJA), a volatile form of JA, prior to infection had no effect on resistance. Expression of the JA responsive gene *PDF1.2* was induced in leaf tissue after MeJA treatment (Edgar et al, 2006). Therefore both SA and JA/ET play a role in Fusarium interaction with Arabidopsis. Like SA, ABA is believed to antagonize JA and ET signalling. Application of ABA reduces *PDF1.2* expression and in the presence of ABA with either MeJA or ethylene, expression of *PDF1.2* cannot be induced. However, ethylene and MeJA induce *PDF1.2* expression in the absence of ABA. Conversely, *aba2-1* mutants express higher levels of JA responsive genes (*HEL, CHI,* and *PDF1.2*) compared to WT. *MYC2* is an ABA-responsive gene and *jin1-9/myc2* TDNA insertion mutants have higher expression of JA responsive genes. Furthermore, *jin1/-9 myc2* mutants are more resistant to FOC. *myc2* mutants had reduced fungal RNA compared to WT plants (Jonathan et al, 2004). In contrast to Berrocal-Lobo and Molina who showed that *coi1-1* seedlings infected on plates were more susceptible to FOC and FOL, soil-infected *coi1-1* mutants were more resistant to FOC and there were no differences between WT and other JA mutants (*aos*, *jar1-1*, *opr3*) (Thatcher et al, 2009; Cole et al, Chapter 3). Plant defense hormone

signaling against *F. oxysporum*, like most rules for defense, do not always follow the prescribed pattern. And in fact both SA and JA play a role in defense.

F. oxysporum is an excellent model pathogen for the study of soil-borne disease. With the addition of full genome sequencing of many isolates, there will more information about the pathogenicity of *F. oxypsorum*. FOL was the first isolate to be sequenced along with *Fusarium graminearum* and *Fusarium verticilium*. From these data, supernumerary chromosomes and areas with high numbers of transposable element encoding sequences that seem to be important for pathogenic activity were discovered. Currently several new strains are being sequenced and already homologues for SIX effectors have been found in FOC. Expectantly more will be discovered about *F. oxysporum* pathogenicity through bioinformatics. In addition to learning more about the pathogenicity, hopefully new discoveries will be made in finding what controls host specificity. What prevents isolates of *F. oxysporum* from infecting many plant species is still unknown. Not only is it important to be thinking from the prospective of the pathogen but also to be working with the idea of how the plant defends itself against the pathogen. How is the plant recognizing the effectors from *F. oxysporum* whether it be via PRRs, or R proteins and what is activated downstream of these receptors?

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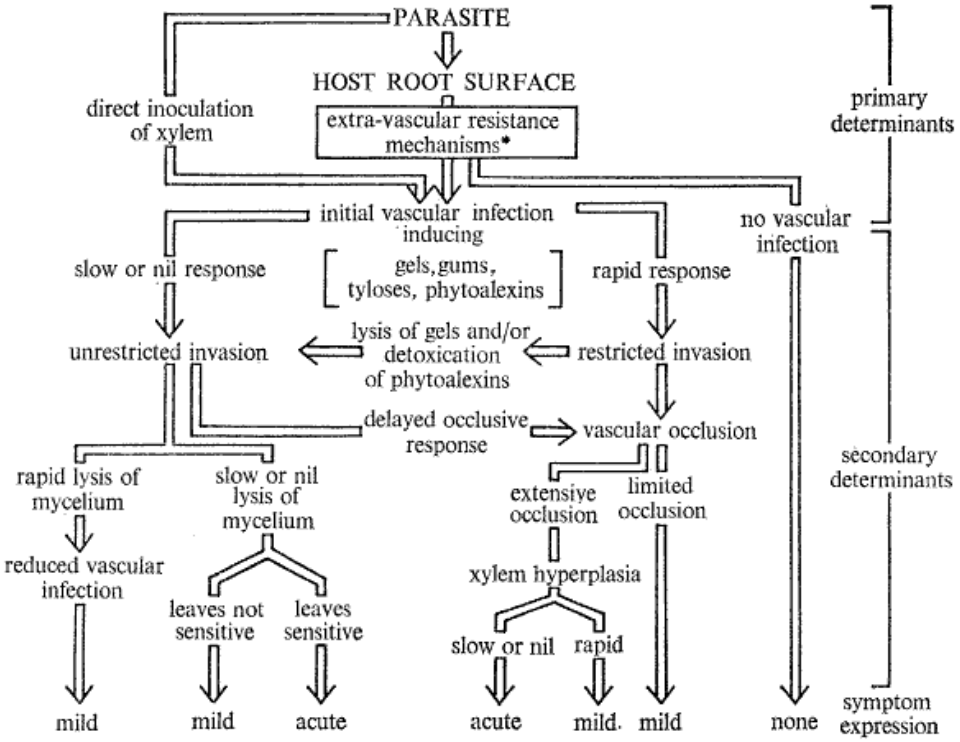

FIGURE 6. Scheme showing some alternative sequences of determinant responses that can occur during the vascular phase of a wilt disease. Differentiation between a high-resistance and a low-resistance host could theoretically occur at any dichotomy. * Differentiation in the pre-vascular phase has been discussed elsewhere (Talboys 1964).

Figure 1-1. Flow diagram looking at the sequence of events for primary and secondary determinants. Figure taken from Talboys. 1972. PNAS.

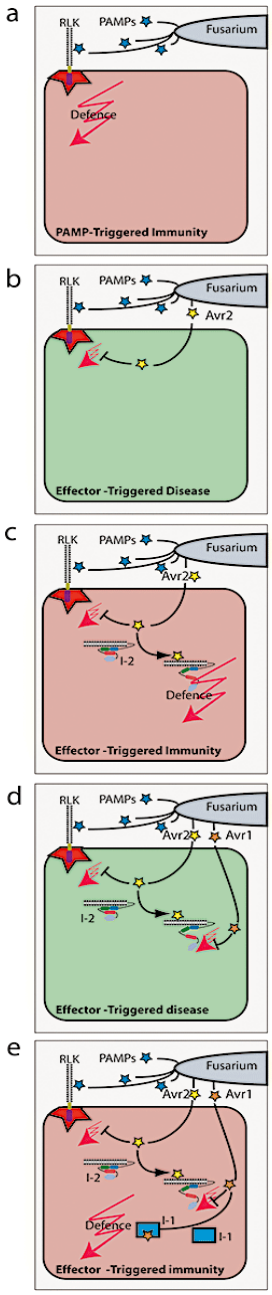

Figure 1-2. Model depicting the interaction between FOL Avr (SIX) proteins and tomato I receptors. Figure from Takken and Rep. 2010. Mol. Plant Pathol.
(A) Depicts a priming MTI response to FOL that is mediated by RLKs and RLPs. (B) AVR2 is secreted by FOL into tomato cell and activates effector triggered defense. (C) AVR2 is recognized by I-2, which leads to resistance in tomato to FOL. (D) FOL strain that now secretes AVR1 in addition to AVR2 can evade resistance in tomato. (E) Tomato plants that makes I-1 receptor now recognizes AVR1 and again triggered a defense response and the plant is resistant.

Chapter 2

An S domain 1 receptor-like kinase confers resistance to *Fusarium oxysporum* f sp *matthiolae*

Abstract:

Fusarium wilt, caused by the fungal pathogen *Fusarium oxysporum,* evokes considerable crop damage each year. The use of resistant varieties of crops and the integration of resistant genes is an effective method of control. Previously, six RESISTANCE TO FUSARIUM OXYSPORUM (RFO) loci were identified in a cross between Arabidopsis accessions Columbia 0 and Taynuilt-0. Map-based cloning was used to identify a third resistance gene, *RFO3*, located on chromosome three. *RFO3* encodes an S domain 1 receptor like kinase with a predicted extracellular bulb-type lectin domain, a PAN/APPLE domain, and an intracellular kinase domain. *RFO3* confers isolate-specific resistance to the *F. oxysporum* f sp *matthiolae* pathogen. Based on grafting and root staining, *RFO3* expression in roots provides resistance by preventing *F. oxysporum* colonization.

Introduction:

Analogies have been made between self incompatibility and host pathogen responses. In self incompatibility, it is the detection of self that prevents inbreeding, which lowers fitness. Meanwhile in host defense, it is the detection of nonself or modified self that prevents the invasion of a pathogen (Sanabria et al, 2008; Hodgkin et al, 1988). In plants, there are many different forms of self incompatibility and in Brassica self incompatibility is controlled by the *S DOMAIN RECEPTOR KINASE (SRK)*, a stigma-localized transmembrane receptor. The ligand for *SRK* is *SP11/SCR*, a pollen glycoprotein. In Brassica, there are multiple alleles for both *SRK* and *SP11*. During a

self incompatible response, if matching alleles of SCR and SP11 interact, the pollen does not hydrate on the stigma surface and pollen tube formation is blocked. At the cellular level, SP11 interacts with SRK, which causes downstream signaling to occur. SRK first dimerizes, which then leads to the interaction with the M locus protein kinase (MLPK). Both kinases contribute to the phosphorylation of the U box E3 ubiquitin ligase, ARC1. ARC1 ubiquitinates Exo70A1, which prevents the release of compatibility factors (Ivanov et al, 2010).

Associations have been made between close relatives of SRK and defense responses. The expression of Brassica homolgs *S GENE FAMILY RECEPTOR 1 and 2* (*SFR1* and *2*) are induced by bacterial pathogen *Xanthomonas campestris* and defense hormone salicylic acid (SA). Likewise, the expression of homologues in Arabidopsis, *ARK1* and *3*, are induced by *X. campestris* and SA (Pastuglia et al, 1997; Pastuglia et al, 2002). *SRK, SFRs*, and *ARKs* all belong to the S Domain 1 (SD1) family of RLKs. Furthermore, it has been theorized that S domain family members that are expressed in vegetative tissues are involved in cell to cell signaling such as host defense. The first direct evidence of an S domain family member involved in defense was *Pi-d2* in rice, which belongs to the SD 2 family and provides resistance to blast disease (Chen et al, 2006).

Gene for gene resistance, first described by Flor (1942), illustrates the fundamental idea by which a resistance gene (R gene) from a plant interacts with an avirulence gene (AVR gene) from a pathogen, thereby leading to resistance in the plant host. Since Flor (1942), the interaction between plant and plant pathogens has become

more complex. Many reviews describe the two-tiered level of defense in plant immunity (Dodds & Rathjen, 2010; Jones & Dangl, 2006). The first tier of defense is activated by microbe-associated molecular patterns (MAMPs). MAMPs are molecular patterns that are required for microbe survival and are made by a certain class of pathogens. MAMP triggered immunity (MTI) in plants is mediated through pattern recognition receptors (PRRs) that recognize MAMPs (Zipfel et al, 2004; Miya et al, 2007). While most known PRRs provide broad resistance, there are cases where resistance is only to a small subset of pathogens. For example, the receptor-like kinase, *Xa21,* is unique in that it is classified as a PRR but provides a race specific defense response to *Xanthamonas oryzae* pv *oryzae* (Song et al., 1995). This characteristic of pathogen-specific resistance is more common for the second tier of defense, effector triggered immunity (ETI). ETI is mediated through intracellular proteins. The most common class consists of nucleotide binding leucine-rich repeat proteins (NB-LRRs). These receptors either directly recognize effectors that are secreted by pathogens or recognize modifications to host proteins due to the presence of the pathogen. The downstream activation of defense-related genes, the release of phytoalexins, the building up of cell walls, and an increase in callose deposition is similar in ETI and MTI. MTI and ETI were to be considered completely separate responses but as more evidence emerges, in fact the distinction is imprecise, and the interactions are more complex.

Fusarium oxysporum is a filamentous fungus that causes root rot and vascular wilt disease in a number of agriculturally important crops including cotton, banana, and tomato. *F. oxysporum* enters the host through root tips where it eventually colonizes the

xylem. Only during late stages of the disease can *F. oxysporum* be found in the shoot of infected plants. Symptoms of vascular wilt consist of an initial epinasty and a stunting of the rosette. Subsequently, older leaves begin to yellow starting in the vasculature followed by spreading throughout the leaf. The first signs of yellowing or chlorosis of leaves are typically unilateral. The yellowing of leaves eventually reaches younger leaves and finally the plant collapses and dies. Disease symptoms are believed to be caused by the blockage of water and nutrients from the root to the shoot (Michielse & Rep, 2009; Di Pietro A et al*,* 2003).

There is specificity in the interaction between *F. oxysporum* and plant hosts. Isolates of *F. oxysporum* can only infect one or a few closely related plant species. Diener and Ausubel (2005) demonstrated that only *F. oxysporum* strains that infect other Cruciferous plants can be studied with Arabidopsis. These *F. oxysporum* isolates include *raphani* (FOR, radish pathogen), *matthiolae* (FOM, garden stock pathogen), and *conglutinans* (FOC, cabbage pathogen). Previously, Diener and Ausubel (2005) were able to map *RESISTANCE TO FUSARIUM OXYSPORUM (RFO)* loci in Arabidopsis using FOM. In their study, a resistance gene, *RESISTANCE TO FUSARIUM OXYSPORUM 1 (RFO1)*, was identified as a wall-associated kinase-like kinase that provided resistance not only to FOM but also to FOC. Other work utilizing Arabidopsis as a model host has focused on the effects of hormone signaling on *F. oxysporum*-host interaction. For instance, we have shown that various isolates of *F. oxypsorum* produce jasmonates including JA conjugated to isoleucine, the active form of JA. The fungal derived jasmonates affect colonization of plants roots and can affect plant gene

expression. Moreover, the JA signaling *coi1-1* mutants are resistant to *F. oxysporum* (Thatcher et al, 2007; Cole et al, Chapter 3). Additionally, the overexpression of *THIONIN 2.1* (*THI2.1*), a type of defense protein, was shown to provide resistance to FOM (Epple et al, 1997). A substantial amount of the work on *F. oxysporum* has also been done in tomato where R genes, *Immunity (I)* genes, and effectors have been identified (Takken & Rep, 2010).

Here we describe the cloning and characterization of *RESISTANCE TO FUSARIUM OXYSPORUM 3 (RFO3),* an SD1 receptor-like kinase from Arabidopsis. We have determined that *RFO3* is a pathogen-specific resistance gene. Furthermore, though *RFO3* is expressed in most vegetative tissues, RFO3 expression in root tissue is important for resistance.

Materials and Methods

Fusarium oxysporum infection assays

Cultures of FOX are grown for five to seven days in Oxoid Czapek Dox medium (sodium nitrate 2 g/L, potassium chloride 0.5 g/L, magnesium glycerophosphate 0.5 g/L, ferrous sulphate 0.01g/L, potassium sulphate 0.35g/L, and sucrose 30.0 g/L). Cultures were filtered using sterile gauze to remove hyphae. Spore suspensions were centrifuged and supernatant was removed. The pellet was washed with water three to five times to remove sucrose and salts from the media. After washing, the spores are suspended in 50 ml of water. The concentration of spores was estimated using a hemocytometer. The culture was diluted down to a working concentration using water.

Arabidopsis were grown for three to four weeks in presoaked Jiffy peat pellets at 28-30°C with 12 hours day and night cycles. For root inoculation, pellets were soaked in spore suspension for 1-2 minutes, then removed. Infected plants are then allowed to grow for another 2-3 weeks in growth room and then scored based on health index (Diener & Ausubel, 2005).

Cloning of *RFO3*

RFO3 was amplified from Col-0 genomic DNA using high fidelity Herculase® taq DNA polymerase (Strategene). Primers for amplification were forward primer "GCAGGTACCAGTCAGAGTGATTTTTCCGC" and reverse primer "GCAGGTACCTCAAAACGATTGATTCGAACC", which amplified a 4519 bp region containing *RFO3* on chromosome 3. This PCR fragment was subsequently cloned into the binary vector PZP212 using the KpnI site. The construct was sequenced to verify accuracy and was electroporated into *Agrobacterium tumefaciens* strain GV3101 for plant transformation. Arabidopsis accession Ty-0 was transformed using the floral dip method described in Desfeux et al., 2000. T1 transformed seeds were selected for kanamycin resistance on plant nutrient agar (5 mL 1M KNO3, 2 mL MgSO4, 2 mL Ca(NO3)2, 2.5 mL 20 mM Fe EDTA, 1 mL micronutrients, 2.5 mL KH2PO4, agar 7.5 g/L) containing 50 mg/L kanamycin. Three-4 weeks old T1 transformants were infected with $1X10^6$ conidia/mL of FOM pathogen.

Sequencing of *RFO3* Ty0 genomic DNA and cDNA

The *RFO3*^{T} genomic sequence was obtained by amplifying sections of *RFO3* from Ty0 genomic DNA. The first section was amplified using the forward primer SP1F "TGGGCAAGAGCTCGTTTCAGC" and the reverse primer SP4R "GGTCCTTCTGAGTCATGGTGGAAGC". A second section was amplified using the forward primer SP5F "ACATGCCCAACAAGAGCCTTGACT" and the reverse primer SP7R "TTCGATGCGTTAAGAGCACACAAGA". Primers were based on the Col-0 genomic sequence. Each section was sequenced using additional primers that were located in between the original primer pairs. These primers included for the first section: SP1R "TGCGGAAGGGGATACAGTCTCTCT", SP2F "TGGGCTCTGGTTTAAGGGTGGCT ", SP2R "GCCTTACTCTGTGGAATGACTTTTCCC", SP3F "GGGGATGGTACGGGCTGCGA", SP3R "TCCACTAATCCTTGCCCGGATGC", and for the second section SP4F "ACGCCGAGGCAAGAGAAGTGC" and SP5R "GTAGCTCATCTTGCTTCCATCACTGT", SP7F "AGAACCGAGTTCGCGAGGTGA". The first section aligned to *RFO3*^{Ta} and the second section aligned with *RFO3*^{Tb}. To complete the sequence, primers on the end of either fragment were used to amplify a third section that was in between the first and second fragments. The three sections were aligned to obtain the full genomic sequence of both RFO3 genes in Ty0.

Full length *RFO3*^{Ta} cDNA was amplified from Ty-0 cDNA made using Invitrogen SuperScript III First-Strand Synthesis system. Primers used to amplify cDNA were the reverse primer "GGGGTACCTCATCTTGCTTCCATCACTGT" and the forward primer "GGGGTACCATGTGGTCAAATTGCATCTTTCT". Once full-length cDNA was

amplified, primers that were previously used for amplifying the Ty-0 genomic sequence were used to amplify cDNA to create overlapping fragments that were then sequenced and aligned. The full cDNA of *RFO3*^{Ta} is 2334 bp in length.

*RFO3*p::*GUS* reporter construct

A promoter *GUS* fusion was made by PCR-amplifying the *RFO3* promoter from the end of the previous gene to the ATG of *RFO3*. The primers used were the forward primer “CGGAATTCTAGTTGTTTTTGATGAAGACAA” and the reverse primer “CGGAATTCAATTTCAGATTTTCTGAAACTTG”, the final amplicon was 976 bp in length. The promoter was cloned into the binary vector pORE R2 (Arabidopsis Biological Resource Center). This vector contains β glucuronidase (*gus A* gene) (Coutu C et al., 2007). The construct was sequenced for fidelity and orientation. The construct was then transformed into Agrobacterium strain GV3101 and subsequently transformed into Arabidopsis accession Ty-0. T1 transformants were selected on plant nutrient agar containing 50 µg/ml kanamycin.

Transformants were stained using a solution of 0.1M sodium phosphate buffer, pH 7, containing 0.3 mg/ml 5-bromo-4-chloro-3-indoxyl-β-D-glucuronic acid. Plants were left overnight in the staining solution at 37°C. Samples were then destained using increasing concentrations of ethanol over 2-3 days. Samples were rehydrated before images were taken using a dissecting scope and recorded with a digital camera (Dino-Eye Eyepiece, AM423XC, BigC, Torrance, CA).

Grafting *RFO3*^{Col0} and *RFO3*^{Ty0} rootstocks and scions

NIL1E2 and Ty0 were used for grafting experiments. Seedlings were grown vertically on the surface of plant nutrient agar containing 1.5% Sigma Phytogel for 7-10 days in Conviron growth chamber with 8 hours light 16 hours dark at 22°C. Seedlings were cut in half at the hypocotyls and rootstocks and scions were exchanged to create four combinations: 1E2 scion/1E2 rootstock, 1E2 scion/Ty0 rootstock, Ty0 scion/1E2 rootstock, and Ty0 scion/ Ty0 rootstock. Grafted scions and rootstocks were held together using 0.3 mm diameter Silastic laboratory tubing and allowed to grow for an additional 7-10 days in the growth chamber. Grafts were checked for adventitious root formation. Any adventitious roots found were removed. Grafted plants were then transferred to presoaked peat pellets and were grown for another 7 days until they were large enough for infection (Bainbridge et al., 2006).

Root staining to visualize *F. oxysporum* in infected plants

Three-week-old plants were infected with the FOM pathogen. Ten days after inoculation, the roots from mock-and FOM-inoculated plants were isolated from soil. Roots were cleaned to remove as much soil as possible without damage to the roots. The roots were then stained in 0.1M sodium phosphate buffer, pH7 with 0.01% Triton X-100. The staining solution included either 5-bromo-4-chloro-3-indoxyl-α-L-arabinofuranoside (X-ARA, #B-290, Gold Biotechnologies, St. Louis, MO) or 4-nitrophenyl-α-L-arabinofuranoside (NP-ARA, #N-240, Gold Biotechnologies, St. Louis, MO), where 100-fold excess stock solutions

were 20 mg glycoside reagent (2%) in 1 ml dimethylformamide. For visualizing infection, roots were stained with X-ARA overnight at 28° C and destained with two transfers to excess water at 4 ˚C for several hours to overnight. Blue precipitate was observed using a low-magnification binocular microscope and recorded with a digital camera (Dino-Eye Eyepiece, AM423XC, BigC, Torrance, CA). To quantify infection, cleaned root systems were incubated for 20 hrs at 28 ˚C on a rotisserie tube mixer in 40-fold volume NP-ARA staining solution and then frozen at -20˚C. Optical density (OD) of all samples at 410 nm (OD_{410nm}) and 600 nm (OD_{600nm}) was measured using a spectrophotometer (Smart Spec 3000, Biorad, Philadelphia, PA). OD_{600nm} was subtracted from OD_{410} to account for nonspecific light diffraction in samples to give the OD_{410}/gram fresh weight roots. Measurements were standardized so that the mean OD_{410}/gram fresh weight was 1.0 for uninfected wild type.

Results

RFO3^C confers specific resistance to FOM.

In the absence of other major *RFO* QTLs, we confirmed the quantitative resistance of *RFO3*. In a prior study, *RFO3* was one of six *RFO* QTLs segregating in progeny of the F_1 backcross (BC) of Arabidopsis accessions Columbia-0 (Col-0), as donor parent, and Taynuilt-0 (Ty-0), as recurrent parent (Diener and Ausubel, 2005). F_1BC progeny that were heterozygous for Col-0 and Ty-0 alleles of *RFO3*-linked marker nga162 were quantitatively more resistant to FOM than F_1BC progeny that were homozygous Ty-0, which suggested that the Col-0 allele (*RFO3*^{C}) was a dominant

resistance trait. In the subsequent self progeny of one F_1BC plant, namely 6E5, we exclusively observed the segregation of *RFO3*-mediated resistance because 3E2 was heterozygous for Col-0 and Ty-0 alleles at *RFO3* (*RFO3*$^{C/T}$) but was homozygous Ty-0 at other major *RFO* loci. Among self progeny of 6E5, resistance to FOM and the *RFO3*-linked marker MOA2.2 clearly cosegregated, and both *RFO3*^{C} and the Ty-0 allele (*RFO3*^{T}) were codominant because *RFO3*^{C} homozygotes (*RFO3*$^{C/C}$) expressed significantly stronger resistance to FOM than did *RFO3*$^{C/T}$, which expressed stronger resistance than the Ty-0 homozygotes (*RFO3*$^{T/T}$, Figure 2-1A).

Fine mapping of resistance confined *RFO3* to a 220 kilobasepair genomic interval on chromosome 3 that included 66 TAIR10 annotated. Using the original F_1BC population, we assigned a map position for *RFO3* between markers CIW11 and nga162 (Diener and Ausubel, 2005). Among the tested Col-0 and Ty-0 recombinant plants, 1E2.A6 and 2C3.B9, in particular, had recombination breakpoints between markers that are tightly linked to *RFO3* (Figure 2-1B). To assign *RFO3* genotypes to 1E2.A6 and 2C3.B9, we crossed the two recombinants to Ty-0 and evaluated the Rfo phenotype in the resulting F_1 progeny. If 1E2.A6 or 2C3.B9 were *RFO3*$^{C/T}$, resistant (*RFO3*$^{C/T}$) and susceptible (*RFO3*$^{T/T}$) plants would cosegregate with the *RFO3*-linked marker MVC8 in a 1:1 ratio. Resistance to FOM and the *RFO3*^{C}-linked allele of MVC8 indeed cosegregated among the F_1 progeny of 1E2.A6 and 2C3.B9 (Figures 2-1D and 2-1E, respectively). Furthermore, among the F_2 progeny of the selfed F_1 progeny of 1E2.A6 or 2C3.B9 that were *RFO3*$^{C/T}$, wilt resistance and MVC8 again cosegregated (Figures 2-1F and 2-1G, respectively). Thus, both 1E2.A6 and 2C3.B9 were *RFO3*$^{C/T}$ and had

recombination breakpoints flanking either side of RFO3 that were no more than 220 kbp apart.

We wanted to know if *RFO3*^{C} resistance was specific to FOM. Therefore, a near isogenic line (NIL) 1E2 was created that contains a short segment of Col-0 on chromosome three where *RFO3* is located and the rest of the genome is Ty-0. Line 1E2 was infected with FOM, FOC races 1 and 2, and FOR. These *formae speciales* have previously been shown to infect Arabidopsis (Diener & Ausubel, 2005). Disease symptoms for 1E2 appeared similar to Ty-0 after infection with FOC race 1, 2, and FOR. Only when infected with FOM was line 1E2 more resistant than Ty-0 (Figure 2-2).

***RFO3* is an SD1 receptor-like kinase.**

Of the 66 predicted ORFs, there were several candidate genes, including a putative disease resistance gene, two glycosyl hydrolase proteins, and lectin protein kinase family protein. We took F3 lines from the original Col-0/Ty-0 cross with breakpoints within our defined 250 kilobase pair region and tested them for the association to resistance to narrow down the interval that contained *RFO3*. The final interval for *RFO3* was defined by SSLP markers MVC8 and MSL1.59K containing 27 ORFs and 89 kilobase pairs (Figure 2-3A). Twenty-two ORFs were cloned from Col-0 BACs into PZP212 binary vector. Seven ORFs were transformed into susceptible Ty-0 and infected with the FOM pathogen. Of the 27 ORFs remaining in the interval, only one of the original strong candidate genes remained (Figure 2-3B).

AT3G16030, was PCR-amplified from Arabidopsis accession Col-0 DNA and subsequently cloned into the binary vector, PZP212. *AT3G16030* was then transformed into the FOM-susceptible accession, Ty-0. Fourteen T1 transgenics where tested for enhanced resistance to FOM. *AT3G16030* T1 transgenics showed intermediate resistant to FOM compared to Ty-0. Five out of fourteen (36%) T1 transgenics were susceptible, seven of fourteen (57%) were intermediate, and one of fourteen (7%) were resistant. In comparison, eleven out of fifteen (73%) Ty-0 plants were susceptible, 4 of fifteen (27%) were intermediate resistant, and 0 of 15 (0%) were resistant (Figure 2-3C and D). Seeds were collected from T1 transgenic 1D and 19 homozygous T2 plants were inoculated with FOM to test for enhanced resistance in the next generation. Similar to the T1 plants, the T2 generation of *AT3G16030*^{C} plants were significantly more resistant than Ty-0 ($P \leq 0.01$). Fourteen of nineteen (74%) of the T2 transgenics were intermediate, four of nineteen (21%) were susceptible, and one out of nineteen (5%) were resistant to FOM (Figure 2-4).

To confirm that *AT3G16030* is *RFO3*, the homozygous SAIL 1212_G06 tDNA insertion mutant for *AT3G16030* was crossed to Ty-0. The F_1 was BC to the Ty-0 parent and the F_2 generation was infected with FOM to look for a loss of linkage to resistance at marker 3.3 on chromosome three near *AT3G16030*. The F_2 population contained 57 plants in total. At marker 3.3, F_2 plants that were Col-0 were just as resistant as those that were Ty-0. Therefore, the association with resistance seen previously near *RFO3* was lost. In the F_2 population, markers 1.2, close to *RFO2,* and marker 1.8, close to *RFO1,* both still showed linkage to resistance. Furthermore, markers that were not near

the *RFO* loci exhibited no linkage to resistance (Figure 2-5). Therefore, *AT3G16030* enhances resistance to FOM within the *RFO3* locus on chromosome three and was designated *RFO3*. Furthermore, there are no other genes within the *RFO3* locus that also provide resistance to FOM.

We infected the homozygous SAIL_1212_G06 tDNA mutants with FOM amid the expectation of an increase in susceptibility. However, infected *rfo3* mutants were not more susceptible. We infer that there is enough resistance in Col-0 background to FOM that it is difficult to perceive the effect from the loss of only *rfo3*. Therefore, we crossed the *rfo3* to homozygous *rfo1* SALK insertion line SALK_077975 to create an *rfo3 rfo1* double mutant. The *rfo1* mutant has been shown to have enhanced susceptibility to FOM and FOC (Diener & Ausubel, 2005). The *rfo3 rfo1* double mutant, when infected with FOM, was more susceptible than either *rfo1* or *rfo3* single mutants. However, *rfo3 rfo1* mutants were not as susceptible as Ty-0 (Figure 2-6). The double mutant was also infected with FOR and FOC race 1, but there was no significant difference in susceptibility of the double mutant compared with either single mutant (Figure 2-6). This is consistent with the result that $RFO3^{C}$ resistance is FOM-specific.

$RFO3^{T}$ has two SD1 RLK genes.

RFO3 is a member of the SD1 family of RLKs based on homology of the kinase domain. SD1 family members have an extracellular bulb type lectin (B lectin) domain that is a predicted carbohydrate binding domain, an S locus domain that is associated with self incompatibility, a PAN/APPLE domain that is known for protein-protein binding, a transmembrane domain, and an intracellular kinase domain (Figure 2-7) (Shiu and

Bleeker, 2001). *RFO3*^{T} was sequenced and in contrast to *RFO3*^{C} where there is a single gene, there are two *RFO3* like genes in Ty-0 located next to each other. The first gene, *RFO3*^{Ta}, is 3148 bp in length and the second gene, *RFO3*^{Tb}, is 3340 bp in length with 390 bp between the two genes. This 390 bp sequence has 76% nucleotide identity to the sequence upstream of *RFO3*^{C}. Based on the sequence analysis of *RFO3*^{T}, it appears the first half of *RFO3*^{Ta} containing the B lectin domain has more sequence identity with *RFO3*^{C}, and the second half of *RFO3*^{Tb}, including the kinase domain is like *RFO3*^{C}. The first half of *RFO3*^{Ta} from 1- 832bp is 93% nucleotide identity to *RFO3*^{C} and the second half from 996- 3148 has 87% nucleotide identity to *RFO3*^{C}. *RFO3*^{Tb}, from 1680- 3340 is 90% identical to *RFO3*^{C}. However, the identity decreases to 85% from 1-940 bp for the first half of *RFO3*^{Tb}. Finally, the sequence at the end of *RFO3*^{Tb} has 93% nucleotide identity to the sequence downstream of *RFO3*^{C} (Figure 2-7b).

Additionally, we were able to isolate cDNA from *RFO3*^{Ta}. The *RFO3*^{C} cDNA is 2583 bp compared to *RFO3*^{Ta}, which is 2334 bp. There is approximately 200 bp missing from exons one and two in *RFO3*^{Ta} compared to *RFO3*^{C} (Figure 2-7b). Also there is a considerable decrease in sequence similarity that makes aligning the two sequences difficult at the end of the second exon. The lectin domain (23- 240 aa) were 87% identical to *RFO3*^{C}. The S locus and PAN domains (241-400 aa) were 50% identical. Finally, the kinase domain (500-776 aa) is 95% identical to *RFO3*^{C}. After sequencing *RFO3*^{Ta} and *RFO3*^{Tb}, there were no obvious changes in either sequence such as a frame shift or nonsense mutation that would make these genes nonfunctional. Therefore, what causes *RFO3*^{T} to be a susceptible allele is unknown.

***RFO3^C* restricts the colonization of roots by FOM.**

An *RFO3* promoter::*GUS* fusion was made to determine in which tissues *RFO3* was more highly expressed. *GUS* staining was detected in vegetative tissues, leaves and roots, but not in reproductive tissues, flowers and siliques. Staining in the leaves and roots appears to be vascular (Figure 2-8A). Also, *RFO3* seems to be expressed more in older senescing leaves compared to younger leaves. This staining pattern was also seen in infected *RFO3*p::*GUS* plants (Figure 2-8B). Given that *RFO3*, is expressed in both the shoot and root, we wanted to know in which tissue *RFO3* expression was essential for resistance to FOM. Using NIL 1E2 and Ty0, seedlings were severed at the hypocotyl and grafted to each other in four combinations: *RFO3^C* scion *RFO3^T* rootstock, *RFO3^T* scion *RFO3^C* rootstock, *RFO3^C* scion and rootstock, and *RFO3^T*scion and rootstock. Successful grafts were transferred to soil and infected with FOM. Grafts with Ty0 rootstocks (*RFO3^C* scion- *RFO3^T* rootstock and *RFO3^T* scion and rootstock) were more susceptible than grafts with *RFO3^C* roots (Figure 2-9). Thus RFO3^C expression in roots confers resistance to FOM.

5-bromo-4-chloro-3-indoxyl-α-L-arabinofuranoside (X-ARA) is a substrate for arabinofuranosidase activity in fungi. Once cleaved, X-ARA creates a blue color that can be used to stain fungi in plant roots and determine the degree of infection. Both Ty-0 and NIL 1E2 were inoculated with FOM and roots were not isolated until ten days post inoculation, when early disease symptoms such as stunting of rosette and epinasty were visible in shoot (Figure 2-10A). As seen in Figure 2-10B and C, resistant 1E2 roots are colonized significantly less by FOM than susceptible Ty-0 roots. In summary,

expression of *RFO3*^{C} in the root is important for preventing colonization of the root and thereby enhancing resistance to FOM.

Homolog of *RFO3* is involved in FOM resistance

Based on the phylogenic tree created by Shiu et al (2004) that organized RLKs from Arapidopsis and rice based on protein sequence identity of kinase domains, the closest homolog of *RFO3* is *AT1G67520*. *AT1G67520* belongs to the SD1family of RLKs in Arabidopsis and is located on chromosome one. *RFO3* and *AT1G67520* have about 85% identity at the N terminus (1-800bp) and 87% identity at the C terminus containing the kinase domain (1480- 2108bp). Since these two genes are closely related, we wanted to determine if the *RFO3* homolog, *AT1G67520*, was also involved in resistance to FOX. Since the *rfo3* single mutant had no phenotype, we anticipated that the single mutant of *at1g67520* would also not have a more susceptible phenotype. Therefore, a homozygous TDNA insertion line for *AT1G67520,* SALK_004748C, was crossed to a *rfo1* SALK line to create a double mutant. This double mutant was then infected with the four FOX pathogens that colonize Arabidopsis roots along with *rfo1* and *at1g67520* single mutants. Enhanced susceptibility compared to *rfo1* and *at1g67520* single mutants was only seen in the double mutant when infected with the FOM pathogen. There was no significant difference in infections between the double mutant and *rfo1* or *at1g67520* single mutants when infected with FOC races 1, 2, or FOR (Figure 2-11).

Discussion

S domain kinases have been implicated in defense response. For example, *Brassica oleracea* genes *SFR2* and *3* have been shown to be induced by bacterial pathogen *X. campestris*. There has also been a B type lectin cloned in rice that provides resistance to rice blast disease. Here we present an SD1 family lectin receptor-like kinase from Arabidopsis that provides resistance to FOM. *RFO3* belongs to the same family of receptors as the well-characterized SD1 receptor SRK. This is advantageous in that a large sum of work has been done to determine protein interactions involved in the signaling mechanism not only in Brassica but also in Arabidopsis. Samuel et al (2008) looked at the interaction between SD1 receptors and a U box family of E3 ubiquitin ligases. It was discovered that like SRK and ARC1 in Brassica, the kinase domains of SD1 family members also interact with the ARM domain of U box proteins form Arabidopsis. Specifically plant U box (PUB) proteins 9, 13, and 38 showed the most consistent interaction with various SD1 family kinases. We are currently working to determine if *RFO3* kinase domain interacts with PUBs. Other receptors involved in defense have been shown to interact with E3 ligases. For example, Xa21 interacts with E3 ligase Xa21 Binding Protein 3 (XB3) for the resistance response to *X. oryzae* pv. *oryzae*. This interaction is between the kinase domain of Xa21 and ankyrin repeats and ring finger domains of XB3 (Wang et al, 2006). Furthermore, FLS2 interacts with PUBs 12 and 13 in a complex with BAK1. Lu et al (2011) found BAK1 phosphorylates PUB12 and 13 and this interaction leads to the ubiquitination and degradation of FLS2. The interaction between PRRs and E3 ligases may be a conserved mechanism to control defense signaling.

The distinctions between PTI and ETI response pathways are not as strict as they once were. In the PTI response, the MAMPs are typically molecular patterns that the pathogen needs to survive or improve fitness. This is in contrast to the ETI response, where the effectors are for virulence and not necessaryily important for survival or fitness of the pathogen (Thomma et al, 2011). However, studies have shown that MAMPs, like flagellin, can be used for virulence by the pathogen (Taguchi et al, 2003). A study by Naito et al (2008) demonstrated that by disrupting the motility of flagellin they could decrease the virulence of bacterial pathogen *Pseudomonas syringae* pv. *tabaci* on tobacco leaves. In addition, PRRs are typically evolutionarily conserved in order to maintain the recognition of the corresponding MAMP. Meanwhile, it has been theorized that R genes are coevolving with pathogen effectors in what is termed an arms race (Bergelsonet al, 2001). In the arms race plants are under pressure to create new variations of R genes and therefore R genes are evolutionarily young. However, there are examples of R genes that are stable and evolutionarily conserved (Van der Hoorn et al, 2002). Finally, all known PRRs are cell surface transmembrane receptors that interact with a ligand. Meanwhile, most R proteins are intracellular NB-LRRs that interact either directly or indirectly with an effector. *RFO3* is a typical PRR because it has an extracellular binding domain presumably for ligand binding and an intracellular kinase domain for signaling. Additionally, we predict that *RFO3* is plasma membrane localized. However, *RFO3* is atypical because like an R gene, it provides resistance only to a single isolate of FOX but no other FOX isolates that infect Arabidopsis. Likewise, *Xa21* in rice also has characteristics of both PRRs and R genes. *Xa21* is also

a receptor-like kinase but like *RFO3* provides race-specific resistance to *X. oryzae* pv *ozyzae* race 6 (Zipfel et al, 2004). Our lab has also seen the blurring of characteristic between PTI and ETI with *RFO2*, which is a receptor-like protein that lacks an intracellular kinase domain and only enhances resistance to FOM. Our study provides further evidence that there is an array of resistance when describing PTI or ETI responses.

In sequencing Ty-0 allele of *RFO3,* we discovered two *RFO3* genes. We were unable to determine the nucleotide change in either gene that causes Ty-0 to be susceptible. However, we were able to isolate a full length cDNA from one of the genes, $RFO3^{Ta}$. Therefore, at least one of the genes from Ty-0 has the potential to produce RFO3 protein. $RFO3^{Ta}$ has the same domains as RFO^{Col0}. Duplication of RFO3 is prevalent in plant species. Among the approximate 300 sequenced accessions released from the 1001 genomes project, about 40% have missing sequence similar to $RFO3^{T}$ where the sequence cannot be aligned. This could either mean that the gene is significantly different from the $RFO3^{C}$ reference sequence or that there are two genes with similar sequence but not identical that are being aligned at the same region of the reference. In addition to the $RFO3^{T}$, we also looked at the homolog of *RFO3*, *AT1G67520*. These two genes are the most similar to each other based on phylogeny utilizing kinase domain protein sequence. We have seen that both *rfo3 and at1g67520* are more susceptible to FOM when crossed with *rfo1*. This would suggest *AT1G67520* is also a resistance gene. We are in the process of creating a triple mutant of *rfo1*, *rfo3*, and *at1g67520* to determine if there is an additive effect of these resistance genes.

Additionally, in the future we will transfer *AT1G67520* into a susceptible background and test for enhanced resistance.

RFO3 was previously identified as an activator of photosynthetic genes and designated *CALLUS EXPRESSION OF RBCS 101 (CES101).* Root tissue and calli normally suppress chlorophyll production and the expression of photosynthetic genes. However, when an activation tag containing quadruple repeat enhancers derived from the cauliflower mosaic virus 35S promoter was placed in front of *AT3G16030*, calli formed from root tissue expressing the tagged version of *AT3G16030* were light green due to chlorophyll production and induction of photosynthetic gene *RBSC-3B* (Niwa et al, 2006). This phenotype is unusual because in plants that are under biotic stress processes related to growth and development such as photosynthesis are usually down regulated in order to redirect resources needed for defense (Berger et al, 2007; Bolton, 2009; Garavaglia et al, 2010). Studies have shown that photosynthesis decreases around the primary site of infection in leaf tissue (Berger et al, 2007). Activation of resistance can cause plants to have reduced fitness. This concept is seen with constitutive resistant mutants that are typically severely stunted. However, Bolton (2009) suggests photosynthesis could also increase in order to keep up with the energy demands of defense response. Therefore, *CES101/RFO3* may have some function in signaling to activate the production of energy within plant cells along with defense. It would be interesting to see if the over expression of CES101/RFO3 enhanced resistance to FOM infection as we would expect. Other members of the SD-1 family

have phenotypes related to plant defense. Therefore, it is not unexpected that *RFO3* would be a resistance gene in addition to regulating photosynthetic genes.

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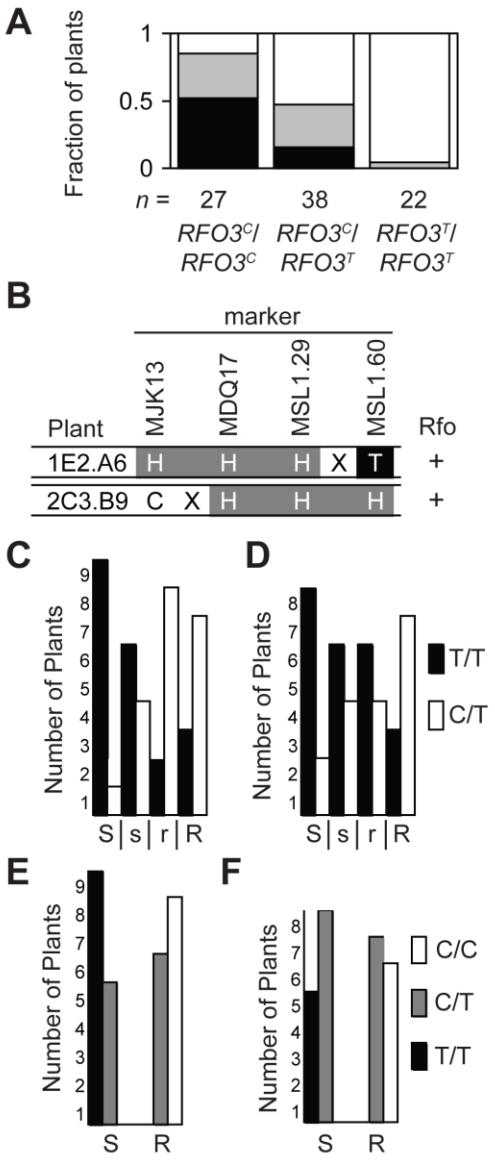


Figure 2-1. Mapping of RFO3
(A) Cosegregation of marker MOA2.2 among progeny of 6E5 with *RFO3*^{C} resistance. *RFO3*^{C} and *RFO3*^{T} are codominant. (B) Map showing lines 1E2. A6 and 2C3.B9 are heterozygous for *RFO3* between markers MJK13.38K and MSL1.2. Cosegregation of *RFO3*^{C} allele at MVC8 and FOM resistance with lines 1E2.A6 (C) and 2C3.B9 (D). Resistance and marker MVC8 co segregate in *RFO3*$^{C/T}$ F_2 progeny of selfed F_1 progeny of 1E2.A6 (E) and 2C3.B9 (F).

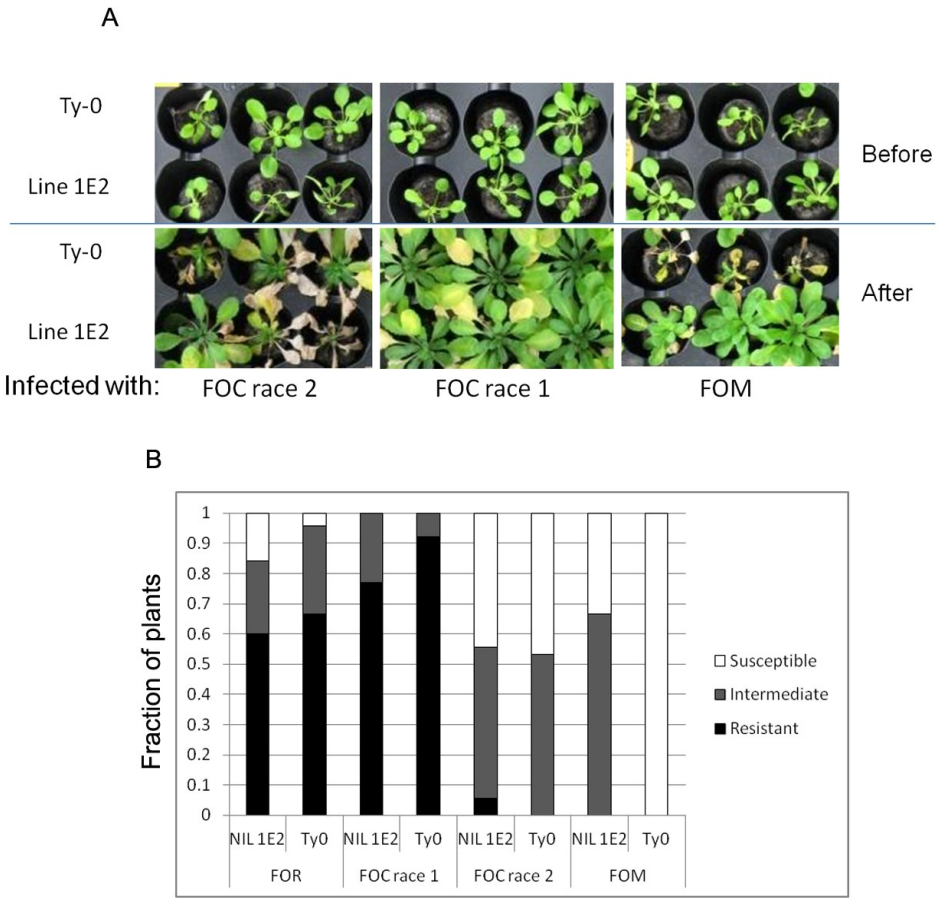


Figure 2-2. RFO3 is an FOM specific resistance gene.
(A) Representative 1E2 and Ty-0 infected plants shown before infection and 2 weeks after infection with FOM and FOC race 1and 2. (B) Quantification of disease symptoms in two weeks after infection using health index (5-0): Resistant (5,4); Intermediate (3,2); Susceptible (1,0). $P<0.01$ for FOM infection.

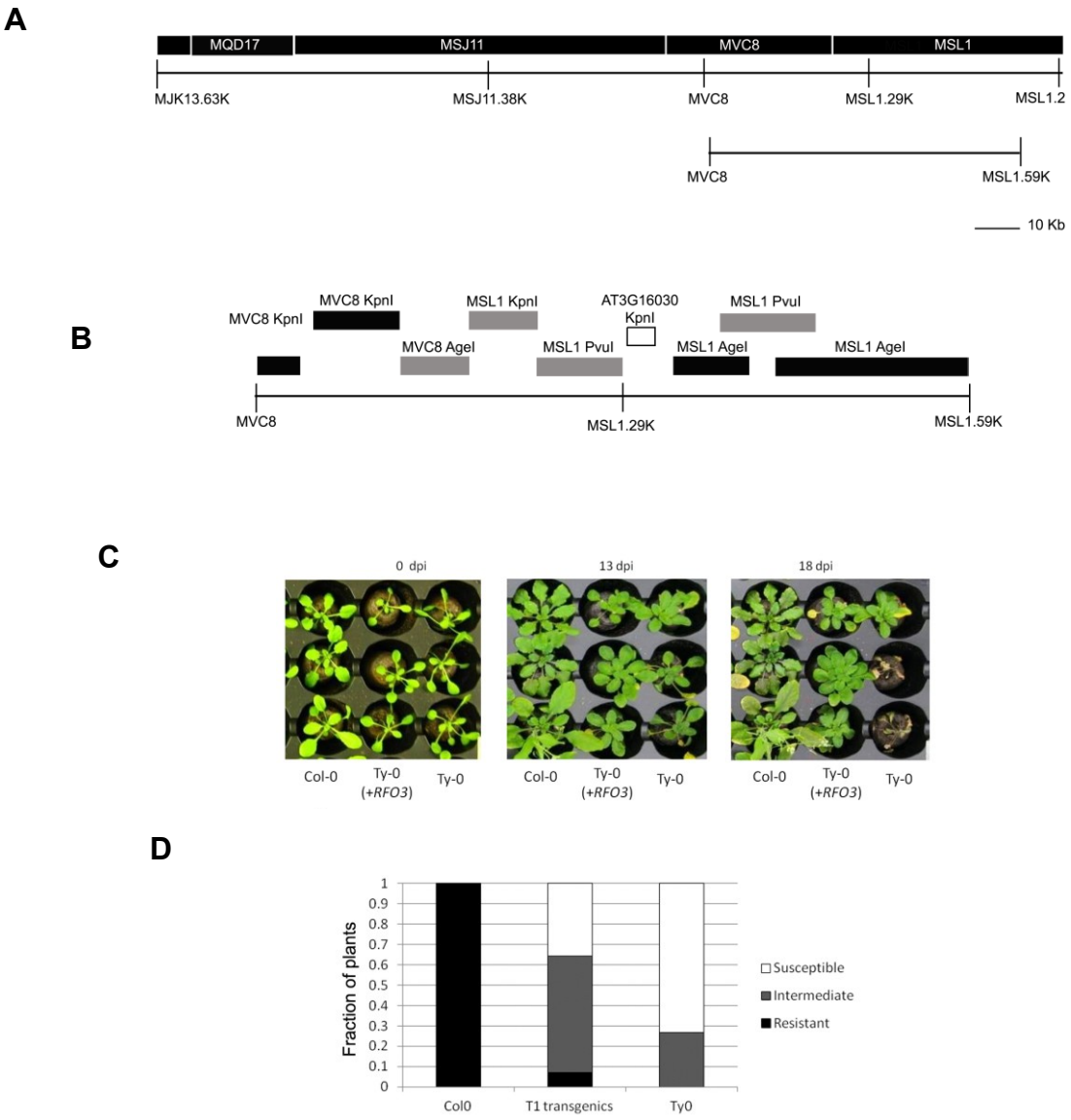


Figure 2-3. Cloning of RFO3.
(A) Initial 250 kbp region containing *RFO3* between SSLP markers MJK13.38K and MSL1.2. BACs spanning the interval are depicted as black boxes. Final interval containing *RFO3* between markers MVC8 and MSL1.59K. (B) Fragments cloned from BACs within final 89 kbp region are shown in gray boxes. Fragments cloned and tested for enhanced resistance are shown in black boxes. *AT3G16030* is depicted as a white box. Figure is not drawn to scale. (C) Representatives of FOM infected *RFO3* T1 transgenics along with Col-0 and Ty-0 controls. (D) Quantification of disease symptoms using health index two weeks after infection. 15 Col0, 14 T_1 transgenics, 15 Ty-0 plants were used in assay. (P≤0.01)

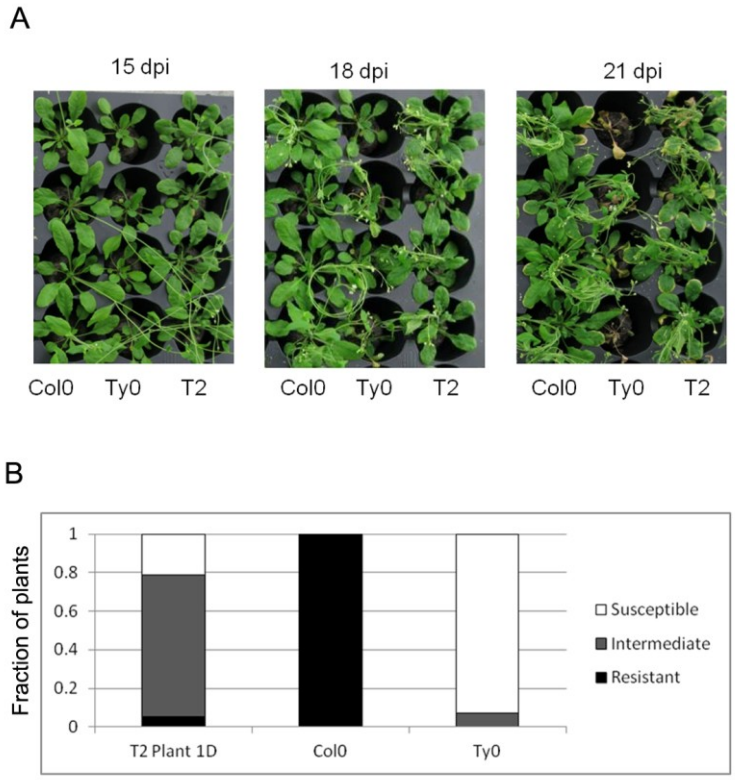


Figure 2-4. Infection of *RFO3* T2 transgenic line 1D.
(A) Representatives of FOM infected Col-0, Ty-0, and *RFO3* T2 transgenic line 1D shown at 15, 18, and 21 dpi. (B) Quantification of disease severity based on scoring using health index. 19 T_2 transgenics, 15 Col-0, 14 Ty-0 plants were used in assay. ($P \leq 0.01$)

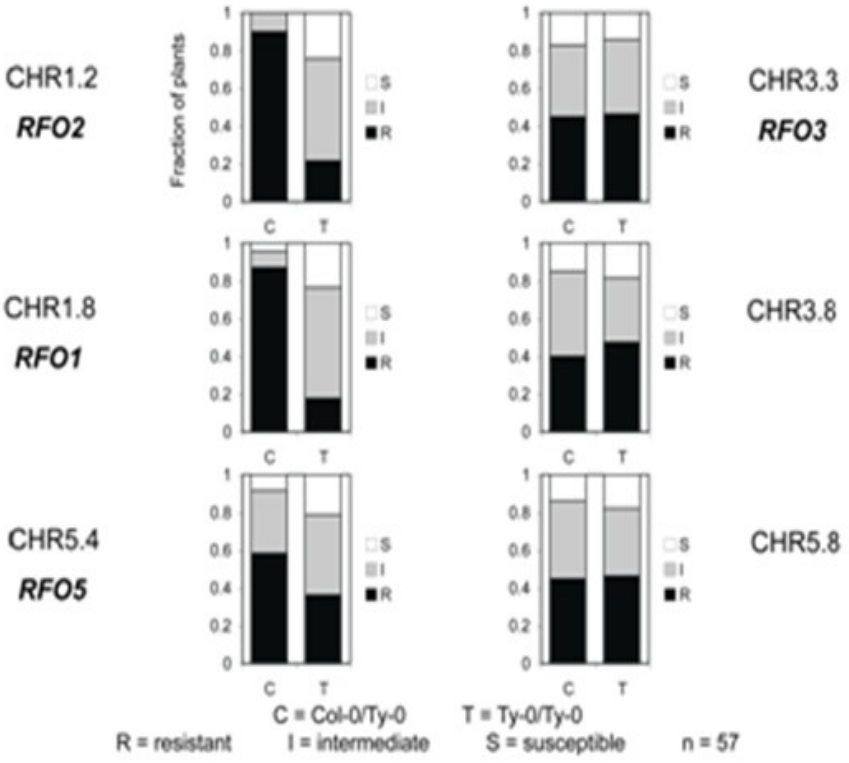


Figure 2-5. Confirmation *AT3G16030* is *RFO3*.
Quantification of resistance detected at markers CHR1.2 near *RFO2*, CHR1.8 near *RFO1*, CHR5.4 near *RFO5*, CHR3.3 near *RFO3.* Additional makers CHR3.8 and CHR3.8 were used as controls. R=Resistant (5,4); I= Intermediate (3,2); S= Susceptible (1,0)

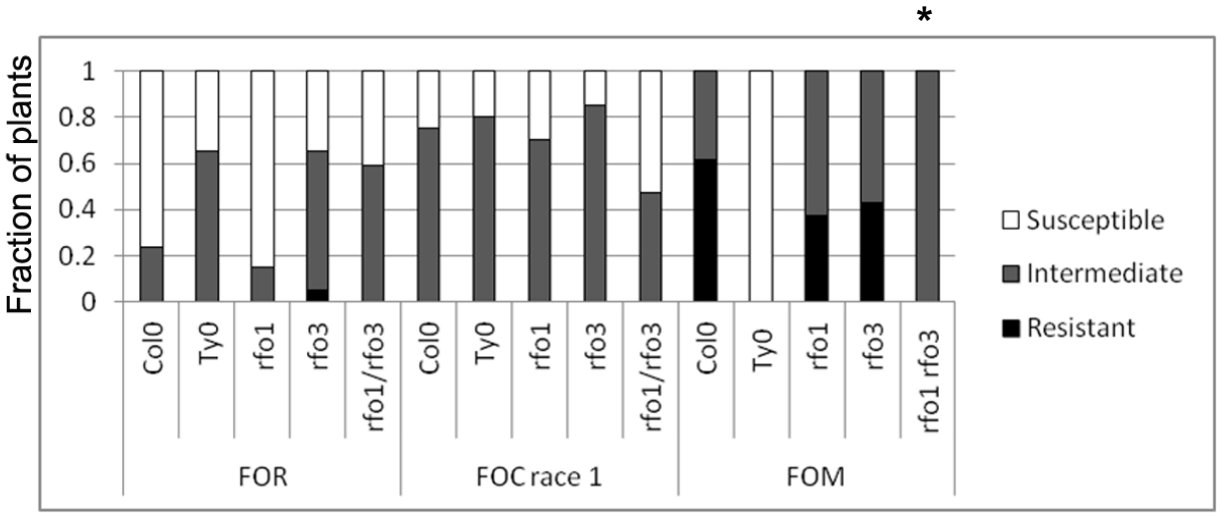


Figure 2-6. Infection of *rfo1 rfo3* double mutant
Scoring of FOM infected *rfo1 rfo3* infected plants based on health index. Resistant plants (5,4); Intermediate (3,2); Susceptible (1,0). Asterisk= P≤0.01

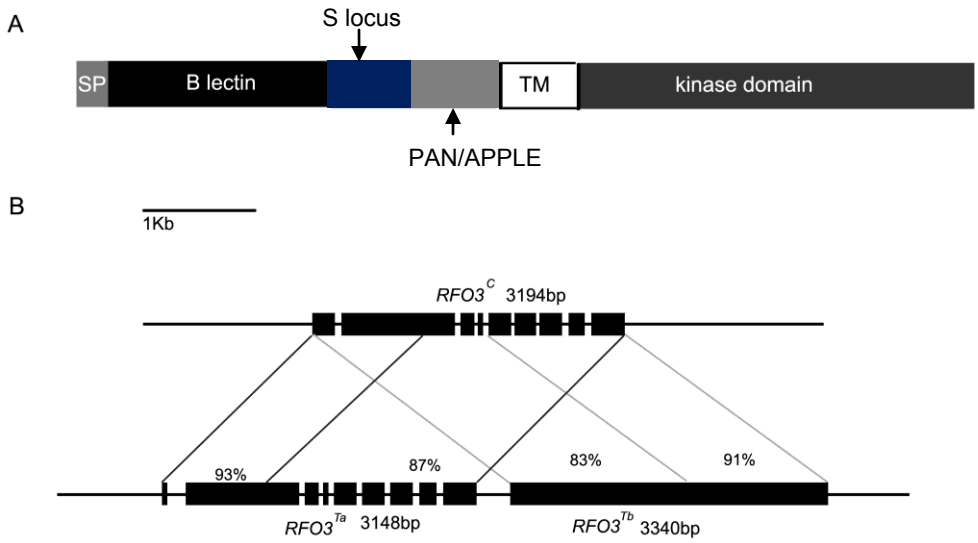
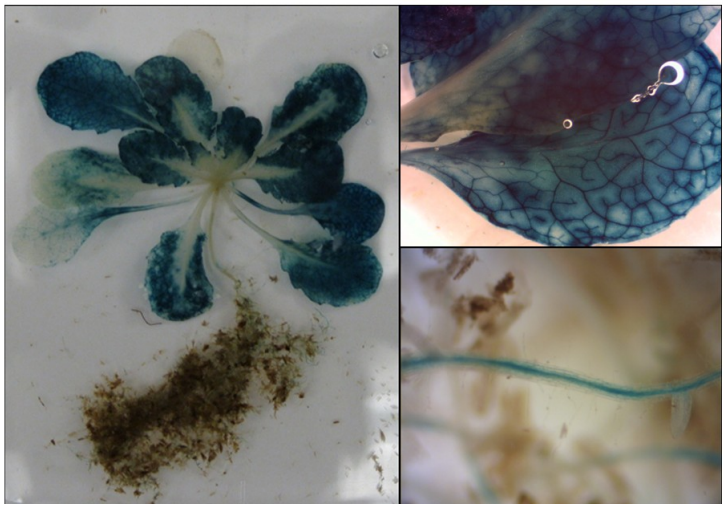


Figure 2-7. Comparison of RFO3C and RFO3T sequence.
(A) Domains of RFO3 SP, signal peptide; B lectin, bulb type lectin; S Locus Domain; PAN/ APPLE domain; TM, transmembrane domain; kinase domain. (B) Diagram of *RFO3*^{C} and *RFO3*$^{Ta\ and\ Tb}$ with comparison of genomic sequence identity.

A



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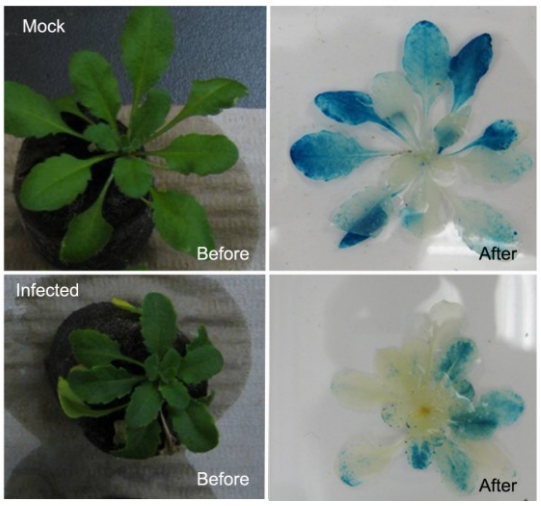

Figure 2-8. *RFO3*p::*GUS* staining
(A) GUS staining is seen mock inoculated *RFO3*p::*GUS* line#8. Top right close up view of stained leaf. Bottom right close up view of staining in root. (B) GUS staining of mock top row and FOM infected bottom row of infected *RFO3*p::*GUS* line #5.

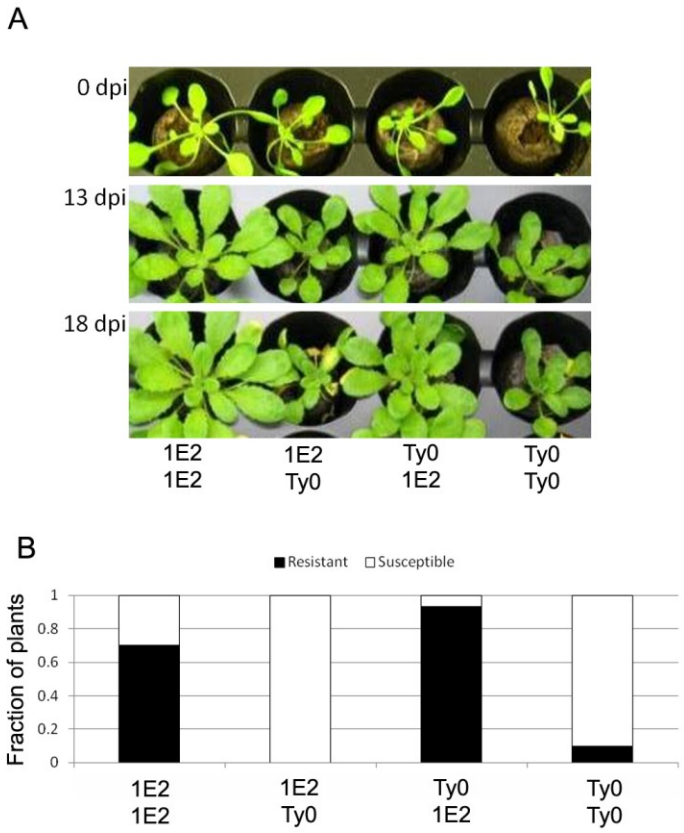

Figure 2-9. Grafting rootstocks and scions of RFO3^{C} and RFO3^{T}.
(A) Representatives of infected grafts 1E2 scions and rootstocks, 1E2 scions and Ty0 rootstocks, Ty0 scions and 1E2 rootstocks, and Ty0 scions and rootstocks shown on 0,

13, and 18 days post inoculation. (B) Fraction of grafts that are resistant (5,4,3) or susceptible (2,1,0) to FOM 21 dpi based on health index.

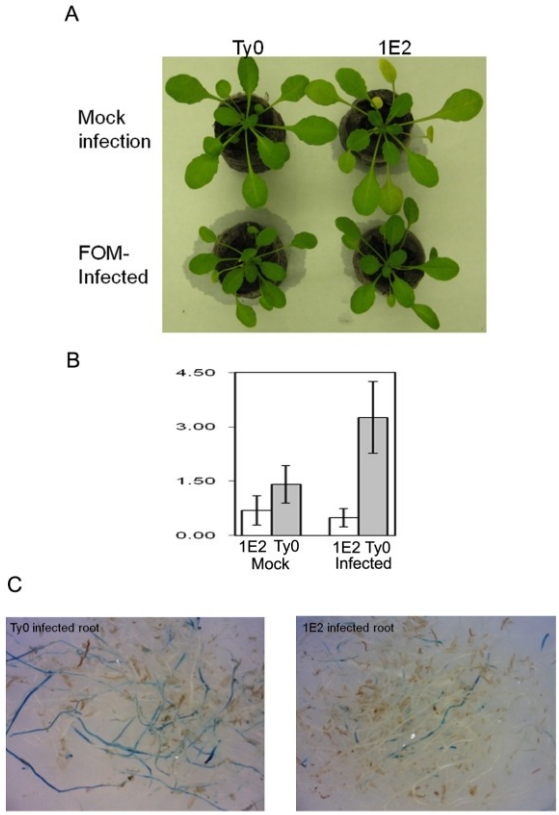

Figure 2-10. Comparison of root colonization by FOM between *RFO3^{C}* and *RFO3^{T}*. (A) 10 day old representatives of FOM and mock inoculated Ty-0 and 1E2 plants prior to root isolation and ARA staining. (B) Quantification of ARA stain using absorbance measurement from spectrophotometer. (C) ARA staining of FOM in the roots on Ty0 (left) and 1E2 (right) infected roots.

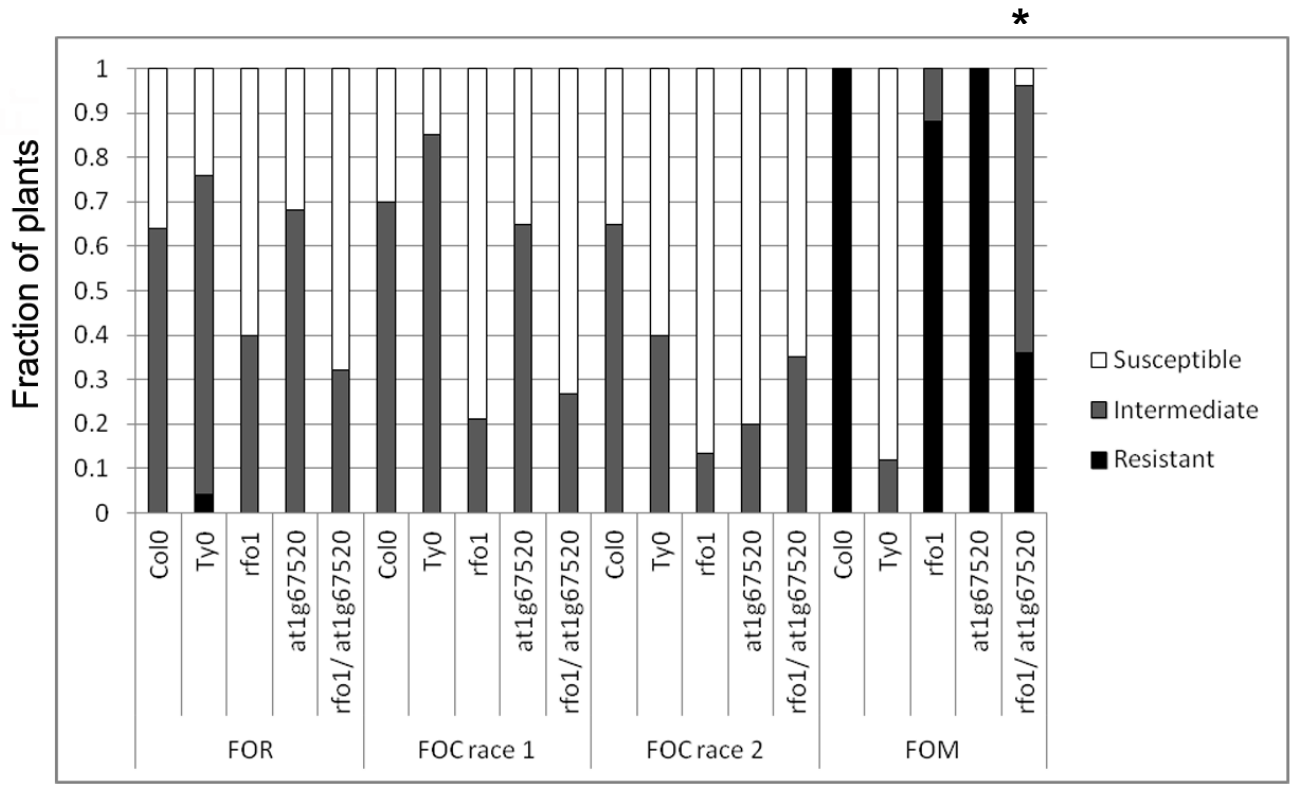

Figure 2-11. Infection of rfo1 at1g67520 double mutant
Scoring of infected *rfo1 at1g67520, rfo1,* and *at1g67520* plants with pathogens FOR, FOC race1, race 2, and FOM. Asterisk= P≤0.05

Chapter 3

Fusarium-derived jasmonates promote fungal colonization of Arabidopsis roots

Abstract

Host-specific forms of the soil-borne fungus *Fusarium oxysporum* cause debilitating vascular wilt diseases, and the relative susceptibility of hosts correlates with persistence of *F. oxysporum* in infected xylem. Our results suggest that persistent vascular infection of Arabidopsis roots by three crucifer-infecting forms of *F. oxysporum* requires host perception of Fusarium-derived jasmonates. Vascular infection in roots and wilt disease symptoms in shoots were suppressed in Arabidopsis plants that were insensitive to jasmonates but were normal in plants that were incapable of producing jasmonates. Instead, we show that some pathogenic forms of *F. oxysporum*, including crucifer-specific pathogens, could be a source of biologically relevant quantities of jasmonates, especially the bioactive jasmonoyl-L-isoleucine, during infection. In contrast, host perception of jasmonates was inconsequential in Fusarium wilt of tomato, and we failed to detect the production of jasmonates by a tomato-specific pathogen. We hypothesize a common role for fungal-derived jasmonates and possibly ethylene among xylem-colonizing fungi.

Introduction

By causing diseases that are difficult to treat, vascular wilt fungi limit cultivation of numerous agricultural crops and the endurance of shade trees (Mace et al. 1981). Notable consequences of vascular wilt are Panama disease, which consumed an estimated 60,000 hectares of banana plantation in Central America in the last century, and Dutch elm disease, which during the past century decimated native and planted

stands of elm trees across North America (Gibbs, 1979; Ploetz, 2000). As a distinctive adaptation, vascular wilt fungi share the ability to colonize the vascular system of their hosts (Mace et al, 1981). Initial colonization of vascular tissue is confined to the lumen of water-conducting xylem vessels that contain low concentrations of organic nutrients (sugars and amino acids) along with inorganic salts. Thus, vascular wilt fungi must be capable of coping with a nutrient-poor environment as well as an active host defense.

Pathogenic forms (or *formae speciales*) of the soil-borne fungus *F. oxysporum* are responsible for host-specific vascular diseases in over one hundred cultivated species (Kistler, 1997). In particular, Fusarium wilt of *Arabidopsis thaliana* is a convenient pathosystem for studying host resistance to, and pathogenesis of, vascular wilt fungi (Diener & Ausubel, 2005; Ospina-Giraldo et al, 2003). Three *formae speciales*, namely *F. oxysporum forma specialis* (f. sp.) *conglutinans* (FOC), *F. oxysporum* f. sp. *raphani* (FOR) and *F. oxysporum* f. sp. *mathioli* (FOM) can be isolated from diseased cabbage (*Brassica* species), radish (*Raphanus sativus*) and garden stock (*Mathiola incana*), respectively (Bosland and Williams 1987). Foliar wilt symptoms in these field hosts, such as stunting, epinasty, yellowing and premature senescence of leaves are reproduced in the related crucifer Arabidopsis (Diener & Ausubel, 2005). Wild accessions of Arabidopsis exhibit a remarkable range of responses to FOC, FOM and FOR, from complete resistance to strong susceptibility. At least six quantitative trait loci (QTLs) contribute to the natural diversity of resistance among Arabidopsis accessions (Diener & Ausubel, 2005). The gene identity of one QTL, *RESISTANCE TO*

F. OXYSPORUM 1 (*RFO1*), as a putative pattern-recognition receptor suggests that quantitative resistance represents variation in innate immunity.

Talboys and others characterized the interactions of vascular wilt fungi and their hosts as having three phases (Beckman 1987; Beckman and Roberts 1995; Talboys 1972). In Fusarium wilt of Arabidopsis, the primary determinative (prevascular) phase is marked by fungal invasion and colonization of undifferentiated tissue at root apices, both root tips and lateral root (LR) primordia (Diener, 2012). In the secondary determinative (vascular) phase, *F. oxysporum* extends into the vascular cylinder from colonized root apices and spreads throughout the vascular system via the xylem. In the expressive phase, symptoms appear in foliage in the absence of pathogen as *F. oxysporum* colonizes the above ground rosette only late in infection if at all (Smith & Walker, 1930). Although infections by the three crucifer-infecting *formae speciales* produce subtle distinctions, wilt symptoms in FOC-, FOM- and FOR-infected Arabidopsis are similar (Diener and Ausubel 2005), and the disease progresses to wilting and death when loss of transpiration becomes severe (Beckman, 1987; Talboys, 1972; Mace et al, 1981).

Previous studies show that the plant hormones, abscisic acid, ethylene, jasmonates and salicylic acid (SA) influence the severity of Fusarium wilt disease. Arabidopsis genotypes that suppress SA biosynthesis (*eds5*) or SA accumulation (*nahG*) enhance symptoms in FOC-infected Arabidopsis, while genotypes that are deficient in abscisic acid biosynthesis (*aba1*) or jasmonate signaling (*coi1*) greatly reduce the severity of symptoms (Anderson et al, 2004; Diener & Ausubel, 2005;

Thatcher et al, 2009; Trusov et al, 2009). Exogenous SA induces resistance to *F. oxysporum* f. sp. *lycopersici* (FOL) in tomato while ethylene insensitivity in *Never ripe* (*Nr*) tomato plants strongly suppresses wilt disease (Lund et al, 1998; Mandal et al, 2009).

Jasmonates, including jasmonic acid (JA), methyl jasmonate (MeJA) and jasmonoyl-L-isoleucine (JA-Ile), are a class of oxylipins that elicit similar responses in plants (Sembdner & Parthier, 1993). However, the active form of jasmonate is JA-Ile, and JA and MeJA must be converted to JA-Ile for their effects to manifest (Fonseca et al, 2009). JA-Ile promotes the association of the jasmonate receptor complex, a heterodimeric protein, composed of the F-box protein COI1 and one of several JAZ transcriptional repressor proteins (Yan et al, 2009). This COI1-JA-Ile-JAZ complex, as part of a larger SCF complex, catalyzes polyubiquitination of the JAZ repressor, which the 26S proteasome subsequently degrades (Chini et al, 2007; Thines et al, 2007). COI1-mediated elimination of JAZ repression of JA-responsive genes explains all physiological responses to jasmonates in Arabidopsis, and thus the loss-of-function *coi1* is insensitive to jasmonates (Browse, 2009).

Plant hormones, such as jasmonates, are engaged in developmental programs as well as responses to environmental cues (Browse, 2009; Sembdner & Parthier, 1993). For instance, Arabidopsis mutants that are either deficient for or insensitive to jasmonates are male sterile because jasmonate signaling normally plays critical roles in stamen development, including filament elongation, anther dehiscence, and pollen maturation (Feys et al, 2004; Von Malek et al, 2002). More generally, in plants,

jasmonate signaling is associated with responses to injury and necrosis (Browse, 2009). In Arabidopsis, for example, jasmonate signaling in response to herbivory and infection by necrotrophic pathogens modulates the accumulation of peptides, such as thionins, and compounds, such as camalexin and glucosinolates, that can antagonize the growth of pests and pathogens (Florack & Stiekema, 1994; Mewis et al, 2006; Zhou et al, 1999; Vignutelli et al, 1998). Because jasmonates are commonly associated with metabolism that deters herbivory and are antimicrobial, jasmonate signaling is commonly regarded as a defense response pathway (Browse, 2009).

The roles for hormone signaling during plant disease are usually expressed in terms of host resistance, and thus plant hormones can either promote or suppress resistance to disease (Glazebrook, 2005; Pieterse et al, 2009; Spoel & Dong, 2008). Salicylic acid (SA) signaling is associated with resistance to biotrophic pathogens and induction of systematic acquired resistance, while ethylene signaling, in addition to jasmonate signaling, is associated with resistance to necrotrophic pathogens. However, because signaling from different hormones can be antagonistic, signaling from both may work at cross-purposes and negate the critical contribution of either or both (Kunkel & Brooks, 2002). Antagonistic signaling by jasmonates and SA best exemplifies this hormone crosstalk during pathogen infection. Loss of jasmonate signaling in Arabidopsis mutants can produce exaggerated SA signaling and enhance resistance to biotrophic pathogens, while loss of pathogen-induced accumulation of SA can heighten jasmonate signaling and resistance to necrotrophic pathogens (Kloek et al, 2001).

From the perspective of a virulent pathogen, successful pathogenesis depends on the interaction between hormone signaling and virulence (Robert-Seilaniantz et al, 2011). The most profound effects of hormones in infected plants are observed in studies that examine interactions with virulent pathogens (Grant & Jones, 2009), so obvious positive or negative effects of hormones in disease arguably show whether hormone signaling promotes or obstructs, respectively, the virulence strategies of pathogens. When hormone signaling is appreciated from the perspective of virulence strategy, jasmonates are generally antithetical to the kind of virulence that necrotrophic pathogens express and conducive to the virulence of biotrophic pathogens. This perspective better explains why hormone signaling in plants often plays an inconsistent role during infection by pathogens with the same lifestyle, or pathogens from the same genus, as different pathogens may express similar but distinct virulence strategies (Jakob et al, 2007).

Even as hormone signaling is an integral part of host response to infection and damage, the stimulation and/or suppression of hormone signaling plays a critical role in pathogen virulence. Indeed, a variety of plant pests and microbial pathogens are capable of producing one or more plant hormones (Grant & Jones, 2009); and, in particular, fungal strains from the genus *Fusarium* alone can produce auxin, abscisic acid, gibberellins, ethylene or jasmonates (Cross & Webster, 1970; Dörffling et al, 1984; Dowd et al, 2004; Tsavkelova et al, 2012). In fact, JA and JA-Ile were first identified as the products of fungi, rather than of plants, as JA was characterized as a senescence promoting activity in cultures of the tropical phytopathogen *Botrydiplodia theobromae*

(Aldridge et al, 1971), and JA-Ile was discovered as an alternative metabolite of the gibberellin-producing fungus *Gibberella fujikuroi* (Cross & Webster, 1970). However, the best-studied microbial jasmonate is probably coronatine, a critical polyketide effector of bacterial leaf speck pathogen *Pseudomonas syringae*. Coronatine affects foliar infection in Arabidopsis in multiple ways, by inhibiting the closure of stomates, suppressing SA signaling, and promoting leaf senescence (Kloek et al, 2001; Melotto et al, 2006).

In this paper, we show that some but not all pathogenic *F. oxysporum* strains, including the three crucifer-infecting strains, produce biologically relevant amounts of jasmonates in axenic culture as well as in Arabidopsis. We show that this Fusarium-derived jasmonate promotes the development of specific foliar symptoms, but more importantly the colonization of the vascular cylinder by *F. oxysporum* in roots. In contrast, we show that the tomato pathogen *F. oxysporum* f. sp. *lycopersici* (FOL) does not produce appreciable amounts of jasmonates, and that perception of jasmonate is inconsequential for Fusarium wilt of tomato. From the observation that fungal pathogens producing jasmonates are associated with gum exudation, we hypothesize a common role for pathogen-derived jasmonates, and alternatively ethylene, in vascular diseases.

Materials and Methods

Standards

(±) JA (Research Products International Corp., Mount Prospect, IL) and (±) $^{2}H_{4}$ JA (C/D/N Isotopes Inc, Quebec, Canada) were purchased commercially. JA-Leu, JA-Ile, and $^{13}C_{6}$-JA-Ile were generously provided by Paul Staswick (University of Nebraska, Lincoln, NE).

Plant and *F. oxysporum* stocks, growing conditions, and infection assays.

Arabidopsis mutants or transgenic lines, in the Columbia-0 (Col-0) genetic background, originated from published sources, *sid2-2*/*eds16* (Wildermuth et al. 2001), *rfo1* (SALK_077975, Diener and Ausubel 2005), *coi1-1* (Feys et al. 2004), or were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH), *aos* (SALK_017756C). *THI2.1*p:*uidA* transgenic seeds were kindly provided by H. Bohlmann (Epple et al. 1995). Gregg Howe (Michigan State University, East Lansing, MI) generously provided tomato seeds from *JAI1*/*jai1* fruit. Arabidopsis seeds were sown on Jiffy 7 1"x1.25" #734 peat pellets while tomato seeds were sown on 1.75"x1.75" #703 pellets (Growers Solution, Cookeville, TN) and grown under cool white fluorescent lighting with moderate intensity during a 12-h daylength with 30˚C daytime and 27˚C nighttime temperatures. Alternatively, Arabidopsis seeds were sown on Plant Nutrient (PN) agar plates (Diener and Ausubel 2005).

FOC race 1 (strain 777), FOR (strain 815) and FOM race 2 (strain 726) originate from Paul H. Williams through H. Corby Kistler (Bosland and Williams 1987; Kistler et al. 1991). Kerry O'Donnell (USDA/ARS, Peoria, IL) provided FOL race 2 (strain 4287, Ma et al. 2010) and FOT (NRRL 26954) while H. C. Kistler provided FOL race 3 (MN-25)

and *Fusarium graminearum* (Gz3639). Fusarium cultures were grown on Czapek-Dox minimal medium (Oxoid Ltd, Hampshire, UK), and conidial suspensions were harvested from 3- to 7-d old shaken cultures, washed 3 times with sterile water. Concentration of suspended conidia was measured using a hemacytometer, and plants were irrigated with 10^6 to 5 x 10^7 conidia/ml (or water as a control) to initiate infection of 2- to 3-wk old plants. Disease severity was scored using an ordinal health index (HI), described in Diener and Ausubel (2005), in which 0 is dead, and 5 is unaffected.

PCR-based genotyping of Arabidopsis plants is described in Diener and Ausubel (2005). Codominant alleles at Salk insertions were PCR-amplified using the T-DNA-specific primer (LBb1, 5'-GCGTGGACCGCTTGCTGCAACT-3') in combination with the gene-specific oligonucleotide primer pairs: for *aos*, SALK_017756-LP, 5'-AACAACAAAATCCTTACCGGC-3' and SALK_017756-RP and 5'-CTAACCGGAGGCTACCGTATC -3', and for *rfo1*, AT1G79670-P5, 5'GAGATTTAATGTGAACAACTCC-3' and 5'-SALK_077975-RP, 5'-CGTTGGTGAATAGTCAATTTCC-3'. For the *coi1-1* CAPS marker (Konieczny and Ausubel 1993), a 612 bp product was PCR-amplified by coi1-1_CAPS-F, 5'-TCGACCGGGAAGAAAGGATTA -3' and coi1-1_CAPS-R, 5'-ACACAGTTTGTGGAAACCCCA -3' and wild-type (*COI1*) but not *coi1-1* DNA is digested by *Xcm*I. Genotyping of *sid2-2* is described in Wildermuth et al. (2001).

Using the PCR-based assay of Li et al. (2004), 20 *jai1*/*jai1*, 48 *JAI1*/*jai1* and 32 *JAI1*/*JAI1* seedlings were obtained from genotyping 100 seedlings from selfed *JAI1*/*jai1*; and, as expected, approximately one-quarter of the germinated seedlings from selfed

JAI1/*jai1* were resistant to 1 mM MeJA. Two-week old tomato seedlings were irrigated with 2 x 10^6 FOL (MN-25) conidia/ml (or water for mock-infection) to initiate infection. Infected seedlings were transplanted to pots with autoclave-sterilized soil. In each pot, *jai1*/*jai1* and *JAI1*/*JAI1* plants were paired: 12 pots had FOL-infected plants, and six had mock-infected plants. Plants were grown for 5 weeks in a Conviron growth chamber under high intensity lighting at 30˚C with a 12-hour daylength. Scoring of plants using a disease index from 0 (unaffected) to 5 (dead) is described in Walker and Foster (1946).

Visualization and quantification of infection in roots

To measure the fresh weights of plant tissue, shoots that were removed at the hypocotyl-root junction in water-saturated soil were weighed. Roots that were cleaned of peat soil in 0.1% Triton X-100 and 20 mM EDTA, pH 8.0, using fine needle-sharp tweezers, and dried of excess solution on wax paper with tissue paper were weighed in microcentrifuge tubes.

Cleaned roots were incubated on a rotisserie tube mixer at 28 ˚C in 40-fold excess volume staining solution, 10 mM EDTA, 0.1 % Triton X-100, 1 mM $K_3Fe(CN)_6$, 0.1 M sodium phosphate, pH 7.0. The staining solution included either 5-bromo-4-chloro-3-indoxyl-α-L-arabinofuranoside (X-ARA, #B-290, Gold Biotechnologies, St. Louis, MO) or 4-nitrophenyl-α-L-arabinofuranoside (NP-ARA, #N-240, Gold Biotechnologies, St. Louis, MO), where 100-fold excess stock solutions were 20 mg glycoside reagent (2%) in 1 ml dimethylformamide. For visualizing infection, roots were stained with X-ARA overnight and destained with two transfers to

excess water at 4 ˚C for several hours to overnight. Blue precipitate was observed using a low-magnification binocular microscope and recorded with a digital camera (Dino-Eye Eyepiece, AM423XC, BigC, Torrance, CA). Twenty x-magnified digital images of all stained root apices were collected. Staining patterns in images were assigned to one of four categories, described in Results and Figure 5, and results are presented as fraction of category of root apices among all stained root apices of a genotype. To quantify infection, cleaned root systems were incubated for 20 hrs at 28 ˚C on a rotisserie tube mixer in 40-fold volume NP-ARA staining solution and then frozen at -20˚C. Optical density (OD) of all samples at 410 nm (OD_{410nm}) and 600 nm (OD_{600nm}) was measured using a spectrophotometer (Smart Spec 3000, Biorad, Philadelphia, PA). OD_{600nm} was subtracted from OD_{410} to account for nonspecific light diffraction in samples to give the OD_{410}/gram fresh weight roots. Measurements were standardized so that the mean OD_{410}/gram fresh weight was 1.0 for uninfected wild type.

Quantification of jasmonates in culture filtrates

Still cultures in 100 ml Czapek-Dox medium, which were kept dark at ambient temperature for 3 wk, then centrifuged, filtered and stored at -80˚C. Internal standards $^{2}H_4$ JA (934 pmol) and $^{13}C_6$ JA-Ile (978 pmol) were added to 1 ml aliquots of thawed culture filtrates, acidified to pH 2 with 0.1N HCl, and extracted three times with 1 ml ethyl acetate. Phases were separated by low speed centrifugation and the pooled organic phases were dried in a vacuum centrifuge. Extracts were resuspended in 10 µl

methanol and 110 µl water, and 50 µl aliquots were injected onto a reverse phase HPLC column (C18 Asentis Express, 15 cm x 2.1 mm x 2.7 µm, Supelco Analytics, Sigma-Aldrich, St. Louis, MO) equilibrated in 80% buffer A (water containing 10 mM HCO_2NH_4 and 0.1 mM cetyl trimethylammonium bromide)/20% buffer B (CH_3CN/aqueous 10 mM HCO_2NH_4, 90/10, v/v) and eluted (200 ul/min) with an increasing concentration of buffer B (min/% B; 0/20, 2/20, 52/80, 55/20, 60/20). The effluent from the column was passed to a flow splitter and a proportion of the flow (about 20%) was passed to an Ionspray™ source connected to a triple quadrupole mass spectrometer (PE-Sciex ABI III$^+$) operating in the negative ion tandem mass spectrometric multiple reaction monitoring (MRM) mode in which the intensity of specific parent □ fragment ion transitions (JA m/z 209 - 59, $^2H_4$ JA m/z 213 - 61, JA-Ile/JA-Leu m/z 322 - 130, $^{13}C_6$ JA-Ile m/z 328 - 136) were monitored under previously optimized conditions (orifice -55 volts, argon collision gas at instrumental GCT setting of 180) using instrument-manufacturer supplied software for data acquisition (Tune version 2.5 and RAD version version 2.6) and analysis (MacSpec version 3.3 and BioMultiView version 1.3.1).

Two closely-eluting peaks in the sample chromatograms with the same transition as JA-Ile/Leu (m/z 322 □ 130) were tentatively identified using JA-Ile and JA-Leu standards and co-chromatography experiments. Under the prescribed chromatographic conditions, the stereoisomers in the JA-Ile standard eluted as two equally intense peaks at 39.2 and 40.8 min, while the stereoisomers in the JA-Leu standard eluted as two equally intense peaks at 40.0 and 41.5 min. The four peaks of JA-Ile/Leu exhibited

near-baseline separation when equal amounts of JA-Ile and JA-Leu standards were injected. The identity of JA-Ile/Leu peaks in sample extracts was confirmed by co-chromatography experiments by addition of authentic JA-Leu or JA-Ile to FOT fungal extract. In these experiments the peaks from the sample co-chromatographed with the early (40.8 min) or later (41.5 min) peak, respectively.

Bioassays with *THI2.1*p:*uidA*

Seedlings were grown for 5 to 7 days on plant nutrient (PN) agar with 0.5% sucrose at 28°C with 12 hours day and 12 hours night. Seedlings were infected with 1 x 10^6 conidia/ml. Seedlings were vacuum-infiltrated with 100 µM MeJA in 0.02% acetone, 100 µM $AgNO_3$ or mock-treated with corresponding solvent and left for 3 d at 28˚C. Seedlings were wounded with a single cut to the cotyledons. Seedlings were stained overnight with 0.2 mg/ml 5-bromo-4 chloro-3-indolyl-β-D-glucuronide (X-Gluc, Gold Biotechnologies, St. Louis, MO) in 0.1 M sodium phosphate buffer, pH 7.0 at 37°C. Seedlings were observed and photographed after destaining in ethanol and rehydration.

Grafting rootstocks and scions

Grafts were essentially performed as described in Turnbull et al. (2002). Wild type and *coi1* were grown on the surface of PN agar plates for 1 wk at 22°C with a short (8 h) daylength to inhibit flowering. MeJA-resistant *coi1* seedlings among *COI1*/*coi1* progeny were transferred to PN agar plates after selection on plates with 30 µM MeJA. After scion and rootstocks were joined, grafts were left on PN agar plates for 10 d

before transplanting to peat pellets. Over the next week, adventitious roots growing from the scion were removed with a scalpel. Four-week old grafted plants were infected with FOC 2 wk after transplanting.

Results

Jasmonates in Fusarium culture filtrates

The accumulation of JA and JA-Ile/Leu was measured in the cultures of six Fusarium strains, the wheat head blight *F. graminearum* and five pathogenic *F. oxysporum formae speciales*, namely FOC, FOM, FOR, FOL and *F. oxysporum* f. sp. *tulipae* (FOT). Jasmonates were extracted from filtrates of three-week old cultures, separated by HPLC and detected by electrospray ionization coupled to multiple reaction monitoring (MRM). A single peak for JA that coeluted with the JA standard and deuterated-JA was detected (Figure 3-1). JA was reproducibly present in cultures of FOC, FOM, FOR and FOT (in Table 1) but absent (< 4.8 pmol/ml) in cultures of FOL and *F. graminearum*. When amounts of JA were quantified, much less JA was measured in the cultures of FOC and FOR than in FOM and FOT cultures(in Table 3-1). The most JA (200 pmol/mL) accumulated in cultures of FOT.

The same samples revealed two closely-eluting peaks in the m/z 322 - 130 chromatograms, corresponding to the natural stereoisomers of JA-Ile and JA-Leu. JA-Ile and JA-Leu standards and the ^{13}C-labeled JA-Ile internal control each eluted as two clearly separated peaks. The early peak (at 40.8 min) in Fusarium extracts coeluted with the later-eluting peak in the JA-Ile standard and internal control, while the later

peak (at 41.5 min) in Fusarium extracts coeluted with the later-eluting peak in the JA-Leu standard (Figure 3-2). The reverse phase separation of the sterioisomers of these compounds observed here is the same as previously reported by Fonseca et al. (2009). We detected and measured both JA-Ile and JA-Leu in all strains that produced JA with the exception of FOR, and failed to detect either amino acid conjugate in FOR, FOL, and *F. graminearum*. The molar amount of JA-Ile/Leu in extracts was consistently higher (from 3- to 32-fold more in Table 3-1) than JA, and the highest JA-Ile concentration was in FOT cultures (6.5 nmol/mL).

F. oxysporum produced biologically relevant amounts of jasmonate in plants

To test whether Fusarium strains could produce biologically relevant amounts of jasmonate in infected plants, we developed a plant line that lacks endogenous jasmonates but responds to an exogenous source of jasmonate activity. Transcriptional expression of *THI2.1*, a jasmonate-inducible thionin gene in Arabidopsis, was monitored using the transgenic reporter *THI2.1*p:*uidA*, created by Epple et al. (1995), which fuses the *THI2.1* promoter to the *Escherichia coli beta*-glucuronidase (GUS) gene *uidA*. *THI2.1*p:*uidA* expresses GUS activity in seedlings in response to jasmonate, and this response depends on *COI1* (Bohlmann et al. 1998). When *THI2.1*p:*uidA* seedlings were treated with methyl jasmonate (MeJA), GUS activity was induced at the meristem and in the first leaves (Figure 3-3A), and similar GUS expression was induced when *THI2.1*p:*uidA* seedlings were treated with silver nitrate (Figure 3-3A). Additionally, *THI2.1*p:*uidA* was induced along the cut edge of a wounded cotyledon (Figure 3-3A).

Endogenous jasmonate biosynthesis mediated the response of *THI2.1*p:*uidA* to wounding and silver nitrate because no GUS activity was induced by these stimuli when *THI2.1*p:*uidA* was crossed into the jasmonate-deficient *aos* background (Figure 3-3A) (Chehab et al, 2008). Nevertheless, *THI2.1*p:*uidA* remained responsive to jasmonate in *aos* because treatment with MeJA still induced GUS activity (Figure 3-3A).

Fusarium strains that yielded appreciable amounts of JA-Ile/Leu in culture filtrates (in Table 3-1) also induced jasmonate-dependent *THI2.1*p:*uidA* expression in infected seedlings. We stained transgenic *THI2.1*p:*uidA* seedlings for GUS activity three days after infecting seedlings, growing on agar medium, with the same Fusarium strains that were evaluated for JA and JA-Ile/Leu production. Whether *THI2.1*p:*uidA* seedlings were wild type or *aos*, similar visible staining with X-Gluc was obtained when seedlings were infected with JA-Ile/Leu-producing Fusarium strains, FOC, FOM or FOT, whereas no staining was evident when seedlings were infected by Fusarium strains with undetectable JA-Ile/Leu in their culture filtrates (Figure 3-3B). *THI2.1*p::*uidA* accounted for all GUS activity in Fusarium-infected seedlings because no staining was observed when wild type or *aos* alone was infected (Figure 3-3B). As previously reported by Berrocal-Lobo and Molina (2004), colonization of Arabidopsis seedlings on agar plates lacked host specificity, and all tested Fusarium strains, including *F. graminearum* (Figure 3-3C), could infect Arabidopsis seedlings. Similar to results with Fusarium-infected seedlings, treatment of *THI2.1*p:*uidA aos* seedlings with culture filtrates of JA-Ile/Leu-producing strains gave visible X-Gluc staining while culture filtrates with undetectable JA-Ile/Leu failed to induce appreciable GUS activity (Figure 3-4).

Host perception of Fusarium-derived jasmonate promoted susceptibility

Genetic analysis of host resistance to wilt disease is consistent with the hypothesis that Fusarium-derived jasmonate promotes susceptibility. Specifically, we observed different roles for host biosynthesis of jasmonate (*AOS*) and perception of jasmonate (*COI1*) because *coi1* but not *aos* affected FOC infection. We quantified the stunting of rosette leaves, a symptom of wilt disease, as a reduction in the rosette radius in self-progeny of the *AOS*/*aos COI1*/*coi1* dihybrid. *COI1* alleles but not *AOS* alleles cosegregated with differences in rosette radius, and a reduced rosette radius (and stunting) correlated with the inheritance of *COI1* (Figure 3-5A). Moreover, disease severity was sensitive to gene dosage as *COI1* heterozygotes (±, in Figure 3-5A) had longer rosette leaves than wild-type plants (with two copies of *COI1*). Remarkably, all *coi1* plants expressed no apparent symptoms. Among plants with the same *COI1* genotype, stunting of rosette leaves, as well as other symptoms, was similar whether FOC-infected plants were *aos* mutants or wild type (*AOS*/–) (Figure 3-5A).

We observed a similar correlation in FOM-infected plants as rosette stunting and *COI1* genotype cosegregated in self-progeny of the double mutant *rfo1*/*rfo1 COI1*/*coi1*. *rfo1* plants were infected with FOM because wild-type Col-0 already has complete resistance to FOM (Diener & Ausubel, 2005). Once again, gene dosage of *COI1* correlated with rosette radius (Figure 3-5B), as well as overall disease severity, in FOM-infected plants because heterozygous progeny (*COI1*/*coi1*; ±, in Figure 3-5B) expressed milder symptoms than wild type progeny.

COI1 also promoted susceptibility to FOR, as survival of *coi1* plants was longer than *COI1*/– plants (Figure 3-5C); however, unlike infection with FOC or FOM, infection with FOR killed *coi1* plants. In addition, disease progression in FOR-infected *coi1* and *COI1* plants was strikingly different (Figure 3-5D). Wilt symptoms, such as stunting, epinastic growth and anthocyanin accumulation were strongly suppressed in FOR-infected *coi1* plants (compare *coi1* and *COI1* plants in Figure 3-5D), even in severely infected *coi1* plants on the verge of death (see *coi1* plant in center in Figure 3-5D). In most instances, only a wilting of leaves (notice arrows in Figure 3-5D) preceded, by one to two days, the sudden decline and death of *coi1* plants (see *coi1* plant to the right in Figure 3-5D). In contrast, FOR infections of *aos* and *AOS*/– were indistinguishable in all respects, including the stunting of rosette growth (Figure 3-5E). Because host-derived jasmonate was absent and inconsequential in wilt effected *aos* plants, *F. oxysporum* was the only source of jasmonate for *COI1* activation.

JAR1 is the enzyme used to conjugate Ile to JA to form JA-Ile. JA-Ile was detected in axenic culture of FOC but not in FOR culture. Since *coi1* plants are completely resistant to FOC and there is a delay in susceptibility to FOR, we wanted to know if *JAR1* affected FOR interaction. When infected, *JAR1* and *jar1* plants were equally susceptible to both FOC and FOR (Figure 3-6A). Roots of FOR-infected *JAR1* and *jar1* were stained with X-ARA to see if there was a difference in colonization. The roots of both mutants and wild-type plants stained equally (Figure 3-6B). Therefore, the conversion of JA to JA-Ile has no affect on FOR or FOC infection.

While susceptibility to Fusarium wilt in Arabidopsis strongly depended on perception of Fusarium-derived jasmonate, the production of jasmonate alone was not sufficient to make *F. oxysporum* pathogenic to Arabidopsis. Arabidopsis accession St. Georgen-1 (Sg-1), which is highly susceptible to FOC as well as FOM and FOR (Diener & Ausubel 2005), was completely resistant to soil infected with a high dose of FOT (5 x 10^7 conidia mL^{-1}), despite the fact that FOT produced substantially more jasmonates in axenic culture than FOC (Figure 3-7).

MeJA treatment of plants may have no affect on infection

We have shown that jasmonates made by *F. oxypsoum* have an effect on host susceptibility. We wanted to know if the addition of MeJA could make plants more susceptible to FOR in an isolate that produces lower quantities of jasmonates compared to other measured isolates. Fifty μM MeJA was added to spore suspension of FOR at the time of infection. Plants that were infected with both FOR and MeJA were equally susceptible as plants infected with only FOR (Figure 3-8). From this data, we can determine that the adding MeJA to plants cannot make them more susceptible to FOR infection. However, if jasmonate from *F. oxysporum* is what makes Arabidopsis more susceptible to infection, then why does the addition of MeJA not increase susceptibility. This could be explained because MeJA is a volatile compound and the MeJA could have dissipated before it could affect FOR infection. Also the quantity of MeJA may have been too low to affect infection. This experiment would need to be repeated with various concentrations of MeJA and continuous treatment to see if the addition of MeJA truly has no affect on FOR infection.

Perception of jasmonate in both the root and shoot contributed to susceptibility.

COI1 expression in both the root and shoot contributed to disease susceptibility in reciprocal grafts between rootstocks and scions of wild type (*COI1*) and *coi1*. FOC infections in grafted plants were performed because different phases of infection occur in roots and shoots, and grafts could distinguish, for example, whether *COI1* in roots affected fungal colonization below ground during the two determinative phases or *COI1* in shoots affected development of symptoms above ground in the expressive phase. Plants with *coi1* rootstocks were, like ungrafted *coi1* plants, completely resistant to FOC infection whether grafted scions was *coi1* or wild type, which showed that *COI1* perception in roots was essential for susceptibility (Figure 3-9A and B). Plants with wild-type rootstocks and *coi1* scions were more resistant than ungrafted wild-type plants or grafted plants with wild-type rootstocks and scions (Figure 3-9A and B), which showed that *COI1* perception in shoots also contributed to susceptibility. In fact, absence of jasmonate perception in *coi1* scions altered the normal appearance of wilt disease as some symptoms, such as stunting of rosette leaves, anthocyanin in petioles and epinasty of leaf petioles, were strongly suppressed, and other symptoms such as perivascular yellowing and premature senescence, were delayed (Figure 3-9A). At 21 days after soil was irrigated with FOC, we stained roots of representative grafts with 5-bromo-4-chloro-3-indoxyl-α-L-arabinofuranoside (X-ARA) to examine fungal colonization. X-ARA specifically stains *F. oxysporum* in roots because X-ARA is an indigogenic substrate for an arabinofuranosidase (ARA) activity that *F. oxysporum*

expresses but Arabidopsis does not (Diener, 2012). In comparison to root systems from plants with wild-type rootstocks and scions, which FOC thoroughly colonized, FOC infection was noticeably reduced in roots of plants with wild-type rootstocks and *coi1* scions (Figure 3-9C). Thus, shoot-expressed *COI1* quantitatively influenced fungal colonization in roots, which may explain the observed delay in the death of plants with wild-type rootstocks and *coi1* scions. More strikingly, colonization of *coi1* rootstocks by FOC was abbreviated and largely restricted to root tips whether these rootstocks were grafted to *coi1* or wild-type scions (Figure 3-9C), which clearly showed that root-expressed *COI1* was essential to attain more than sparse colonization of the root system.

Early FOC infection promoted root growth

Prior to the development of symptoms, such as yellowing and premature leaf senesence, infection enhanced root mass of Arabidopsis plants. Because FOC produces jasmonates, and exposure to a superoptimal amount of MeJA is known to inhibit the growth of Arabidopsis roots (Feys et al, 1994), we anticipated that FOC infection would inhibit root growth. Starting with wild-type and *coi1* plants with comparable root and shoot masses (at 0 dpi in Figure 3-10A), shoot mass of both genotypes similarly increased at 4 and 7 dpi, whereas the root mass of wild type grew significantly more than the root mass of *coi1* at 4 and 7 dpi (in Figure 3-10A). This difference was clearer when data was expressed on a proportional basis (in Figure 3-10A). This difference in wild-type and *coi1* roots appeared to be a consequence of FOC

infection and not a consequence of jasmonate perception *per se*. When a similar experiment was performed that included both FOC- and mock-infected plants, more root mass in the FOC-infected plants was measured in both wild type and *coi1*, at 7 dpi (Figure 3-10B). More modest growth by FOC-infected *coi1* was presumably due to stronger resistance to infection. It was concluded that Fusarium-derived jasmonate did not promote susceptibility by inhibiting root growth. Rather, infection promoted by jasmonate resulted in enhanced root mass early in infection.

COI1 promoted fungal colonization of the vascular cylinder

Absence of jasmonate perception (*coi1*) suppressed colonization of the vascular cylinder in the secondary determinative phase of FOC infection. As already observed in grafted plants, root infection was extensive in wild type but sparse in *coi1* late in the infection, by which time the wild-type plants showed severe disease symptoms. To determine when *COI1* was required, root infection in wild type and *coi1*, as indicated by X-ARA staining, was compared during the initial week after the soil was irrigated with FOC. In the first few dpi, root apices, both meristematic root tips and LR primordia, appeared to be similarly invaded and colonized in wild type and *coi1*. However, by 4 to 5 dpi, when wild type remained asymptomatic, X-ARA staining in *coi1* roots rarely extended from the apices into the vascular cylinder, whereas fine vascular staining was associated with colonized root tips and LR primordia in wild type. To quantify this difference in FOC infection, we categorized, according to the extent of fungal colonization, all stained root ends at 5 dpi in three whole root systems of *coi1* and wild

type (Figure 3-11A). X-ARA staining of root apices could be divided into four common patterns, or categories that are depicted in Figure 3-11A: In category i, X-ARA incompletely stained undifferentiated tissue at root apices. In category ii, X-ARA stained apices throughout and possibly differentiated tissues adjacent to apices. In category iii, staining extended broadly into the vascular cylinder for short distances from infected apices. In category iv, fine-staining extended from apices for longer distances. *F. oxysporum* infection at meristematic root tips and LR primordia could be similarly divided into the four categories, however, differences were observed with these two types of apices. X-ARA stained similar numbers of meristematic root tips in three *coi1* (44, 48 and 71) and three wild-type (49, 55,and 63) root systems while fewer LR primordia of *coi1* (13, 15 and 15) than wild type (23, 24 and 29) were stained. Because the total number of LR primordia was not determined, this difference may represent either fewer LR primordia in *coi1* or less infection. Among meristematic root tips, categories i, ii and iii were similarly represented in *coi1* and wild-type root systems (Figure 3-11A), which implied that entry and initial colonization of root tips in the first determinative phase was normal in *coi1*. However, among three *coi1* root systems, no FOC-infected meristematic apex and only one LR primordium was placed in category iv (Figure 3-11A). In contrast, a total of 20 meristematic tips and 22 LR primordia were represented by category iv in three wild-type root systems. Thus, jasmonate perception was critical for extensive growth of FOC into xylem at the transition from primary to secondary determinative phase.

In the second week after infection, wild-type or *rfo1* plants exhibited obvious stunting and had substantially more FOC infection in roots than plants that were also *coi1*. X-ARA staining was extensive in FOC-infected roots that were *COI1*, while few infected *coi1* roots had much staining extending away from root tips. To quantify infection, measurements were made of the relative amount of yellow (OD_{410nm}) 4-nitrophenol product liberated by Fusarium-expressed ARA when roots were incubated with the colorless ARA substrate 4-nitrophenyl-α-L-arabinofuranoside (NP-ARA) (Diener 2012). In one experiment FOC and FOR infection in roots of wild type and *coi1* were compared, and in another, infection in roots of *rfo1* and *rfo1 coi1*. In both experiments, 7- to 10-fold more Fusarium-derived ARA was present in FOC-infected *COI1* than in *coi1* plants (Figure 3-11B); and, 3- to 5-fold more ARA was present in FOR-infected *COI1* (Figure 3-11B).

Mutations in SA biosynthesis or response do not suppress resistance of *coi1*

coi1 suppressed the enhanced susceptibility of mutants that abolish (*sid2*) or attenuate (*pad4*) pathogen-induced accumulation of salicylic acid. In segregating progeny of the *COI1*/*coi1 SID2*/*sid2* dihybrid, wild-type *COI1* and *SID2* genotypes correlated with susceptibility and resistance to FOC, respectively (Figure 3-12A). Again, *COI1* exhibited an incomplete dominance because stunting produced rosette leaves of heterozygotes (±) with a length that was intermediate between the homozygotes. Similarly, *COI1* and *PAD4* genotypes correlated with resistance and susceptibility among the progeny of the *COI1*/*coi1 PAD4*/*pad4* dihybrid. Among progeny that

perceived jasmonate (*COI1*/–), absence of pathogen-induced salicylic acid (in *sid2* homozygotes in Figure 3-12A) or attenuation of SA accumulation (in *pad4* homozygotes in Figure 3-12B) enhanced susceptibility. However, neither *sid2* nor *pad4* compromised resistance in double mutants *coi1 sid2* or *coi1 pad4* that could also not perceive jasmonates. Interestingly, loss of *SID2* did not appreciably affect wilt disease that was instigated by FOR (Figure 3-12C), and the partial resistance of *coi1* to FOR was not dependent on *SID2* as *coi1 sid2* double mutants were significantly more resistant than *COI1*/– *sid2* plants (Figure 3-12D).

Fusarium wilt in tomato was unaffected by jasmonate sensitivity.

Wilt disease was comparable in FOL-infected wild-type (*JAI1*) and jasmonate-insensitive *jai1* tomato plants. As previously reported, deficiency in *JAI1,* the tomato ortholog of Arabidopsis *COI1* (Li et al, 2004), makes *jai1* seedlings insensitive to growth inhibition by MeJA; however, the gross appearance of mock-infected *jai1* plants was indistinguishable from wild type (Figure 3-13A). As well, FOL-infected *JAI1* and *jai1* plants expressed symptoms, such as epinastic growth of petioles and premature senescence of older leaves, with similar severity at 21 dpi (Figure 3-11B and C) and yielded similar distributions of disease index scores at 35 dpi (Figure 3-13D).

Discussion

Prior studies are ambiguous about the role of jasmonates in the interaction of *F. oxysporum* and Arabidopsis. Because conventional wisdom holds that jasmonate

signaling controls necrotrophic pathogens, and *F. oxysporum* is regarded as necrotrophic, jasmonate signaling might be expected to contribute to resistance (Laluka and Mengistea 2010). Rather, prior studies contain evidence that jasmonate signaling promotes either resistance (Aboul-Soud et al. 2004; Berrocal-Lobo and Molina 2004; Epple et al. 1997; McGrath et al. 2005) or susceptibility to *F. oxysporum* (Thatcher et al. 2009; Trusov et al. 2009). Confusion arises from conflating observations from different Fusarium-instigated disease syndromes, namely foliar rot and root vascular infection (Berrocal-Lobo and Molina 2008; Thatcher et al. 2009; Tierens et al., 2001; Trusov et al., 2009). Under favorable conditions, *F. oxysporum* infects and rots leaves or whole seedlings; however, this post-harvest disease, unlike root vascular infection, lacks pathogen specificity because *formae speciales* from both crucifers and non-crucifers are equally aggressive with Arabidopsis (Berrocal-Lobo and Molina 2004). Furthermore, natural quantitative variation in susceptibility to foliar rot among Arabidopsis accessions lacks correlation with susceptibility to root vascular infection (Diener and Ausubel 2005; Llorente et al. 2005). The two disease syndromes affect host genotypes differently because *F. oxysporum* elaborates distinct virulence strategies depending on the type of infection. Thus, *COI1* is critical for, on the one hand, resistance to foliar rot disease and, on the other hand, susceptibility to wilt disease.

Some but not all *formae speciales* produced biologically relevant amounts of jasmonates. Miersch et al. (1999) detected 22 JA-related compounds, including JA and JA-Ile, in culture filtrate of FOM. We quantified JA and JA-Ile in culture filtrates of FOM and several Fusarium pathogens that, to our knowledge, were not previously examined.

In particular, we found that two crucifer-infecting *formae speciales* (FOC and FOM) and the tulip pathogen (FOT) similarly produced both JA and JA-Ile. However, in contrast to Miersch et al. (1999), who measured 20-fold more JA (492 ng/ml) than JA-Ile (25 ng/ml), we detected 6-fold less JA than JA-Ile in the filtrates of FOM as well as less JA than JA-Ile in FOC and FOT. The discrepancy between the two studies may be attributed to differences in the way in which FOM cultures were grown or intrinsic differences in FOM strains as we detected the accumulation of JA and not JA-Ile in the culture filtrates of FOR. Our *THI2.1*p:*uidA* bioassays only detected jasmonate activity in filtrates that contained the COI1 ligand (JA-Ile) or in leaves and meristems that were colonized by JA-Ile-producing *formae speciales* but not in seedlings colonized by FOR, which suggests that FOR does not produce an alternative active COI1 ligand that would be missed by the specific MRM assays used here for JA and JA-Ile. The *in vitro* differences that distinguish FOC and FOM from FOR correlate with differences in virulence strategy as FOR infection proved to be less dependent on jasmonate signaling to promote wilt disease.

Substantial quantities of JA-Leu also accumulated, which was unexpected because previous analysis of fungal cultures has only reported the identification of JA-Ile as a JA-amino acid conjugate. Although JA-Leu is a natural product of plants, in general, JA-Leu is reported to have equivalent or weaker hormone activity than JA-Ile when the two are compared in physiological tests, and JA-Ile typically gives stronger *in vitro* affinity to COI1-JAZ complexes than other amino acid conjugates. However,

JA-Ile, JA-Leu and JA-Val may produce different affinities in different COI1-JAZ complexes and thus produce distinct hormone responses (Katsir et al. 2008).

Fusarium-derived jasmonate promotes fungal growth in roots and symptom development in shoots through distinct root- and shoot-specific processes that depend on *COI1*. In roots, *COI1* promoted fungal colonization of the vascular cylinder as *coi1* rootstocks largely restricted FOC infection to root tips whether they were grafted to *coi1* or wild-type scions. Early in infected plants, before symptoms became evident, FOC infection extended from far fewer FOC-colonized *coi1* than wild-type root apices. Later, when wild-type growth was clearly stunted, substantially less FOC infection was measured in *coi1* than in wild-type roots. Absence of *COI1* was not an absolute barrier to vascular infection as some vascular colonization was seen in FOC infected *coi1* plants, and *coi1* roots remained susceptible to FOR infection. Thus, root-expressed *COI1* promoted the persistence of *F. oxysporum* in the vascular cylinder, which suggests that Fusarium-derived jasmonate acts as a critical virulence factor during the secondary determinative phase (Figure 3-14B). *COI1* might also promote, in the primary determinative phase, the colonization of root apices as fewer FOC-colonized *coi1* than wild-type root apices, especially LR primordia, were observed. Although Thatcher et al. (2009) conclude to the contrary that there is “no difference in the degree of fungal colonization of the wild-type and *coi1* plants until later stages of infection, when host necrosis [is] well developed”, our observations were in accord with their results. To explain the resistance of *coi1*, Thatcher et al. (2009) suggest that FOC produces a toxin (X in Figure 3-14A) requiring root-expressed *COI1* and that this toxin

subsequently affects at a distance symptoms such as chlorosis and necrosis in foliar tissues (Figure 3-14A). Inexplicably, their conclusion rests on the quantification of *F. oxysporum* DNA not in roots but in leaves, where little if any FOC would be expected until late in infection. We note that when leaves from FOC-infected plants were stained with X-ARA, we were unable to detect FOC in leaves and thus also observed no difference in FOC-infected *coi1* and wild-type leaves (S. Cole, *unpublished data*).

COI1 expression in the shoot also promoted virulence in the secondary determinative phase. As Thatcher et al. (2009) also observed, lack of *COI1* in scions delayed chlorosis and eventual death of FOC-infected wild-type rootstocks. Because we observed that enhanced resistance of *coi1* scions correlated with reduced FOC infection in wild-type rootstocks, we hypothesize that a *COI1*-dependent process in shoots also promotes colonization of roots. However, it remains unclear whether shoot-expressed *COI1* affects translocation of a factor (X in Figure 3-14B), such as auxin, from the shoot to roots or, rather, alters the transpiration of water, for example, from roots to shoot (Sun et al. 2009; Melotto et al. 2006).

The obvious effect of shoot-expressed *COI1* is in the expressive phase of infection. Transcript expression studies reveal an early and prominent role for jasmonate-induced gene expression in foliage of FOC-infected plants (Anderson et al. 2004; Kidd et al. 2011). Intuitively, others interpreted jasmonate-related expression as a response to endogenous jasmonate and presumed a positive role in host resistance (Anderson et al. 2004; Vignutelli et al. 1998). However, the fact that wilt disease, in all respects, was indistinguishable in *aos* and wild-type suggests that endogenous

jasmonate biosynthesis plays no role, either positive or negative. Instead, loss of jasmonate perception in *coi1* scions of FOC-infected plants (Thatcher et al. 2009) or FOR-infected *coi1* strongly curtailed specific wilt symptoms, such as darkening of leaves, epinastic growth in petioles, and stunting of rosette leaves. Chronic accumulation of jasmonates from repeated touching or injury of leaves similarly stunts Arabidopsis rosette leaves by a *COI1*-dependent process, and derepression of transcriptional regulators of anthocyanin (purple pigment) biosynthetic genes is *COI1*-dependent (Qi et al. 2011; Zhang and Turner 2008). We attribute these foliar symptoms to perception of Fusarium-derived jasmonate (Figure 3-14B), which presumably translocates via the xylem from root to shoot, because foliar symptoms were normal in FOC- or FOR-infected *aos*.

First and foremost *COI1* antagonized host immunity in the root vascular cylinder. Similar to the resistance conferred by recessive *coi1*, natural resistance genes, such as *RFO1*, *RFO2* and *RFO3*, restrict the colonization of the vascular cylinder by FOM (Diener 2012; Y. Shen, S. Cole and A. Diener, *unpublished data*). In fact, wild-type Col-0 was completely resistant to FOM, even as FOM produced jasmonate, and *COI1* could promote susceptibility to FOM in *rfo1*. Wild-type Col-0, in fact, expresses considerable, albeit partial, immunity to FOC and FOR as well, though a sufficient dose of FOC or FOR kills Col-0 plants (Diener and Ausubel 2005). For instance, FOC colonizes significantly more of the root system when just one resistance gene (*rfo1*) is removed (Diener 2012). Thus, it would appear that the relative strength of opposing

forces, *COI1*-dependent susceptibility and natural immunity, determine whether *F. oxysporum* will colonize roots in the secondary determinative phase.

Although there is precedent for jasmonate signaling suppressing SA signaling (Kunkel and Brooks 2002), Fusarium-derived jasmonate induced susceptibility independent of SA signaling. SA signaling can affect resistance to wilt disease because SA-related mutants are more susceptible to *F. oxysporum* (Diener and Ausubel 2005; Kidd et al. 2009; Trusov et al. 2009). However, previous genetic analysis shows that loss of SA accumulation in *nahG* or *eds5* has no apparent effect on the strong resistance of *coi1* (Kidd et al. 2009; Trusov et al. 2009). Similarly, we found that the strong wilt resistance of *coi1* was unperturbed by SA-related mutants *sid2* and *pad4*. Because reduced SA signaling in SA-related mutants exaggerates JA signaling (Kunkel and Brooks 2002), the enhanced susceptibility of SA-related mutants may in fact be a consequence of an exaggerated response to Fusarium-derived jasmonates, which brings into question whether SA signaling normally affects resistance in wild type. Indeed, *sid2* appeared no more susceptible to FOR than wild type.

Histological analysis has long associated resistance to Fusarium wilt with pectin-like gum deposition in xylem vessels and tylose formation in large vessels (Beckman 1987; Mace et al. 1981). In resistant plants, polysaccharide from and growth of neighboring parenchymal cells fill vessels that have arrested hyphae, whereas the same obstructions are described as incomplete in the vessels of susceptible plants. These static images leave an impression that host plants effect resistance by filling vessels with gum and that the rapidity of this response is the difference between

resistance and susceptibility (Beckman and Roberts 1995; Talboys 1972). However, while a pectic polymer may be an effective barrier to the passive movement of viruses and bacteria in xylem vessels, how it would it be a barrier to filamentous fungi, such as *F. oxysporum*, which express diverse polysaccharide-degrading enzymes and readily penetrate, for example, agar or cellophane, is unclear. On the contrary, deposition of polysaccharide may be beneficial for the growth of *F. oxysporum* in a nutrient-poor xylem sap.

Gum deposition and exudation, or gummosis, is a common response in plants to damage to or infection of xylem vessels (Nussinovitch 2009). Depending on the plant species, jasmonate and/or ethylene can induce gummosis (Saniewski et al. 2006; VanderMolen et. al. 1983). Gum exudation is also a symptom of specific diseases, including Fusarium bulb rot, which afflicts FOT-infected tulip bulbs (Saniewski et al. 2006). We showed that FOT can produce substantial quantities of both JA and JA-Ile/Leu in culture filtrates, and others show that MeJA-treatment of tulip bulbs phenocopies the gum exudation associated with FOT infection (Saniewski et al. 2004). Coincidentally, JA was first identified as a plant growth inhibitor in culture filtrates of *Lasiodiplodia theobromae* (Miersch et al. 1987), a fungal pathogen that is responsible for dieback diseases in peach and other fruit trees (Saniewski et al. 1998; Saniewski et al. 2006). A common symptom of Lasiodiplodia-instigated diseases is gum exudation, which MeJA-treatment of peach stems phenocopies (Saniewski et al. 1998). A molecular and genetic description of gummosis, which unfortunately is lacking, would be

useful for addressing whether jasmonate similarly promotes gum deposition in Fusarium-infected xylem.

In tomato, host perception of ethylene, and not jasmonate, is critical for wilt disease. (Lund et al. 1998; Talboys 1972). In our tests with jasmonate-insensitive tomatoes (*jai1*), we observed no difference in host response to FOL, and failed to detect either JA or JA-Ile/Leu in FOL filtrates. In contrast, the ethylene-insensitive tomato mutant *Never ripe* (*Nr*) exhibits remarkably strong resistance to Fusarium wilt disease (Lund et al. 1998). A number of wilt disease symptoms in tomato are attributed to ethylene, such as petiolar epinasty, foliar abscission, and chlorosis and necrosis (Mace et al. 1981), and ethylene evolves from infected susceptible but not resistant tomato plants (Gentile and Matta 1975). Considering the evidence that ethylene mediates occlusion of xylem vessels in response to pathogen elicitors, Lund et al. (1998) cited the loss of gum deposition in infected vessels as a possible explanation for strong wilt tolerance in FOL-infected *Nr* (VanderMolen et al. 1983). Interestingly, *Nr* has no appreciable effect on Fusarium crown and root rot, instigated by *F. oxysporum* f. sp. *radicis-lycopersici* (FORL), whereas *jai1* is more susceptible to FORL (Kavroulakis et al. 2007). The critical contribution of hormones to pathogen virulence and not host resistance would explain these contradictory effects of ethylene and jasmonate in distinct tomato diseases that are caused by isolates of the same *F. oxysporum* species complex.

Acknowledgments

S. C. was supported by a NIH graduate training program in genetic mechanism at UCLA. A. D. is grateful to the Dean of Life Sciences, University of California, Los Angeles for financial support.

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Table 3-1. JA, JA-Ile and JA-Leu in axenic cultures of pathogenic *Fusarium*

Strain	[JA][a]	[JA-Ile]	[JA-Leu]
FOM	120.2 ± 29.2[b]	738.9±160.1	413.3±200.4
FOC	10.4±3.4	120.6±27.1	74.4±58.7
FOR	8.7±3.8	nd[d]	Nd
FOT	200.8±76.9	6503.5±1000	5835.4±3424
FOL	nd[c]	Nd	Nd
FG[e]	Nd	Nd	Nd

[a] concentration in units of pmol/ml

[b] the range (±) of values is the standard error of the mean (n = 3; α = 0.05)

[c] not detected: Limit of detection of JA was 4.8 pmol/ml.

[d] not detected: Limit of detection of JA-Ile/Leu was 6.19 pmol/ml.

[e] *Fusarium graminearum*

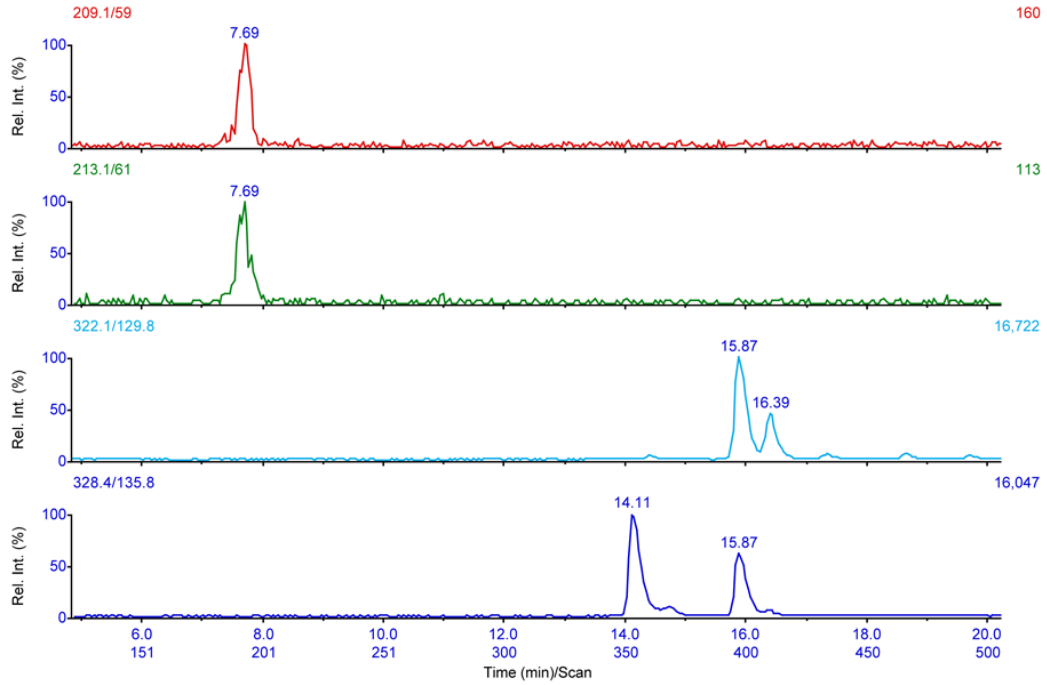
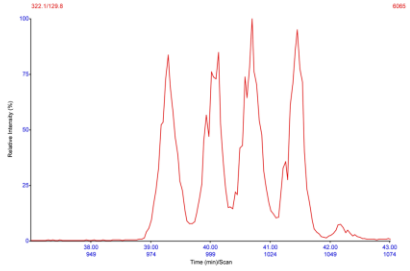
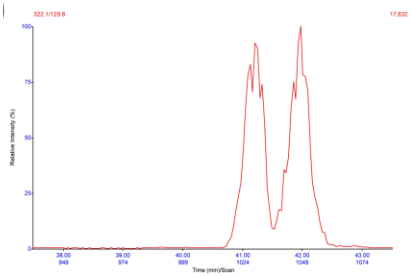


Figure 3-1. Jasmonates (JA and JA-Ile) in extracted axenic culture of FOM separated by reverse phase HPLC and detected by on-line tandem mass spectrometry.
FOM-derived JA was detected as a single peak (m/z 209➔59 transition) that coeluted with $^{2}H_4$ JA (m/z 213➔61 transition) at 7.69 minutes. FOM-derived JA-Ile was detected as a single peak (m/z 322➔130 transition) at 15.87 minutes, with almost base-line separation from a closely eluting component at retention time 16.39 min assigned as JA-Leu (see Figure 3-2). $^{13}C_6$ labeled JA-Ile (m/z 328➔136 transition) eluted as a double peak at 14.11 and 15.87 minutes, the latter of which co-eluted with the peak assigned to FOM-derived JA-Ile.

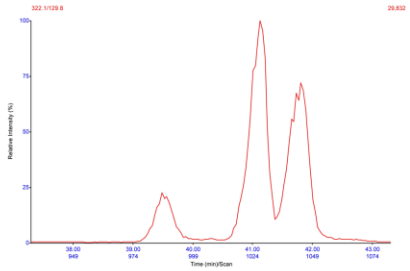
A


322.1/129.8
6065
Relative Intensity (%)
Time (min)/Scan

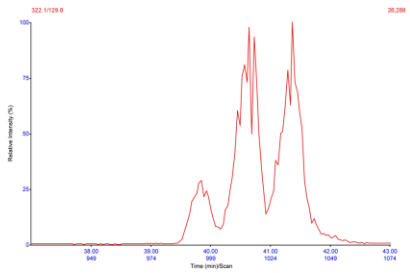
B


322.1/129.8
17,832
Relative Intensity (%)
Time (min)/Scan

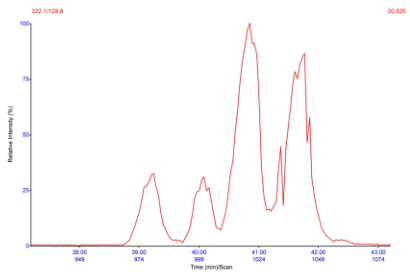
C


322.1/129.8
29,832
Relative Intensity (%)
Time (min)/Scan

D


322.1/129.8
26,288
Relative Intensity (%)
Time (min)/Scan

E


322.1/129.8
20,525
Relative Intensity (%)
Time (min)/Scan

F

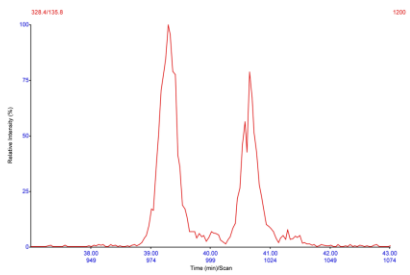

328.4/135.8
1200
Relative Intensity (%)
Time (min)/Scan

Figure 3-2. Cochromatography to verify the assignments of the JA-Ile JA-Leu peaks in FOX-derived samples.
JA- Leu has the same m/z transition (328➔130) as JA-Ile but eluted at a slightly longer retention time. (A) Equal amounts of unlabeled standards for JA-Ile and JA-Leu. (B) Extracted axenic FOX culture. (C) JA-Ile added to extracted axenic FOX culture. (D) JA-Leu added to extracted axenic FOX culture. (E) JA-Ile and JA-Leu added to extracted axenic FOX culture. (F) $^{13}C_6$ labeled internal standard of JA-Ile.

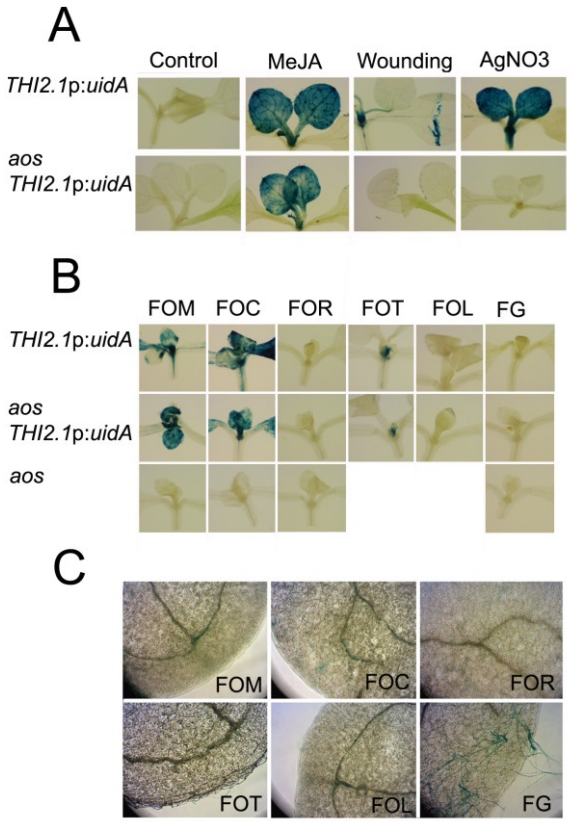


Figure 3-3. Fungal JA-Ile/Leu activates *THI2.1*p::*uidA* expression.
(A) X-Gluc staining of *THI2.1*p::*uidA* (top row) and *THI2.1*p::*uidA aos* (bottom row) seedlings three days after treatment with MeJA or silver nitrate or wounding. (B) X-Gluc staining of young leaves and meristems of *THI2.1*p::*uidA aos* (left), *THI2.1*p::*uidA* (middle), and *aos* (right) seedlings infected with FOM, FOC, FOR, FOT, FOL, and *F. graminearum*. (C) Cotyledon colonized by *F. graminearum* stained with X-ARA 3 dpi.

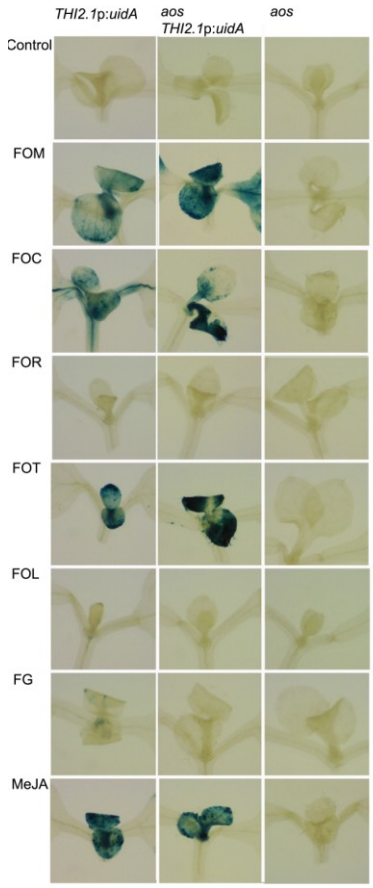


Figure 3-4. Fungal JA-Ile/JA-Leu from axenic culture activates *THI2.1p::uidA* expression.
X-Gluc staining of *THI2.1p::uidA*, *THI2.1p::uidA aos*, and *aos* seedlings 3 days after treatment with axenic cultures of FOM, FOC, FOR, FOT, FOL, *F. graminearum,* and MeJA.

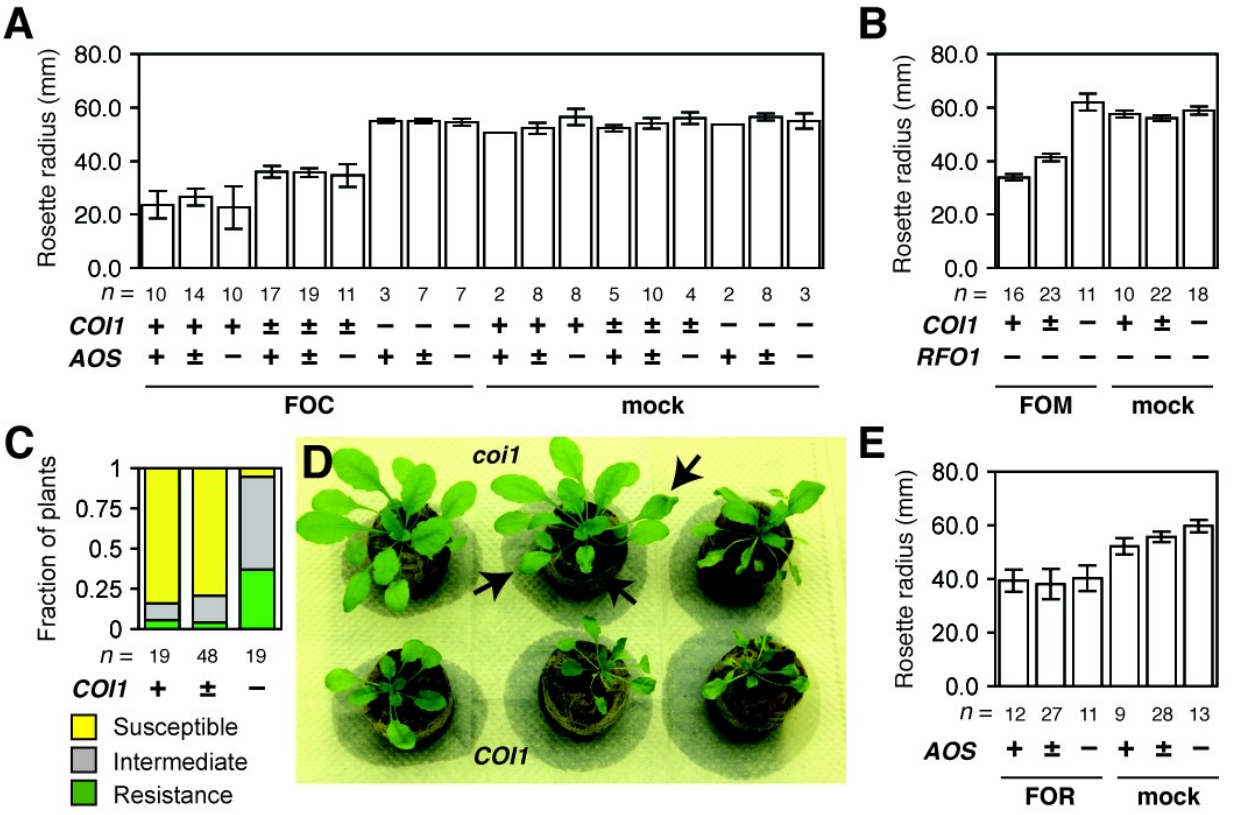


Figure 3-5. Perception of exogenous jasmonates promotes Fusarium wilt. Rosette radius was measured as average length along the midribs of three rosette leaves separated by approximately 120˚. Relevant genotypes were either homozygous wild type (+), heterozygous (±) or homozygous mutant (–). (A) Progeny of *COI1*/*coi1* *AOS*/*aos* dihybrid at 20 dpi with FOC or mock-infected. (B) Progeny of *COI1*/*coi1* *rfo1*/*rfo1* double mutant 20 dpi with FOM or mock-infected. (E) Progeny of *AOS*/*aos* at 20 dpi with FOR or mock infected. Values are the mean of *n* plants, and error bars are standard error (a = 0.05). (C) Wilt disease in *COI1*/*coi1* progeny was scored at 20 dpi using an ordinal health index (HI) (Diener and Ausubel 2005). Plants with 0 ≤ HI < 2 are susceptible, 2 ≤ HI ≤ 3 have intermediate resistance and 3 < HI ≤ 5 are resistant. All mock-infected plants (not shown) were resistant (HI = 5). (D) Foliar symptoms, stunting, epinasty and darkening (anthocyanin accumulation) of leaves, in FOR-infected wild-type plants (bottom row) are suppressed in FOR-infected *coi1* plants (top row). Arrows point to wilting leaves on *coi1* plants in watered soil.

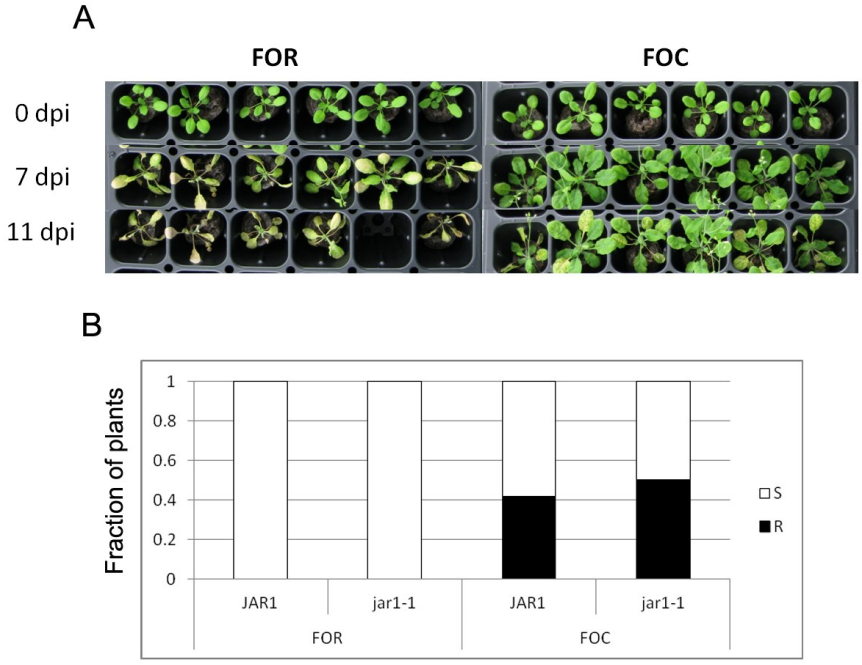


Figure 3-6. Conjugating Ile to JA does not promote Fusarium wilt.
(A) Representatives of *JAR1* and *jar1-1* infected plants with FOR and FOC. (B) Scoring of disease symptoms. In the graph, plants with susceptible phenotype are scored ($0 \leq HI < 2$), plants with resistant phenotype are scored ($3 < HI \leq 5$).

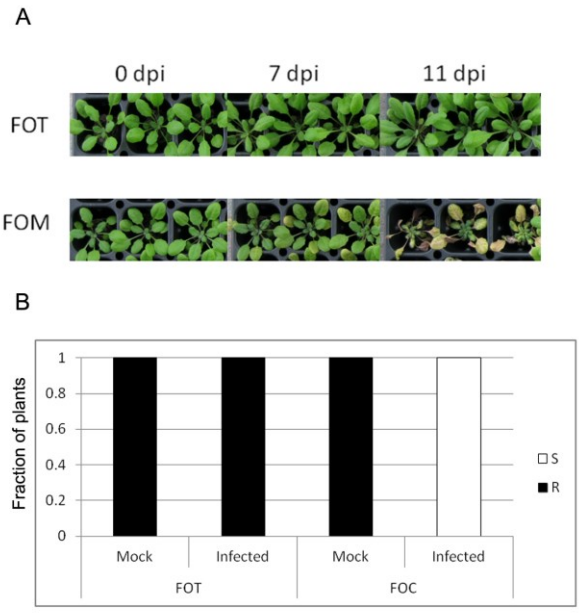


Figure 3-7. Jasmonates, JA-Ile and JA-Leu, do not promote infection of Arabidopsis by non cruciferous isolate FOT.
Representatives of FOT and FOM infected Sg-1 plants. (B) Scoring of disease symptoms. In the graph, plants with susceptible phenotype are scored ($0 \leq HI < 2$), plants with resistant phenotype are scored ($3 < HI \leq 5$). Plants were infected with $5X10^7$ conidia mL^{-1} of FOT and FOM.

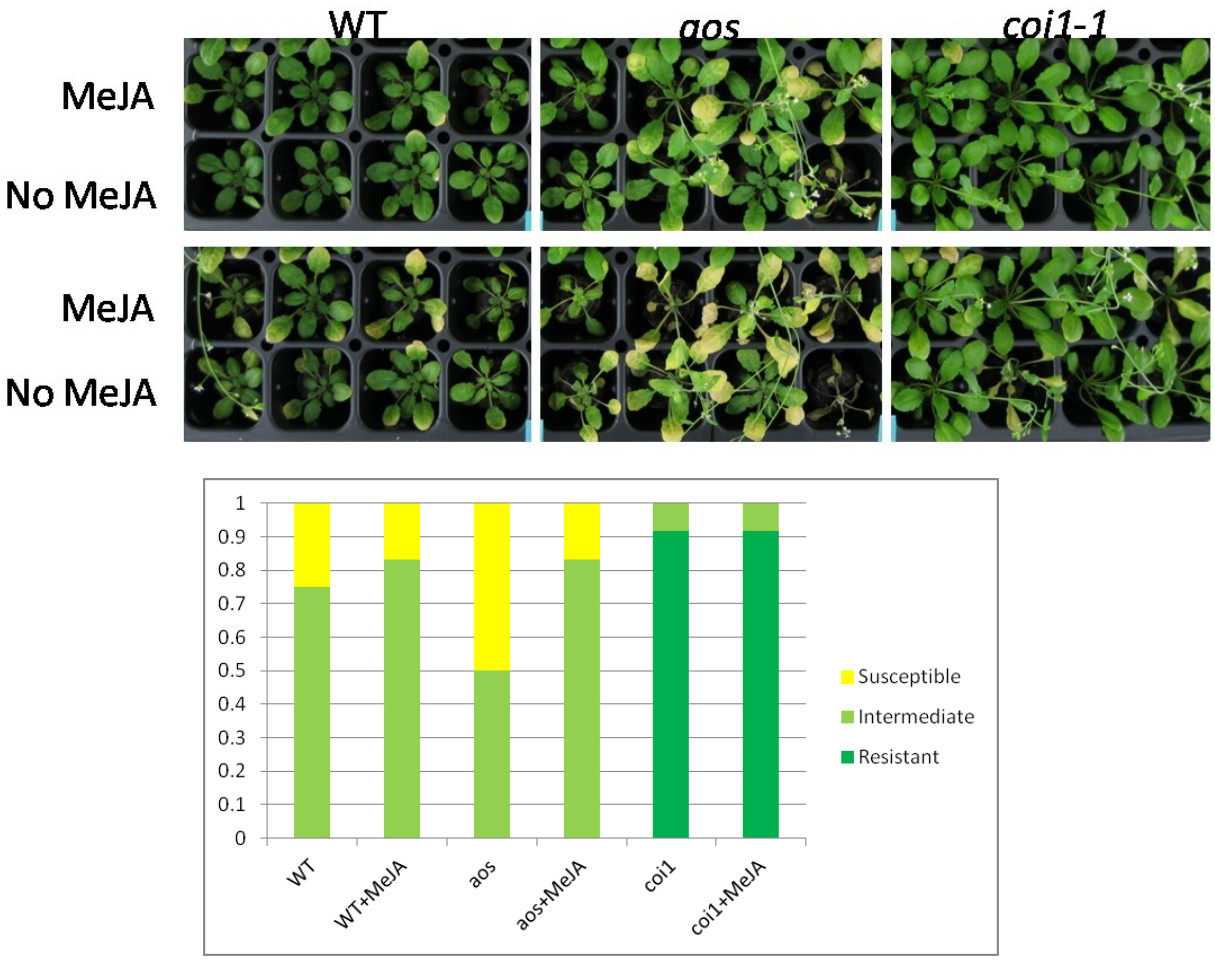


Figure 3-8. Addition of MeJA at the time of infection does not increase susceptibility.
(A) Representatives of MeJA treated and FOR infected plants taken at 0 days and 14 days post inoculation. (B) Disease symptoms were scored using health index (HI). The graph shows fraction of plants that were susceptible (0 ≤ HI < 2, had intermediate resistance (2 ≤ HI ≤ 3) or were resistant (3 < HI ≤ 5) at 21 dpi.

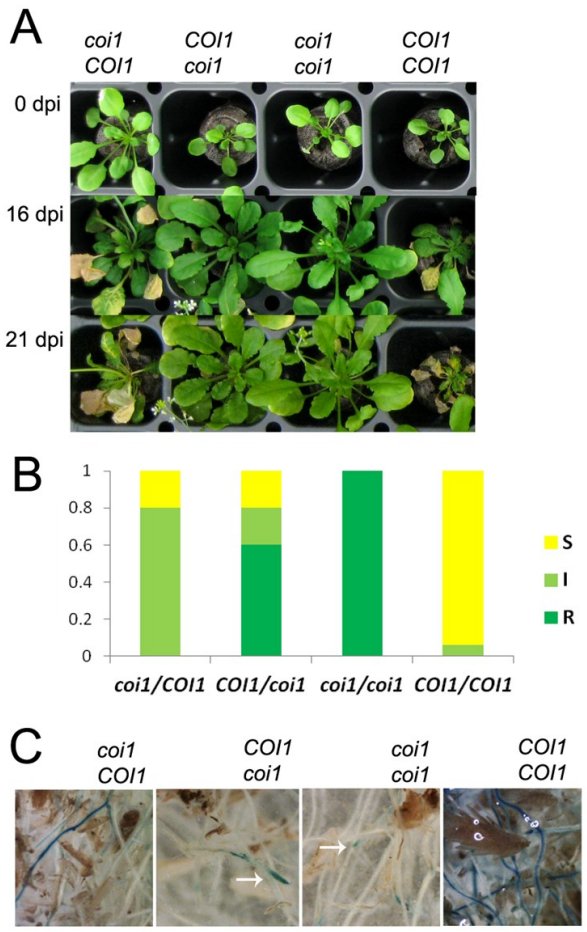


Figure 3-9. Infection of grafted plants with wild-type and *coi1* rootstocks and scion.
(A) Representative grafted plants at 0, 13, and 21 dpi are shown. Genotypes, wild type (WT) or *coi1*, of scion (top) and rootstock (bottom) are given above photographs. (B) Disease symptoms were scored using the HI. The graph shows fraction of *n* grafted plants that were susceptible ($0 \leq HI < 2$, had intermediate resistance ($2 \leq HI \leq 3$) or were resistant ($3 < HI \leq 5$) at 21 dpi. (C) Representative X-ARA staining of FOC infection in grafted roots at 21 dpi. Staining in *coi1* rootstocks was restricted to root tips (arrows).

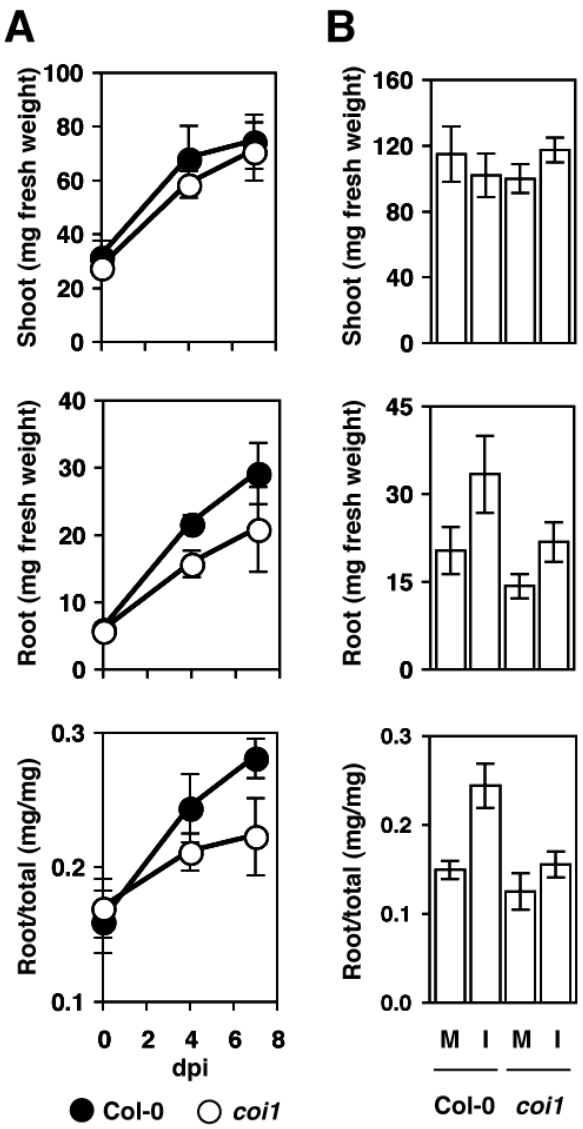


Figure 3-10. FOC infection promoted root growth.
Mean fresh weights (in milligrams) of shoots (top) and roots (middle) of plants in water-saturated soil. Proportional fresh weight of roots (root mass/total mass) is also presented (bottom). (A) Fresh weights of FOC-infected plants were measured at 0 dpi (*n* = 3, for each genotype), 4 dpi (*n* = 4) and 7 dpi (*n* = 5). (B) Fresh weights were measured at 7 dpi for mock-infected (M) wild-type Col-0 (*n* = 4) and *coi1* (*n* = 4) and FOC-infected (I) wild type (*n* = 6) and *coi1* (*n* = 6). Error bars are the standard error of the mean (α = 0.05).

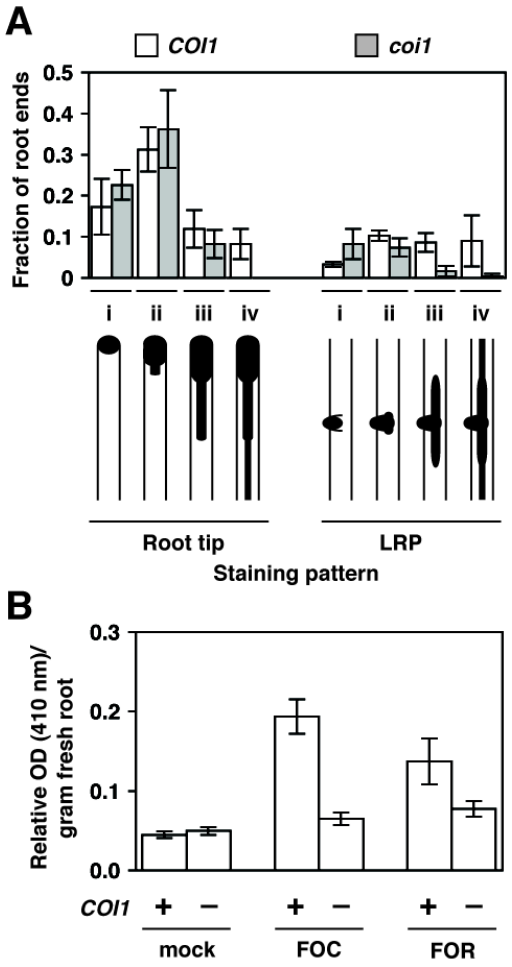


Figure 3-11. *COI1* promotes the fungal colonization of root vascular cylinder. Categories for common X-ARA staining patterns at root apices, meristematic root tips and LR primordia (LRPs), of FOC-infected wild-type (*COI1*) and *coi1* at 5 dpi: (i) partial staining of undifferentiated apices, (ii) staining throughout undifferentiated apices, (iii) broad vascular staining extending for short distances from apices and (iv) fine, extensive vascular staining away from root apices. (A) Fraction of root apices in each category was quantified for three FOC-infected wild-type or *coi1* whole root systems at 4 to 5 dpi. Error bars are the standard error of the mean (α = 0.05). (A) FOC-infected wild-type (COI1) and coi1 roots were stained with X-ARA at 10 dpi. (B) Quantification of FOC or FOR infections (+) of *COI1* (*n* = 4) and *coi1* (*n* = 4) at 11 dpi and infection of *rfo1* (*n* = 4) and *rfo1 coi1* (*n* =4) at 9 dpi using NP-ARA. For all mock-infected roots, *n* = 3. Relative Fusarium-derived ARA activity is expressed as absorbance of 4-nitrophenol product (OD_{410nm})/gram fresh weight roots, where values of mock-infected wild-type roots are set at 1.0. Error bars are the standard error of the mean (α = 0.05).

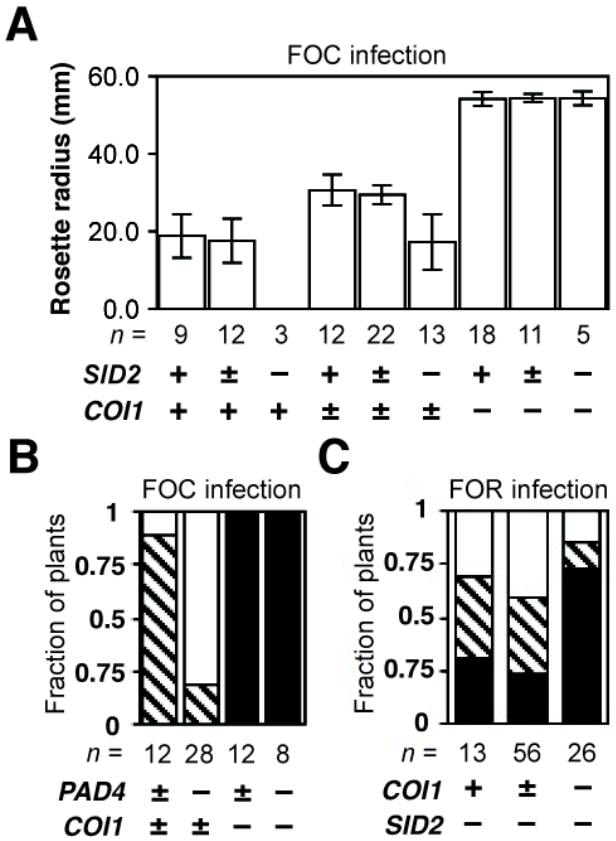


Figure 3-12. *COI1*-dependent susceptibility is independent of SA.
Rosette radius was measured 20 dpi. Relevant genotypes were either homozygous wild type (+), heterozygous (±) or homozygous mutant (–). (A) Progeny of *COI1*/*coi1 SID2*/*sid2* dihybrid were infected with FOC or mock-infected. Number (*n*) of plants with relevant genotype is given below columns, and error bars represent standard error (α = 0.05). Wilt disease was evaluated using a health index 20 dpi, and index scores ranged from 0 (dead) to 5 (unaffected). Scores from 0 to 1.5 are susceptible, from 2 to 3 have intermediate resistance and from 3.5 to 5 are resistant. All mock-infected plants (not shown) were resistant. Fraction of progeny from *COI1*/*coi1 PAD4*/*pad4* dihybrid infected with FOC (B), progeny from *COI1*/*coi1 SID2*/*sid2* infected with FOR (D) or wild type (*SID2*) and *sid2* infected with FOR (C) that were susceptible (open column), resistant (filled column) or had intermediate resistance (hatched column).

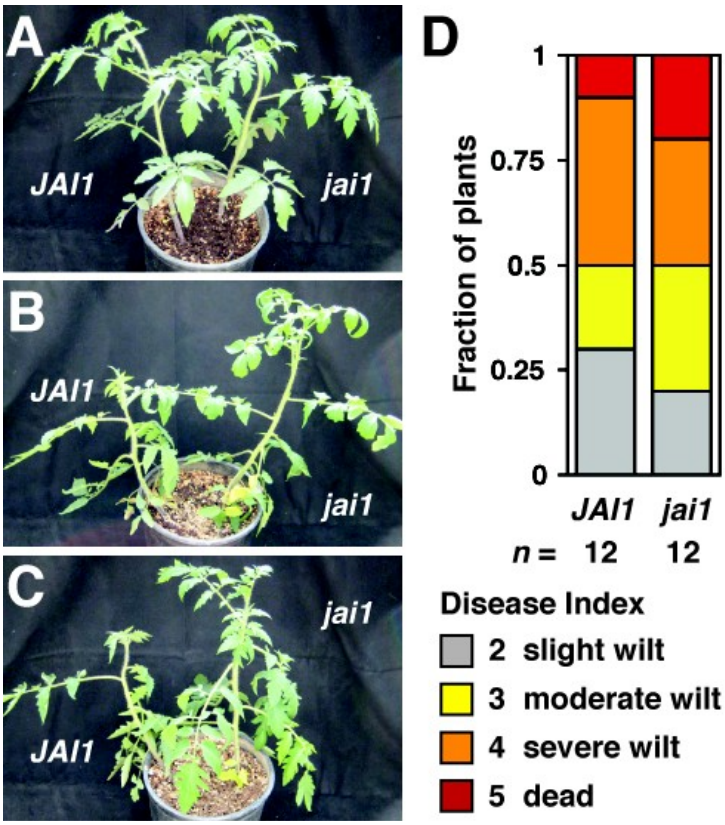


Figure 3-13. Fusarium wilt in tomato *jai1*.
Representative mock-infected (A), or FOL-infected (B and C), wild-type (*JAI1*) and jasmonate insensitive *jai1* tomato plants are shown at 21 dpi. Lower leaves of FOL-infected plants exhibit epinasty and premature senescence when compared to uninfected plants. (D) Symptoms of FOL-infected *JAI1* (*n* = 12) and *jai1* (*n* = 12) plants were scored 35 days after infection using a disease index, from healthy (0), slightly wilted (1), moderately wilted (2), severely wilted (4) to dead (5). Mock-infected *JAI1* (*n* = 6) and *jai1* (*n* = 6) exhibited no wilt symptoms.

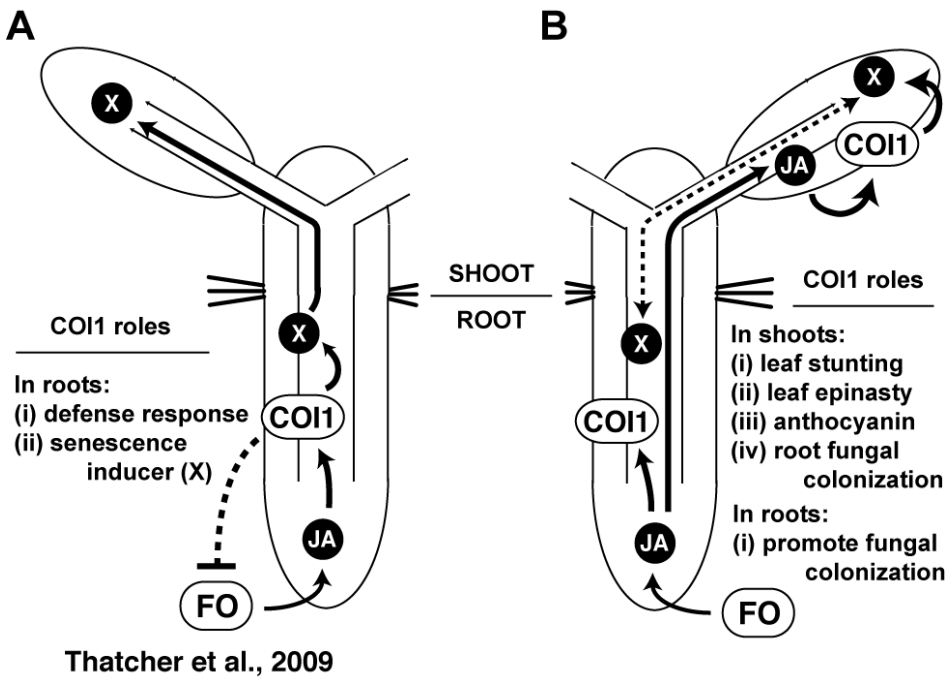


Figure 3-14. Models for *COI1*-mediated susceptibility to *F. oxysporum*.
(A) Thatcher et al. (2009) proposed two functions for root-expressed COI1: Jasmonates, including a Fusarium-derived jasmonate-like activity, activate defense responses (i) and induce senescence-inducing toxin (X) in roots that translocates to shoots where X promotes symptoms (ii). A dashed line is shown for defense responses as no evidence has shown that jasmonate signaling in fact suppresses root infection during wilt disease. (B) Our results indicate that Fusarium-derived jasmonates promote susceptibility through three COI1-mediated events: Shoot-expressed COI1 promotes (1) specific symptoms, stunting (i), epinasty (ii) and darkening (iii) of leaves in the expressive phase and (2) colonization of roots (iv) in one or both determinative phase(s). X represents a factor that mediates the effect of shoot-expressed COI1 on fungal colonization of roots, and a dashed line indicates that the direction of this factor is unknown. Most importantly, (3) root-expressed COI1 promotes the colonization of the root vascular system in the secondary determinative phase.

Chapter 4

Summary

Soil-borne pathogens are difficult to treat and control compared to foliar pathogens. Current treatments for soil-borne pathogens include fumigation, biological control strains of microbes, cultural controls, and integration of resistance genes from resistant cultivars.

In tree nurseries, seedling soil is commonly fumigated prior to transplanting to fields in order to decrease pathogenic microbes such as Fusarium species. Hansen et al (1990) described seedlings grown in soil fumigated with methyl bromide-chloropicrin or dazomet grew larger than seedlings in unfumigated soil. Fumigants are effective against pathogens but many fumigants pose a threat to human health and or the environment. For example, methyl bromide was a typical fumigant used to control pests in agriculture. However, in 1993 the Montreal Protocol identified methyl bromide as contributor to ozone depletion and was phased out by 2005 in developed countries and will be phased out in 2015 in developing countries (EPA, 2012). Not only is methyl bromide damaging to the environment, it's also highly toxic to humans and can cause toxicity to lungs and nervous system, which can lead to long term neurological problems or death (Barry et al, 2012). Other compounds that are less toxic have been tested and are used for restricting disease symptoms and increasing the number of symptomless plants. Botanical extracts from clove, pepper/mustard, and Cassia improved resistance to *F.oxysporum* f sp *melonis*, and f sp *chrysanthemi*. These botanical extracts were developed for foliar pathogens but are also affective for multiple *F. oxysporum* strains (Bowers & Locke, 2000).

In addition to fumigants, there are also cultural controls used to prevent the spread of soil-borne pathogens. These cultural controls include farming crops in soil without a history of disease, use of disease-free seeds, proper watering of crops, crop rotation, and use of cover crops. Proper watering is important because over watering leads to stress in plants and makes them more vulnerable to pathogens. Additionally, allowing soil to go fallow can reduce the amount of *F. oxyporum* because without roots in the soil, the fungus is deprived of nutrients (Scott et al, 2012). Furthermore, cover crops like some mustard species and brassica crops can release compounds that block pathogens or improve the growth of beneficial microbes (Koike et al, 2003).

One of the ways biological control strains of microbes are believed to prevent disease is by blocking sites of infection on the root surface. Also, nonpathogenic strains of Fusarium have been shown to out compete pathogenic strains for nutrients such as carbon. Nonpathogenic strains of Fusarium can also induce a defense response in plants that then reduce infection of pathogenic strains of FOX (Fravel et al, 2003).

Integration of dominant resistance genes is one of the most environmentally safe and effective methods for controlling soil-borne diseases. These dominant genes are relatively easy to breed into desirable crops. However, such genes only provide temporary resistance because pathogens are constantly evolving to evade the resistance provided by these genes. Once a pathogen has overcome the resistance gene that gene is no longer effective in stopping that specific pathogen. In natural plant populations, plants are also under pressure to still recognize pathogens and mount a defense response. This interaction between pathogen factors and resistance gene

products was described as the arms race by Jones & Dangl (2006). This problem is currently seen with banana where *F. oxysporum* f sp *cubense* race 4 infects Cavendish bananas and is no other cultivar of banana exists that is currently both resistant to the new race of *F. oxysporum* and has other desirable food related phenotypes of Cavendish bananas (Ploetz, 2000). The use of multiple genes like those identified in quantitative trait loci analysis could be a more effective method to control resistance to a pathogen because even if the pathogen overcomes one of the resistance genes, there are other resistance loci against that pathogen. Furthermore, some of these genes could recognizes MAMPs and provide a broad range of resistance against a group of pathogens instead of strain specific resistance.

In Chapter 2, we identified *RESISTANCE TO FUSARIUM OXYSPORUM 3 (RFO3).* This is the third resistance gene that our lab has cloned. The first was *RFO1* (Diener & Ausubel, 2005) followed by *RFO2* (Shen & Diener, unpublished data). *RFO1* is a wall-associated kinase-like kinase and *RFO2* is a receptor-like protein with an extracellular leucine-rich repeat domain and a short intracellular C terminus. *RFO3* belongs to the S domain 1 (SD1) family of receptor-like kinases. Other members of SD1 family have been shown to be induced by pathogens but *RFO3* is the first member shown to provide resistance. The most well characterized member of this family is *SRK1* in Brassica, which is involved in self incompatibility. While *SRK1* is specifically expressed in the stigma, *RFO3* is expressed in vegetative tissues. It was hypothesized that members of the SD1 family that were expressed in vegetative tissues are involved in development and defense responses (Sanabria et al, 2008). *RFO3* is unlike a typical

pattern recognition receptor (PRR) because it recognizes *F. oxysporum* f sp *matthiolae* (FOM) specifically and not related strains *F. oxysporum* f sp *conglutinans* (FOC) or *F. oxyspoum* f sp *raphani* (FOR) that also infect Arabidopsis. We also showed that the resistance by *RFO3* was root-specific. Furthermore, roots that were $RFO3^{C}$ were colonized significantly less than roots that were $RFO3^{T}$.

Arabidopsis is an excellent model plant system to work with for *F. oxysporum* in the lab. However, Arabidopsis is not a crop plant and therefore is not agriculturally important. In the future it would be interesting if the resistance genes that our lab has identified were transferred to other crops and were still capable of providing resistance to *F. oxysporum*. Out of the three genes cloned thus far the most useful would be *RFO1* as it provides broader range of resistance to *F. oxysporum* and *Verticillium longisporum* (Diener & Ausubel, 2005; Johansson et al, 2006). *RFO2* and *3* are specific to FOM and therefore least likely to be effective against multiple *F. oxysporum* isolates that affect agricultural crops. In addition to discovering if our work is translational to crop plants, we still do not understand the details of the microbe-associated molecular patterns (MAMPs) or ligands that these *RFO* genes recognize. Furthermore, while SD1 family members have been shown to interact with the plant U box family of E3 ligases, it is still unknown what the signaling mechanisms are for *RFO* genes.

In Chapter 3 we show that several strains of *F. oxysporum* produce jasmonates in axenic culture. The tulip pathogen *F. oxysporum* f sp *tuilpae* produced the largest quantity of jasmonates of all *F. oxysporum* strains tested. Jasmonic acid (JA) conjugated to leucine (JA-Leu) was also detected in *F. oxysporum* strains that produced

JA and JA conjugated to isoleucine (JA-Ile). This is the first time to our knowledge that JA-Leu was detected in a FOX culture. JA-Leu is active in JA signaling but is not as effective as JA-Ile (Staswick & Tiryaki, 2004). The exact role of JA-Leu is still unknown. We also made attempts to measure jasmonates produced in plant roots by the fungus but were unsuccessful. However, *F. oxysporum*-derived jasmonates were shown to be biologically relevant as seen with the activation of JA-responsive gene *THI2.1* in cotyledons and meristems of *F. oxysporum*-infected seedlings.

Other fungi have been shown to produce hormones including *Botrytis cinerea, Ustilago maytis,* and *Gibberella fujikuroi,* among many others. *B. cinerea* makes abscisic acid (ABA) and the first biosynthetic gene was identified as *bcaba1*, along with a possible gene cluster in the *B. cinerea* genome that would suggest a biosynthetic pathway for fungal-derived ABA (Siewers et al, 2004; Siewers et al, 2006). The plant growth hormone gibberellins (GAs) were first discovered in *G. fujikuroi*. Most of the steps and enzymes used in the biosynthetic pathways for GA production in fungi and Arabidopsis are known. And although the structures of GAs are the same in fungi and plants the enzymes in the pathway differ (Hedden et al, 2002). In *F. oxysporum*, the enzymes used in the biosynthetic pathway to produce jasmonates is yet unknown. However, Mierch et al. (1999) found several intermediates made by FOM that are intermediates in plants, which would suggest that fungal-derived JA in *F. oxysporum* is made in a similar fashion as in plants. With more *F. oxysporum* genomes being sequenced and assembled, including FOC race 1 that based on our data synthesizes jasmonates and FOL and F. graminearum, which we know do not produce jasmonates.

A comparison of these three genome sequences could allow for easier identification of potential genes in hormone biosynthetic and signaling pathways. Moreover, genes involved in secondary metabolism tend to cluster as in microbial genomes, e.g. GAs in *G. fujikuroi* and ABA in *B. cinerea*, which will hopefully aid in identifying those genes involved with jasmonate biosynthesis in *F. oxysporum*.

In Chapter 3, we also showed that *coi1* plants were more resistant to FOM and FOC infection. This resistant phenotype was not seen in *aos* or *jar1* plants. Interestingly while *coi1* mutants were resistant to FOC and FOM infection, FOR infection of *coi1* mutants were initially resistant but after a period of time they still succumbed to infection. In addition to the resistant phenotype, *coi1* roots were not colonized as well as *COI1* roots. Therefore, we hypothesize that jasmonates produced by *F. oxysporum* are used to aid in the colonization of roots. The mechanism by which fungal-derived JA makes plants more susceptible to *F. oxysporum* infection is not understood. Studies have shown that MeJA affects the localization of auxin transporters, PIN1 and PIN2 (Sun et al, 2011). This could mean that somehow *F. oxypsorum* produced jasmonates may affect auxin. However, using infected and mock-inoculated *PIN1::GFP* roots, it was unclear if *F. oxypsorum*-derived jasmonates affected PIN1 localization (Cole, unpublished data). Jasmonates also affect gel formation or gummosis (Skrzypek et al, 2005). Gels are ways to block pathogen progession through roots. However, gels could also be helpful to this pathogen if the pathogen can break down the gels and use them as an energy source. However, staining for pectin with the fluorescent stain

Coriphosphine O did not give clear result between infected and uninfected roots. Therefore, it is unclear if *F. oxysporum* derived JA is used for gel formation.

The study of soil-borne plant disease is important because they cause significant crop damage every year. According to the National Cotton Council of America, the average annual cotton yield loss between 1952-2002 due to cotton seedling diseases caused by *Rhizoctonia solani, Pythium spp., Fusarium spp.* was 2.85%. And between 1992 and 1998 approximately 4.5 million bales amounting to 1.5 billion dollars was lost due to cotton seedling diseases. Due to movement of crops, seeds, and agriculture equipment it is easier to spread soil-borne pathogens to new uninfested areas than ever before. Moreover, as the world population grows, we will need to feed more people and reducing crop yield loss caused by pathogens like *F. oxysporum* is important. More research is needed to understand how pathogens particularly soil-borne pathogens influence plant hosts in order to increase colonization and induce disease. Additionally, from the plants' perspective, we need to find methods to control resistance in the plant to reduce or even prevent disease.

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