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Identifying and Characterizing Arabidopsis Jasmonate Signaling Components Manipulated by Pseudomonas Syringae

Neva Laurie-Berry
Washington University in St. Louis

WASHINGTON UNIVERSITY

Division of Biology and Biomedical Sciences

(Molecular Genetics)

Dissertation Examination Committee:
Barbara Kunkel, Chair
Barak Cohen
Tuan-hua Ho
Eric Richards
Craig Pikaard
James Skeath

IDENTIFYING AND CHARACTERIZING ARABIDOPSIS JASMONATE SIGNALING COMPONENTS MANIPULATED BY *PSEUDOMONAS SYRINGAE* *DC3000*

by

Neva Laurie-Berry

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ABSTRACT OF THE DISSERTATION

Identifying and characterizing Arabidopsis jasmonate signaling components manipulated by *Pseudomonas syringae* DC3000

by

Neva Laurie-Berry

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Jasmonate signaling is critical for susceptibility of *Arabidopsis thaliana* to the bacterial pathogen *Pseudomonas syringae*, as demonstrated by the reduced susceptibility of *coi1* mutant plants. The infection responses of another jasmonate-insensitive mutant, *jin1*, had previously been unknown. This work demonstrates that susceptibility to *P. syringae* requires activity of the transcription factor JIN1/MYC2. Reduced susceptibility correlates with reduced sensitivity to the *P. syringae* phytotoxin coronatine, a molecular mimic of the endogenous jasmonate JA-Ile.

The reduced susceptibility of *jin1* mutant plants correlates with increased salicylic acid (SA)-dependent signaling. Analysis of *jin1* plants carrying the *sid2* mutation, resulting in decreased SA synthesis during infection, reveals that the decreased bacterial growth in *jin1* mutants requires SA signaling, while the decrease in disease symptom development does not.

To identify additional jasmonate signaling mutants with altered responses to *P. syringae* infection, over 23,500 mutagenized seedlings were screened for altered sensitivity to coronatine. Seven mutants were identified. Four are less sensitive to coronatine and allelic to known jasmonate signaling mutants. Three exhibit enhanced sensitivity to coronatine. Two of these, *coh-23* and *coh-36*, were further characterized and have increased sensitivity to both coronatine and jasmonate.

The *coh-23* mutant plants have an uncertain response to *P. syringae* infection, exhibiting reduced susceptibility that may result from a second unlinked mutation.

The *coh-36* mutant plants are more susceptible to *P. syringae* infection. They support slightly higher levels of bacterial growth early in infection. Later in infection, they develop more severe symptoms than wild-type plants, despite supporting similar levels of bacteria. This supports the observation from the *jin1 sid2* double mutants that bacterial growth and disease symptom development can be separated.

The mutation in *coh-36* plants was mapped to a region of chromosome 2 not previously implicated in jasmonate signaling, strongly suggesting that the *coh-36* mutant represents a novel component in this pathway. A candidate gene has been identified. Tests are underway to determine whether its function is disrupted in *coh-36* mutants.

The gene affected by the *coh-36* mutation is proposed to be a negative regulator of a jasmonate signaling pathway required primarily for symptom development.

Chapter 1

Introduction

Plant pathology is an important, if sometimes overlooked, area of biological research. Plant pathogens and pests, ranging from viruses and bacteria to fungi, nematodes and insects, have a significant impact on agriculture and horticulture (Agrios 1997). Clearly, there is a strong incentive to understand the science underlying these interactions between plants and pathogens. Our research investigates this process using the interaction between the model plant *Arabidopsis thaliana* and the bacterial pathogen *Pseudomonas syringae*, so this introduction will focus primarily on these organisms.

General plant defenses

Most micro-organisms in the environment are unable to infect plants and produce disease, largely due to a system of innate constitutive and induced defenses present in plants. These defenses include physical barriers such as a thick waxy cuticle layer and reinforced cell walls (de Wit 2007; Grant *et al.* 2006; Chisholm *et al.* 2006), as well as defenses triggered by perception of microbe-associated molecular patterns (MAMPs). MAMPs are molecules common to many microbes, such as flagellin and chitin (de Wit 2007; Bent *et al.* 2007; Chisholm *et al.* 2006). Recognition of these molecules through pattern recognition receptors results in production of basal anti-microbial defenses. These basal defenses include closing of stomata to block pathogen entry (Melotto *et al.* 2006), production of reactive oxygen species to directly damage invading microbes, and activation of phospholipases that trigger production of defense signaling hormones (Ryan *et al.* 2007; Bent *et al.* 2007; Grant *et al.* 2006; Chisholm *et al.* 2006).

A successful pathogen must overcome these general defenses, as well as any defenses induced specifically upon recognition of the pathogen. Most induced defenses are mediated through the activity of one of two plant hormones: salicylic acid (SA) and jasmonate. Jasmonate-mediated defenses are primarily active against fungal and insect pathogens. This hormone signaling pathway will be discussed in more detail below. For most bacterial pathogens, such as *P. syringae*, induced defenses are mediated through the action of SA (Grant *et al.* 2006; Chisholm *et al.* 2006). SA-induced defense signaling can result in localized cell death known as the hypersensitive response (HR) (Nomura *et al.* 2005) and expression of a set of pathogenesis-related (PR) genes whose exact effects on pathogens are uncertain (Ryan *et al.* 2007).

Effects of *P. syringae* virulence factors

In order to both overcome these defenses and create a hospitable environment within the plant host, a pathogen relies on a variety of virulence factors that alter the host physiology. In the case of *P. syringae*, these virulence factors can be divided into two categories: effectors and toxins. Effectors are proteins injected into the host cell cytosol using the type three secretion system, a syringe-like structure produced by the bacteria (da Cunha *et al.* 2007; Nomura *et al.* 2005). *P. syringae* strains are classified into approximately fifty different pathovars based on host range (Nomura *et al.* 2005), and various strains are estimated to produce between 20 and 50 different effectors, which have a variety of effects on the host plant's physiology (Lindeberg *et al.* 2006). Some of the *P. syringae* effector proteins that have been best studied, such as AvrPto, act to

suppress the plant's basal defenses (de Wit 2007; Grant *et al.* 2006; Chisholm *et al.* 2006; Nomura *et al.* 2005). Another set of effectors, including AvrPtoB and AvrPph E, act to suppress SA-mediated defenses and the HR (Nomura *et al.* 2005). Some, like AvrRpt2, may suppress both basal and induced defenses (Nomura *et al.* 2005), as well as altering plant hormone physiology (Chen *et al.* 2007). Other effectors subvert various host processes, from ubiquitination to hormone signaling, to improve pathogen virulence (da Cunha *et al.* 2007; Bent *et al.* 2007; Grant *et al.* 2006; Chisholm *et al.* 2006). Many others have functions that are, as yet, unknown (da Cunha *et al.* 2007; Bent *et al.* 2007; Grant *et al.* 2006; Chisholm *et al.* 2006).

The primary toxin produced by several pathovars of *P. syringae* is coronatine (COR), a small molecule that is presumed to be secreted by the bacteria into the apoplastic space between the plant cells (Nomura *et al.* 2005). COR is synthesized by the bacteria as two separate moieties, (CFA) and (CMA), which are then ligated in the bacterial cell prior to secretion (Bender *et al.* 1999). Production of COR is important for *P. syringae* virulence, as demonstrated by the reduced ability of COR-deficient bacteria to successfully colonize host plants (Brooks *et al.* 2004; Mittal and Davis 1995; Cui *et al.* 2005). COR is required for suppression of SA-mediated defenses and production of disease symptoms (Brooks *et al.* 2005; Uppalapati *et al.* 2007).

The phytotoxin coronatine acts as a jasmonate mimic

Based on structural similarities, COR has long been hypothesized to serve as a mimic of jasmonates, a plant hormone family involved in development, response to

wounding, and defense against insects and many necrotrophic pathogens (Zhao *et al.* 2003; Cui *et al.* 2005; Uppalapati *et al.* 2005; Weiler *et al.* 1994). Additionally, exogenous application of COR and jasmonates have similar effects on plants, including inhibition of root growth, production of the stress pigment anthocyanin, and induction of various genes (Uppalapati *et al.* 2008; Thilmony *et al.* 2006).

Further evidence for the activity of COR as a jasmonate-mimic comes from studies of *P. syringae* infection of plants unable to respond to jasmonates. Plants lacking COI1/JAI1, an F-box protein that is hypothesized to serve as a jasmonate receptor (Katsir *et al.* 2008) in Arabidopsis and tomato, respectively, serve as very poor hosts for *P. syringae* infection (Kloek *et al.* 2001; Zhao *et al.* 2003; Feys *et al.* 2004; Nomura *et al.* 2005). Bacteria are unable to grow to high levels in these plants and fail to produce visible disease symptoms (Kloek *et al.* 2001; Zhao *et al.* 2003; Nomura *et al.* 2005), similar to COR-deficient bacteria infecting wild-type plants (Brooks *et al.* 2004; Brooks *et al.* 2005).

Jasmonate synthesis and signaling

Thus, in order to understand the activity of COR and its significance in pathogenesis, it is important to study jasmonate signaling and responses in the plant to identify processes and outcomes that are required for bacterial growth and disease development. Jasmonic acid (JA) is synthesized from linolenic acid through a series of biochemical conversions in the chloroplast and cytosol (Browse 2005). Several Arabidopsis mutants have been identified that block this process at various stages; these

include a triple mutant of *fatty acid desaturase* (*fad3, 7,* and *8*) genes unable to produce the trenoic acid precursor molecules (McConn and Browse 1996) and *opr3*, a mutation in the enzyme that produces JA from its immediate precursor 12-oxophytodienoic acid (OPDA;Stintzi and Browse 2000; Fig 1). All of these mutant plants are unable to produce JA and thus are deficient in all developmental processes requiring JA, including pollen formation (Browse 2005), as well as defense responses mediated by JA (McConn *et al.* 1997; Vijayan *et al.* 1998).

A large portion of the JA in the plant exists in the form of conjugates, such as methyl-JA (MeJA) and JA-amino acid conjugates (Staswick and Tiryaki 2004; Fig 1). The only JA-amino acid conjugate believed to have substantial activity is JA-Ile (Staswick and Tiryaki 2004), although the presumptive COI1 receptor complex is able to bind a few other JA-amino acid conjugates (Katsir *et al.* 2008). These conjugates are produced through the activity of a GH3 family member known as JAR1 (Staswick *et al.* 2002; Staswick and Tiryaki 2004), and plants carrying a mutation in this gene are impaired in a subset of jasmonate-dependent responses, most notably defense against several fungal pathogens (Staswick *et al.* 1998). This requirement for JAR1 function supports the hypothesis that JA-amino acid conjugates, particularly JA-Ile, are the primary active forms of JA (Katsir *et al.* 2008).

Mutational analysis has also provided some insight into the signaling pathway involved in jasmonate perception and response. The Arabidopsis *coronatine-insensitive 1* (*coi1*) mutation results in plants entirely unable to respond to coronatine or jasmonate (Feys *et al.* 1994), which are thus male-sterile (Feys *et al.* 1994) and highly susceptible to

several fungal pathogens and insect pests (Thomma *et al.* 1998; Reymond *et al.* 2004). This complete block in signaling agrees well with the recent evidence that the SCF complex containing COI1 acts as the primary jasmonate receptor (Katsir *et al.* 2008; Fig 1). The *jasmonate-insensitive 1* (*jin1*) mutation, in contrast, results in fully fertile plants impaired in only some aspects of jasmonate signaling (Berger *et al.* 1996). The JIN1/MYC2 transcription factor is believed to act downstream of COI1 to positively regulate root inhibition and expression of some jasmonate-responsive genes (Lorenzo *et al.* 2004; Boter *et al.* 2004; Fig 1).

More recently, a family of proteins have been identified as negative regulators of jasmonate signaling. These jasmonate ZIM-domain (JAZ) proteins are hypothesized to be targets of COI1-mediated ubiquitination (Fig 1), recognized only in the presence of an active jasmonate bound to COI1 (Chini *et al.* 2007; Thines *et al.* 2007; Katsir *et al.* 2008). In the absence of active jasmonates, JAZ proteins interact with JIN1, and likely additional transcriptional regulators involved in other aspects of jasmonate-mediated responses, and prevent the transcription factor from activating its target genes (Chini *et al.* 2007; Thines *et al.* 2007). According to this model, in the presence of a JA-amino acid conjugate COI1 binds to JAZ proteins, resulting in their ubiquitination and degradation, freeing JIN1 and other unknown transcription factors to rapidly initiate jasmonate-dependent responses (Chini *et al.* 2007; Thines *et al.* 2007; Chung *et al.* 2008; Fig 1).

There are 12 *JAZ* family members in Arabidopsis, and differences in expression patterns have been observed between various *JAZ* genes (Chung *et al.* 2008; Chini *et al.* 2007; Agnes Demianski, unpublished data). This, combined with the observation that

COI1 is capable of binding multiple forms of JA, suggests that the JAZ proteins may complex with COI1 in order to determine the specificity of a given jasmonate response. For example, JAZ3 binds to JIN1 (Chini *et al.* 2007), and JIN1 is proposed to mediate a subset of responses induced by JA-Ile (Laurie-Berry *et al.* 2006; Chapter 2). This hypothesis suggests that other JAZ family members may interact with transcription factors required for responses to other forms of JA, such as JA-Val or JA-Leu.

Various other mutants with altered jasmonate-related responses have been identified, but the specific role of most of these in jasmonate signaling is uncertain. Many of these were identified based on screening for abnormal expression of jasmonate-responsive genes. These include: *cev1*, which constitutively expresses the gene encoding vacuolar storage protein 1 (*VSP1*; Ellis and Turner 2001); several *cet* mutants that constitutively express *thionin* (Nibbe *et al.* 2002); and *cex1*, which has constitutive expression of both of these genes along with a jasmonate-responsive defensin gene called *PDF1.2* (Xu *et al.* 2001). This approach has proved less than ideal for identifying genes with specific effects in jasmonate signaling as both *cev1* and several of the *cet* mutants also constitutively express SA-mediated and other defenses, indicating that the mutations in these lines result in a more general activation of defense responses that is not specific to jasmonate signaling (Nibbe *et al.* 2002; Ellis *et al.* 2002).

Interactions between jasmonate signaling and other hormone responses

The various hormone signaling pathways in the plant interact with one another to form a network that allows the plant to respond appropriately to any given situation

(Glazebrook *et al.* 2003). Thus, a thorough examination of jasmonate signaling cannot be conducted without considering other hormones that interact with this pathway. Jasmonate signaling has interactions with several other pathways, some of which will be discussed here.

The interaction that has been best studied is that with SA signaling. As mentioned above, jasmonate and SA signaling pathways regulate the plant's two main defense responses, active against necrotrophic primarily fungal pathogens and biotrophic primarily bacterial pathogens, respectively (Delaney *et al.* 1994; De Vos *et al.* 2005). The primary interaction between these two signaling pathways is mutual inhibition (Spoel *et al.* 2003; Beckers and Spoel 2006; Thaler *et al.* 2002). This is a logical arrangement as it allows the plant to selectively activate the appropriate set of defenses for a given threat, while repressing responses that are irrelevant, or possibly detrimental, to the current situation (Spoel *et al.* 2007). This inhibition appears to be mediated, at least in part, at the level of hormone synthesis (Nickstadt *et al.* 2004; Spoel *et al.* 2003). The specific mechanisms by which this occurs have not been identified, but some genes, such as the transcription factor *WRKY70*, have been implicated in the process (Li *et al.* 2006). While mutual inhibition is the most typical interaction observed for jasmonate and SA signaling, the interplay between these two pathways is likely to be more complicated, depending on the timing of the hormone signals and the relative concentrations of each (Schenk *et al.* 2000; Beckers and Spoel 2006; Thaler *et al.* 2002; Mur *et al.* 2006).

Another hormone that has long been associated with jasmonate signaling is ethylene, a gaseous molecule involved in germination, fruit ripening, flower and leaf

senescence, and defense (Guo and Ecker 2004). Most anti-fungal defenses require coordinated activation of jasmonate and ethylene signaling, as resistance is lost in plants lacking genes critical to either pathway (Thomma *et al.* 1998; Thomma *et al.* 1999). Additionally, activation of several defense genes, including *PDF1.2*, requires signaling through both pathways (Lorenzo *et al.* 2003; Penninckx *et al.* 1998; Fig 1). The coordination of ethylene and jasmonate signaling is likely to involve the transcription factor *Ethylene Response Factor 1* (*ERF1*; Fig 1), although the exact role of this gene in the process is unclear (Lorenzo *et al.* 2003).

More recently, jasmonate signaling has been associated with abscisic acid (ABA), a hormone involved in seed germination, regulating stomatal aperture to control transpiration, and responses to abiotic stresses (Busk and Pages 1998). This connection was discovered primarily through the identification of the *jin1* mutation in the *AtMYC2* gene (Lorenzo *et al.* 2004). This transcription factor had previously been implicated in ABA signaling (Abe *et al.* 2003), and *jin1* mutants exhibit altered ABA signaling as well as jasmonate responses (Abe *et al.* 2003; Yadav *et al.* 2005). This impact of ABA on jasmonate signaling appears to antagonize the positive interactions between jasmonate and ethylene signaling in induction of defenses such as PDF1.2 (Anderson *et al.* 2004). ABA signaling is also required for jasmonate synthesis in response to infection by the fungal pathogen *Pythium irregulare* (Adie *et al.* 2007), suggesting that ABA may have a large impact on jasmonate signaling. Interestingly, MeJA has similar effects to ABA in guard cells (Munemasa *et al.* 2007), raising the possibility that interaction between these hormones may extend beyond pathogen infections.

Contributions of this thesis

This thesis presents my work on jasmonate signaling mutants, with a particular focus on how the various mutations alter the process of *P. syringae* growth and disease symptom production in the plant. Expression of marker genes is used to attempt to elucidate how each mutant is affecting jasmonate and SA signaling during the infection process, suggesting that *JIN1* and the gene defined by the novel *coh-36* mutant regulate both expression of jasmonate-dependent genes and inhibition of SA-mediated defenses.

In Chapter 2, I examine the role of *JIN1* in susceptibility to *P. syringae* by working with *jin1* mutant plants. This chapter shows that *jin1* plants are less susceptible to infection by *P. syringae* pv. *tomato* DC3000 and that this reduced susceptibility correlates with reduced sensitivity to COR, both in roots and leaves. The correlation between sensitivity to COR and susceptibility to DC3000 is also shown in other jasmonate mutants, including *coi1*, *jar1*, and *axr1*. I examine gene expression in *jin1* plants during infection, demonstrating that expression of some jasmonate-responsive genes is decreased in these mutants, while expression of a jasmonate- and ethylene-responsive gene and of a SA-responsive gene are elevated. The role of these elevated SA-mediated defenses is examined by studying *jin1 sid2* double mutants. The phenotypes of these plants reveal that the reduced bacterial growth observed in *jin1* mutants is dependent upon elevated SA, while the reduction in visible disease symptoms is not. I used these data to develop an integrated model for jasmonate and salicylic acid signaling and interactions during DC3000 infection. This work was published as "The *Arabidopsis thaliana JASMONATE INSENSITIVE 1* gene is required for suppression of salicylic acid-

dependent defenses during infection by *Pseudomonas syringae*" by Laurie-Berry *et al.* 2006 in Molecular Plant-Microbe Interactions, 19: 789-800.

Chapter 3 includes work done to extend this work to include additional jasmonate signaling mutants. Various mutants and transgenic lines with altered expression of jasmonate signaling and synthesis were examined with respect to jasmonate sensitivity and/or DC3000 infection. The majority of the mutants tested were determined to have no effect on these phenotypes. Because the existing mutants provided little insight into jasmonate signaling during the DC3000 infection process, I conducted a screen for novel mutants with abnormal sensitivity to COR. The screen process is described in this chapter, along with the initial results. Two novel mutants with increased sensitivity to coronatine, called *coh-23* and *coh-36*, are described, along with their preliminary characterization. One of these mutants, *coh-23*, was back-crossed once and then further characterized with respect to infection and jasmonate sensitivity. Some analysis of these results is discussed, and a possible model is presented for how the gene affected by the *coh-23* mutation may impact jasmonate signaling.

Chapter 4 consists of more detailed characterization of the *coh-36* hypersensitive mutant. Following two back crosses, *coh-36* mutants exhibit increased sensitivity to MeJA treatment and increased susceptibility to DC3000, consistent with the hypothesis developed in Chapter 2. An examination of gene expression is presented, with results suggesting that the coh-36 mutation impacts both jasmonate-induced genes and markers of SA-mediated defense. The gene affected in the *coh-36* mutant is mapped to a defined interval on chromosome 2, a region that has not previously been implicated in jasmonate

signaling. Preliminary data is presented on a gene in this region that is a possible candidate for the affected gene.

In Chapter 5, I discuss the significant results from the previous three chapters and their implications to our understanding of the role of jasmonate signaling in Arabidopsis during infection by *P. syringae*, as well as presenting some of the questions that remain to be answered. I also include a description of future experiments to better understand both the *coh-23* and *coh-36* mutants.

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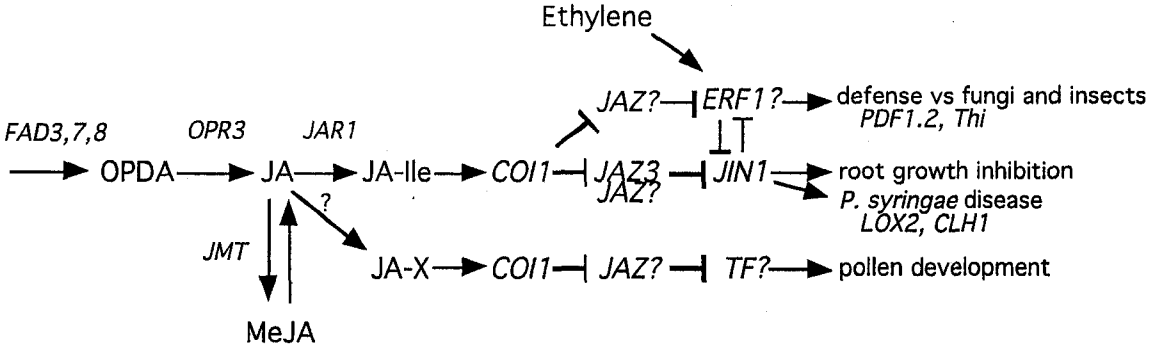
Figure 1

A current model for the jasmonate signaling pathway.

Synthesis of JA occurs through a series of precursor molecules derived from fatty acids. *FAD3, 7*, and *8* are involved in an early step in this process, resulting in the precursor linolenic acid (Browse *et al.* 1985). This process produces the immediate jasmonate precursor OPDA, which is converted to JA by *OPR3* (Stintzi and Browse 2000). *JAR1* and *JMT* encode enzymes that catalyze the formation of JA-amino acid conjugates and methyl jasmonate (MeJA), respectively (Seo *et al.* 2001; Staswick and Tiryaki 2004; Staswick *et al.* 2002). The possibility of additional active jasmonates is also proposed. *COI1* is placed downstream of all active forms of jasmonate because it is required for all known jasmonate-dependent responses (Feys *et al.* 1994; Kloek *et al.* 2001) and encodes an F-box protein believed to act as the jasmonate receptor (Katsir *et al.* 2008; Devoto *et al.* 2002). The proteins encoded by the *JAZ* family of genes are proposed to be ubiquitinated by COI1, resulting in their degradation (Chini *et al.* 2006; Thines *et al.* 2006). JAZ3 interacts with and inhibits *JIN1* (Chini *et al.* 2006), so it has been placed as a negative regulator in the pathway leading to *JIN1*. Specific functions have not yet been determined for other *JAZ* family members, as indicated by the question marks. Because *jar1* mutants with significantly reduced levels of JA-Ile are fully fertile (Staswick *et al.* 1998), we place pollen development in a pathway stimulated by an unknown form of jasmonate and regulated by an unknown transcription factor. The other jasmonate-dependent responses under consideration (defense against necrotrophic pathogens,

susceptibility to *Pseudomonas syringae*, and inhibition of root growth) are all placed in the branched pathway controlled by JA-Ile. *JIN1* is required for jasmonate-mediated inhibition of root growth, susceptibility to *P. syringae*, and induction of genes including *LOX2* and *CLH1* (Berger *et al.* 1996; Laurie-Berry *et al.* 2006; Nickstadt *et al.* 2004; Lorenzo *et al.* 2004). A different set of jasmonate-mediated responses, including defense against necrotrophic pathogens and insect pests and expression of genes involved in these defenses, do not require *JIN1* activity and are instead controlled by a different transcription factor, likely *ERF1* (Lorenzo *et al.* 2003). These *JIN1*-independent responses are coordinately regulated by jasmonate and ethylene signaling (Lorenzo *et al.* 2003). *JIN1*-dependent and *ERF1*-dependent signaling are also controlled through mutual inhibition, as demonstrated by increased expression of *ERF1*-responsive genes in *jin1* mutants and increased expression of *JIN1*-responsive genes in *erf1* mutants (Laurie-Berry *et al.* 2006; Lorenzo *et al.* 2003; Lorenzo *et al.* 2004; Boter *et al.* 2004).


Ethylene
JAZ?
ERF1?
defense vs fungi and insects
PDF1.2, Thi
FAD3,7,8
OPR3
JAR1
OPDA
JA
JA-Ile
COI1
JAZ3
JAZ?
JIN1
root growth inhibition
P. syringae disease
LOX2, CLH1
?
JMT
JA-X
COI1
JAZ?
TF?
pollen development
MeJA

Chapter 2

The *Arabidopsis thaliana JASMONATE INSENSITIVE 1* gene is required for suppression of salicylic acid-dependent defenses during infection by *Pseudomonas syringae*

Neva Laurie-Berry, Vinita Joardar, Ian H. Street, and Barbara N. Kunkel

ABSTRACT

Many plant pathogens suppress anitmicrobial defenses using virulence factors that modulate endogenous host defenses. The *Pseudomonas syringae* phytotoxin coronatine (COR) is believed to promote virulence by acting as a jasmonate analog, as *COR-insensitive* 1 (*coi1*) *Arabidopsis thaliana* and tomato mutants are impaired in jasmonate signaling and exhibit reduced susceptibility to *P. syringae.* To further investigate the role of jasmonate signaling in disease development, we analyzed several jasmonate-insensitive *A. thaliana* mutants for susceptibility to *P. syringae* pv. *tomato* strain DC3000 (*Pst*DC3000) and sensitivity to COR. *jasmonate insensitive 1* (*jin1*) mutants exhibit both reduced susceptibility to *Pst*DC3000 and reduced sensitivity to COR, while *jasmonate resistant 1* (*jar1*) plants exhibit wild-type responses to both COR and *Pst*DC3000. A *jin1 jar1* double mutant does not exhibit enhanced jasmonate insensitivity, suggesting that *JIN1* functions downstream of JA-amino acid conjugates synthesized by JAR1. Reduced disease susceptibility in *jin1* mutants is correlated with elevated expression of *Pathogenesis related 1* (*PR-1*) and is dependent on accumulation of salicylic acid (SA). We also show that *JIN1* is required for normal *Pst*DC3000 symptom development through an SA-independent mechanism. Thus, *Pst*DC3000 appears to utilize COR to manipulate *JIN1*-dependent jasmonate signaling both to suppress SA-mediated defenses and to promote symptom development.

INTRODUCTION

To successfully cause disease, a plant pathogen must first create a suitable environment for growth within the host. This process includes suppression of general plant defense responses induced upon microbial attack (Heath 2000; Ponciano *et al.* 2003; Thordal-Christensen 2003) and stimulation of the release of water and nutrients into the apoplast (Alfano and Collmer 1996; Ponciano *et al.* 2003). General antimicrobial defenses include accumulation of the signaling molecules salicylic acid (SA) and jasmonic acid (JA) and its derivatives (collectively referred to as jasmonates), induction of pathogenesis-related genes, and production of antimicrobial compounds (Felix *et al.* 1999; Glazebrook *et al.* 1997; Hammond-Kosack and Jones 1996). Little is known about the specific mechanisms utilized by pathogens to evade or inhibit these defenses, but several secreted pathogen virulence factors have been implicated in this process (Alfano and Collmer 1996; Kunkel and Brooks 2002; Ponciano *et al.* 2003).

One such virulence factor is coronatine (COR), a phytotoxin required for full virulence of several strains of the bacterial plant pathogen *Pseudomonas syringae* (Bender *et al.* 1999). *P. syringae* strains unable to produce COR are compromised in their ability to grow and cause disease on host plants, including *Arabidopsis thaliana* (Brooks *et al.* 2004; Mittal and Davis 1995). While the exact mode of action of COR is not fully understood, several lines of evidence suggest that it may alter host physiology by mimicking one or more jasmonates (Feys *et al.* 1994; Staswick and Tiryaki 2004; Weiler *et al.* 1994). This hypothesis is suggested by the structural resemblance between the coronafacic acid moiety of COR and several jasmonates (Bender *et al.* 1999; Feys *et al.*

1994; Weiler *et al.* 1994) and supported by the similarities between their effects on plant tissue, including inhibition of root elongation in *A. thaliana*, production of the stress pigment anthocyanin, production of ethylene, and leaf senescence (Bender *et al.* 1999; Feys *et al.* 1994; Staswick and Tiryaki 2004; Weiler *et al.* 1994). There is also genetic evidence that jasmonates and COR act through the same signaling pathway in *A. thaliana* and tomato, as the jasmonate-insensitive *A. thaliana* and tomato mutants, *coronatine insensitive 1* (*coi1*) and *jasmonic acid insensitive 1* (*jai1*), respectively, are also insensitive to COR (Feys *et al.* 1994; Zhao *et al.* 2003).

The observations that *coi1* and *jai1* mutants exhibit severely decreased susceptibility to *P. syringae* infection reinforce the importance of COR in *P. syringae* virulence (Feys *et al.* 1994; Kloek *et al.* 2001; Zhao *et al.* 2003). These data also suggest that an intact jasmonate signaling pathway is required for full susceptibility to infection by COR-producing strains of *P. syringae*. One hypothesis, based on mounting evidence of mutual antagonism between jasmonate and SA pathways, is that COR promotes susceptibility to *P. syringae* infection by stimulating jasmonate signaling in plants, thereby inhibiting SA-mediated defenses that normally limit growth of *P. syringae* within host tissue (Kunkel and Brooks 2002; Brooks *et al.* 2005). This hypothesis is supported by observations of increased expression of SA-induced defense-related genes in COR-insensitive mutants of both *A. thaliana* and tomato (Kloek *et al.* 2001; Zhao *et al.* 2003).

The *A. thaliana coi1* mutants used to assess the role of jasmonate signaling in pathogenesis are pleiotropic, exhibiting defects in multiple jasmonate-dependent processes, including pollen development (Feys *et al.* 1994), defense against insects and

necrotrophic pathogens (Penninckx *et al.* 1996; Thomma *et al.* 1998), and induction of jasmonate-responsive genes (Benedetti *et al.* 1998; Benedetti *et al.* 1995; Feys *et al.* 1994; Penninckx *et al.* 1996; Thomma *et al.* 1998). Thus, *COI1* appears to be a master regulator of jasmonate-dependent responses, which makes sense as it encodes an F-box protein potentially involved in targeting multiple components of jasmonate signaling for degradation (Xie *et al.* 1998). As a result, *coi1* mutant plants may not be appropriate for examining specific aspects of jasmonate signaling required for susceptibility to *P. syringae*. Thus it is important to assess disease susceptibility in other jasmonate signaling mutants, especially those impaired in only a subset of jasmonate-mediated responses.

Several additional jasmonate-insensitive mutants have been identified in *A. thaliana*, including *jasmonate resistant 1* (*jar1*; Staswick *et al.* 1992), *jasmonate insensitive 1* (*jin1*; Berger *et al.* 1996), *jasmonate insensitive 3* and *4* (*jai13/4*; Lorenzo *et al.* 2004), *auxin resistant 1* (*axr1*; Tiryaki and Staswick 2002), *enhanced disease susceptibility 8* (*eds8*; Glazebrook *et al.* 2003; Ton *et al.* 2003), and several *jasmonate under-expressing* mutants (*jue1/2/3*; Jensen *et al.* 2002). However, to date, only *jar1, jin1, axr1, and eds8* mutants have been genetically well characterized (Berger *et al.* 1996; Lorenzo *et al.* 2004; Staswick *et al.* 1992; Staswick and Tiryaki 2004; Staswick *et al.* 2002; Glazebrook *et al.* 1996). Studies using the *jar1-1* mutant demonstrated that this mutation has no detectable impact on plant susceptibility to virulent *P. syringae* (Clarke *et al.* 2000; Kloek *et al.* 2001; Nickstadt *et al.* 2004; Pieterse *et al.* 1998). This result may not be surprising as *JAR1* encodes an enzyme involved in JA modification rather than a component of the jasmonate signaling pathway (Staswick and Tiryaki 2004; Staswick *et*

al. 2002). *JIN1* encodes a MYC family transcription factor involved in mediating a subset of jasmonate-induced responses (Boter *et al.* 2004; Lorenzo *et al.* 2004). Like *COI1*, this gene is required for full susceptibility to *P. syringae* pv. *tomato* (Nickstadt *et al.* 2004), but the mechanism(s) underlying this phenotype is not well understood. *AXR1* encodes an enzyme required for converting the ubiquitin-like RUB protein into an activated form necessary for proper function of the ubiquitin-ligating SCF complex and is involved in multiple hormone signaling pathways (del Pozo *et al.* 2002; Tiryaki and Staswick 2002). *axr1* mutant plants have recently been demonstrated to have reduced disease susceptibility to *P. syringae* pv. *tomato* infection (Kunkel *et al.* 2004). In contrast, *eds8* mutant plants exhibit enhanced susceptibility to *P. syringae* (Glazebrook *et al.* 1996). While the relevant gene has not been cloned, gene expression profiling experiments have suggested a role for *EDS8* in jasmonate and/or ethylene signaling (Glazebrook *et al.* 2003).

In this study, we further investigate the role of jasmonate signaling in *P. syringae-A. thaliana* interactions. We demonstrate that *JIN1* is required both for full susceptibility to *P. syringae* pv. *tomato* and full sensitivity to COR and that the decreased disease susceptibility of *jin1-1* mutant plants is dependent on accumulation of SA during infection. These results suggest that COR-mediated stimulation of a *JIN1*-dependent jasmonate signaling pathway suppresses SA-dependent defenses, leading to susceptibility to *P. syringae* pv. *tomato* infection.

RESULTS

jin1 plants exhibit reduced susceptibility to *P. syringae*

To further investigate the role of jasmonate signaling in susceptibility to COR-producing *P. syringae*, we infected *A. thaliana jin1-1* (Berger *et al.* 1996) mutant plants with the virulent *P. syringae* pv. *tomato* strain DC3000 (*Pst*DC3000). These experiments revealed that *jin1-1* plants have reduced susceptibility to this pathogen, developing mild chlorosis and few to no water-soaked disease lesions (data not shown). To determine whether decreased symptom production in *jin1-1* plants is correlated with decreased bacterial growth in plant tissue, *Pst*DC3000 levels were measured over the course of infection (Fig. 1A). In wild-type Col-0 plants, bacteria multiplied more than three orders of magnitude in the first two days following infection and continued to increase over the next two days. Similar to wild-type plants, bacteria in *jin1-1* plants multiplied by at least three orders of magnitude during the first two days of infection. However, over the next two days, these bacterial populations failed to increase any further. Thus, *jin1-1* plants support bacterial growth levels that are significantly lower than wild-type plants. This difference in bacterial growth correlates well with the milder disease symptoms observed in *jin1-1* plants as compared to wild-type and is consistent with similar observations by Nickstadt *et al.* (Nickstadt *et al.* 2004).

For comparison, we also included two other jasmonate-insensitive mutants in this experiment, *jar1-1* and *coi1-20* (Kloek *et al.* 2001; Staswick *et al.* 1992). Consistent with previous reports, levels of bacterial growth in the *jar1-1* mutant were similar to wild-type at all time points examined (Fig. 1A; Clarke *et al.* 2000; Kloek *et al.* 2001; Nickstadt *et*

al. 2004; Pieterse *et al.* 1998). In contrast, bacteria multiplied only tenfold in *coi1-20* mutant plants over the four day course of infection; this extremely low amount of bacterial growth is consistent with the complete absence of visible disease symptoms in these plants (Kloek *et al.* 2001). The reduced disease susceptibility in *jin1-1* plants is not as pronounced as that observed in the *coi1-20* mutants. This can likely be explained by the fact that strong *coi1* alleles, such as *coi1-20* used in these studies, appear to block all jasmonate signaling, while *jin1-1*, although likely to be a null allele (Lorenzo *et al.* 2004), only affects a subset of jasmonate responses (Berger *et al.* 1996). Two additional alleles, *jin1-7* and *-8*, both of which contain early T-DNA insertions and are believed to be null alleles (Lorenzo *et al.* 2004), exhibited reduced disease susceptibility essentially identical to that observed in *jin1-1* (data not shown).

The *jin1-1* mutation was originally isolated in a *gl1* (glabrous) background lacking trichomes (Berger *et al.* 1996). As we utilize dip inoculation in our infection experiments, it is possible that the absence of trichomes may contribute to the reduced disease susceptibility phenotype by altering epiphytic colonization and/or entry into the leaf by *Pst*DC3000 (Beattie and Lindow 1994). To determine whether the reduced disease susceptibility observed in *jin1-1 gl1* plants was enhanced by the absence of trichomes, these plants were crossed to wild-type Col-0 (see methods). The glabrous and reduced disease susceptibility phenotypes segregated in the F2 progeny as single, unlinked recessive traits, while jasmonate-insensitivity cosegregated with reduced disease susceptibility (data not shown). Homozygous *jin1-1 GL1* plants exhibited reduced susceptibility phenotypes essentially identical to the original *jin1-1 gl1* lines when

infected with *Pst*DC3000 (Fig. 1B and symptom data not shown), confirming that the decreased response to infection is not significantly impacted by the absence of trichomes. A single, homozygous *jin1-1 GL1* line was selected for use in all further experiments to avoid possible complications from the *gl1* mutation.

JIN1 and _JAR1_ function in the same signaling pathway mediating JA inhibition of root growth

Although *jin1-1* and *jar1-1* mutant plants respond differently to *Pst*DC3000 infection (Fig.1), seedlings of both mutants exhibit intermediate levels of insensitivity to the inhibitory effects of methyl jasmonate (MeJA; Berger *et al.* 1996; Staswick *et al.* 1992). These similarities and differences between *jin1-1* and *jar1-1* mutant phenotypes suggest that jasmonate signaling may be more complex than previously described. Both *jin1-1* and *jar1-1* mutations behave similarly to known null alleles (Lorenzo *et al.* 2004; Staswick *et al.* 2002) but lead to only partial loss of jasmonate signaling (Berger *et al.* 1996; Staswick *et al.* 2002), which might suggest functional redundancy between these two genes. However, this seems unlikely, as the two genes encode very different products (Staswick and Tiryaki 2004; Staswick *et al.* 2002; Lorenzo *et al.* 2004) that are unlikely to act at the same step in jasmonate signaling. The similarity of their MeJA insensitivity phenotypes raises the possibility that they may, however, function in the same pathway leading to inhibition of root growth. If JIN1 and JAR1 function in the same pathway leading to inhibition of root growth, one would predict that a *jin1-1 jar1-1* double mutant would not exhibit enhanced MeJA insensitivity at the level of root growth, as some

degree of sensitivity would be maintained via one or more additional jasmonate signaling pathways. Alternatively, the two genes may encode components of separate jasmonate signaling pathways, both of which are involved in physiological responses to MeJA application, such as inhibition of root elongation, but only one of which (*JIN1*) is required for *P. syringae* disease development. In this model, a *jin1-1 jar1-1* double mutant would be predicted to exhibit increased insensitivity to MeJA treatment, perhaps similar to that observed in *coi1* mutants.

To distinguish between these two possibilities, we crossed *jin1-1* and *jar1-1* single mutants and isolated F2 offspring homozygous for both mutations (see methods). Because both of these alleles behave similarly to null alleles (Lorenzo *et al.* 2004; Staswick *et al.* 2002), the double mutants are presumed to lack functional JIN1 and JAR1 proteins, allowing us to examine their relative roles in jasmonate signaling. To assess jasmonate sensitivity in the resulting double mutants, we assayed root inhibition in response to exogenous application of MeJA. *jin1-1* and *jar1-1* single mutants each exhibited a characteristic, reproducible degree of root inhibition when grown on media containing MeJA (Fig. 2). On 10μM MeJA, wild-type Col-0 seedlings developed roots that were approximately 30% of their length on media lacking the hormone. In contrast, root growth in *jin1-1* and *jar1-1* mutants was less severely inhibited by MeJA, respectively exhibiting ~60% and ~80% of their untreated lengths. *jin1 jar1* double mutants exhibited a degree of root inhibition similar to that of *jar1-1* single mutants (~75%), demonstrating that the two mutations do not produce additive effects. Thus, *JIN1* and *JAR1* appear to act in the same pathway with respect to this phenotype.

jin1 jar1 double mutant plants resemble *jin1-1* plants with respect to reduced disease susceptibility

The above data suggest that *JIN1* and *JAR1* act in a single signaling pathway affecting root sensitivity to MeJA. However, the different responses of *jar1-1* and *jin1-1* mutants to *Pst*DC3000 suggest that *JIN1* mediates disease susceptibility via a mechanism that does not require *JAR1*. To confirm that loss of *JAR1* does not affect *Pst*DC3000 susceptibility in the context of the *jin1-1* mutation, we examined the disease response of the *jin1 jar1* double mutant. The double mutant plants responded similarly to *jin1-1* single mutants when infected with *Pst*DC3000, both in terms of symptom development (data not shown) and levels of bacterial growth (Fig. 1B). This demonstrates that the *jar1-1* mutation does not alter the plant's response to infection with virulent *Pst*DC3000, even in the context of a *jin1-1* mutation, confirming that *JAR1* does not play a significant role in the process of *Pst*DC3000 infection.

jin1-1 plants exhibit reduced sensitivity to coronatine

The reduced disease susceptibility of *jin1-1* and *coi1* mutants to infection with COR-producing *P. syringae* led us to hypothesize that *jin1-1* mutants would also be less sensitive to COR than wild-type plants, while *jar1-1* plants, which exhibit wild-type disease susceptibility, would respond normally to COR treatment. This is supported by the strong correlation in *coi1* mutants between COR insensitivity and reduced disease susceptibility to COR-producing bacterial strains (Feys *et al.* 1994; Kloek *et al.* 2001).

Previous studies have shown that *jin1-1* plants have reduced sensitivity to coronalon, a COR analog; however, this study was not quantitative, nor was it confirmed with authentic COR (Schuler *et al.* 2004). To examine the COR sensitivity of these jasmonate signaling mutants, *jin1-1*, *jar1-1*, and *jin1 jar1* double mutant seedlings were grown on media containing 0.1μM COR, and root inhibition was assayed (Fig. 2). As expected, root growth of wild-type plants on COR was severely inhibited to ~30% of their untreated length. Conversely, roots of *coi1-20* seedlings were completely uninhibited by COR, also as expected, and in two of three experiments, they exhibited enhanced elongation in the presence of 0.1 μM COR. *jin1-1*, *jin1-7*, and *jin1-8* seedlings exhibited an intermediate level of root growth on this media (~55% of the untreated length), demonstrating that *jin1* mutants are partially insensitive to COR (Fig. 2 and data not shown). On the other hand, root inhibition of *jar1-1* plants appeared almost identical to that of wild-type plants (~30%), a phenotype which correlates with their fully susceptible response to *Pst*DC3000 (Fig. 1). *jin1 jar1* double mutants also exhibited intermediate levels of sensitivity to COR (~65%), similar to the *jin1-1* single mutant. These results demonstrate a clear correlation between a plant's level of sensitivity to COR and its susceptibility to *Pst*DC3000 infection.

The inhibition assays described above monitor COR sensitivity in seedling roots. However, this may not adequately reflect COR sensitivity in adult leaf tissue where *P. syringae* infection occurs. To examine COR sensitivity in mature plants, a dilute solution of 5nM COR was infiltrated into the abaxial side of leaves. Seven days later, we measured accumulation of the stress pigment anthocyanin in the infiltrated leaves, a

typical response of Col-0 plants to exogenous application of JA or COR (Bent *et al.* 1992; Feys *et al.* 1994; Greenberg and Ausubel 1993). The resulting data indicate levels of COR sensitivity similar to those observed in roots (Fig. 3). Mock-treated wild-type Col-0 plants produced a relatively small amount of pigment, potentially due to environmental stress combined with the wounding response to infiltration. This response to mock treatment was not observed in the mutant lines examined. Following COR treatment, anthocyanin accumulated to significantly higher levels in wild-type plants, primarily in the petiole and midvein of the infiltrated leaf, as well as the abaxial leaf surface. Occasional pigmentation was also observed on the adaxial side of the leaf, concentrated most strongly around the site of infiltration. Similar levels and patterns of anthocyanin production were observed in *jar1-1* mutants following COR application. As expected for a fully jasmonate-insensitive mutant that does not respond to wounding (Titarenko *et al.* 1997) or COR application (Feys *et al.* 1994), *coi1-20* plants did not produce detectable levels of anthocyanin in response to either mock or COR treatment. *jin1-1* mutant plants treated with COR accumulated small amounts of anthocyanin (Fig. 3), localized to the petiole and leaf midvein. These observations are consistent with those from seedling assays (Fig. 2), indicating that *jin1-1* mutants exhibit intermediate sensitivity to COR. Further, these results indicate that COR sensitivity as monitored in seedlings by root inhibition assays accurately reflects sensitivity in adult leaf tissue.

axr1 mutants exhibit decreased coronatine sensitivity, while _eds8_ mutants do not

Two additional jasmonate insensitive mutants have also been shown to have altered responses to *Pst*DC3000 infection: *eds8* and *axr1*. *eds8* plants have reduced sensitivity to MeJA (Glazebrook *et al.* 2003), but unlike *coi1* and *jin1*, exhibit enhanced susceptibility to *P. syringae* infection (Glazebrook *et al.* 1996). To investigate this apparent discrepancy, we examined the response of *eds8* mutants to 0.1μM COR and determined that they have wild-type sensitivity to the phytotoxin (data not shown). Thus, the reduced jasmonate sensitivity of *eds8* mutant plants does not correlate with altered sensitivity to COR.

The recent findings that *AXR1* plays a role in both disease responses to *Pst*DC3000 (Kunkel *et al.* 2004) and jasmonate signaling (Tiryaki and Staswick 2002) suggested that this mutant may also exhibit reduced COR sensitivity, similar to that observed in *coi1* and *jin1* plants. To examine this hypothesis, *axr1-12* (Lincoln *et al.* 1990) plants were tested for COR sensitivity in root inhibition and anthocyanin accumulation assays. Results from the root inhibition assays are presented in Figure 4. Compared to wild-type Col-0, *axr1-12* plants show significantly less inhibition of root growth when treated with COR (~60% of untreated length in Fig. 4). This intermediate level of sensitivity to COR is similar to that observed in *jin1-1* plants (Figs. 2, 4). Similar results were obtained when COR sensitivity was assayed by monitoring anthocyanin accumulation in mature leaves (data not shown). These data suggest that *AXR1* is required for normal COR-induced responses, which is likely to account, at least in part, for the reduced disease susceptibility of *axr1* mutant plants to *Pst*DC3000 infection (Kunkel *et al.* 2004). It is important to note that, despite the role of *AXR1* in both

jasmonate and auxin signaling, *JIN1* appears to be specific to jasmonate as *jin1* mutants exhibit normal sensitivity to auxin (J. Agnew and N. Laurie-Berry, unpublished data), ethylene, and ABA (Lorenzo *et al.* 2004).

jin1-1 mutants exhibit decreased induction of jasmonate-responsive genes following *Pst*DC3000 infection

To further assess the jasmonate signaling defect in the *jin1-1* mutant, we examined expression of two jasmonate-responsive genes, *Lipoxygenase 2* (*LOX2*) and *Coronatine induced 1* (*CORI1*), by RNA blot analysis. *LOX2* is involved in jasmonate biosynthesis and is also regulated by jasmonates (Bell and Mullet 1993). Expression of *CORI1*, which encodes a predicted chlorophyllase, is stimulated by jasmonate or COR treatment and is induced during infection with *P. syringae* (Benedetti *et al.* 1998; Brooks *et al.* 2005; Tsuchiya *et al.* 1999). Figure 5A shows expression of these two genes in wild-type Col-0 and *jin1-1* plants over the course of infection with *Pst*DC3000. In wild-type plants, transcripts of both *LOX2* and *CORI1* are induced within 24 hours after infection, reaching their highest levels 1 to 2 days after infection. Although these genes are also induced upon infection in *jin1-1* plants, levels of both transcripts were markedly decreased relative to wild-type at all time points examined (Fig. 5A). These data are consistent with the identification of JIN1 as a transcription factor mediating expression of a subset of jasmonate-responsive genes (Lorenzo *et al.* 2004) and also with previous observations that *jin1* plants do not exhibit wild-type induction of several jasmonate-

responsive genes following MeJA treatment (Berger *et al.* 1996; Lorenzo *et al.* 2004; Nickstadt *et al.* 2004).

We also examined expression of the jasmonate and ethylene inducible defense gene *Plant Defensin 1* (*PDF1.2*; Penninckx *et al.* 1996). This gene was weakly induced upon *Pst*DC3000 infection, reaching detectable levels one day after infection (Fig. 5B). Expression of *PDF1.2* was much more strongly induced in *jin1-1* plants throughout the infection process. Although this result seems surprising at first, it is consistent with earlier observations that JIN1 negatively regulates expression of this gene in response to MeJA treatment (Boter *et al.* 2004; Lorenzo *et al.* 2004).

Reduced susceptibility to *Pst*DC3000 in *jin1-1* plants correlates with elevated *PR-1* expression and is dependent on SA accumulation

The reduced susceptibility of *coi1* mutants to *P. syringae* appears to result from hyperactivation of the SA-responsive defense pathway (Kloek *et al.* 2001). To determine if *jin1-1* also exhibits enhanced SA signaling, we examined expression of the SA-responsive gene *Pathogenesis-Related 1* (*PR-1*) during the course of infection with *Pst*DC3000 (Fig. 5). In wild-type plants, this defense-related marker is typically induced within 48 hours after dip inoculation with *Pst*DC3000 (Chen *et al.* 2004). In *jin1-1* plants, *PR-1* was more strongly induced than in wild-type plants and in one of three Northern blot experiments was observed as early as 24 hours following infection (Fig. 5A). These results are consistent with observations by Nickstadt *et al* that *jin1-1* mutants accumulated elevated levels of SA 24 hours after infection with *Pst*DC3000 (Nickstadt *et*

al. 2004). These data support the hypothesis that the reduced susceptibility observed in *jin1-1* mutants results from increased expression of SA-dependent defenses.

To directly test this hypothesis, we examined the effect of the *jin1-1* mutation on disease susceptibility in the context of plants impaired in their ability to accumulate SA. If the reduced disease susceptibility of *jin1-1* plants results from hyperactivation of SA-responsive defenses, overall reduction of SA levels in the plant should result in wild-type susceptibility in *jin1-1* plants. A transgenic construct containing the *P. putida* salicylate hydroxylase *nahG* gene, which encodes an enzyme that degrades SA, was introduced into the *jin1-1* line (see methods). Disease responses were examined in the resulting *jin1-1 nahG* double homozygous lines. As expected, wild-type Col-0 plants carrying the *nahG* transgene supported higher levels of bacterial growth (Fig. 6A) and exhibited more severe disease symptoms than wild-type plants, including an increase in chlorosis and a greater number of individual water-soaked disease lesions that coalesced into patches (Fig. 6B). These results are consistent with the role of SA in limiting growth and spread of virulent *P. syringae* in *A. thaliana* plants (Delaney *et al.* 1994; Dewdney *et al.* 2000; Nawrath and Metraux 1999). The presence of the *nahG* transgene also led to significantly more severe disease symptoms in the *jin1-1* background; *jin1 nahG* plants developed chlorosis and disease lesions, neither of which was observed in the *jin1-1* parental line (Fig. 6B). The observed increase in disease symptom severity in *jin1 nahG* plants correlated with increased levels of bacterial growth (Fig. 6A). As *nahG* suppresses the reduced disease susceptibility of *jin1-1* mutants, this phenotype appears to be dependent on accumulation of SA. However, *nahG* plants have been shown to accumulate high

levels of catechol upon SA degradation, a phenomenon that promotes bacterial growth (van Wees and Glazebrook 2003), making it potentially difficult to interpret results using these plants.

To verify the apparent SA-dependence of reduced susceptibility in *jin1-1* plants, we investigated the disease susceptibility of *jin1-1* mutants carrying the *SA-induction deficient 2* (*sid2-2*) mutation. The *sid2-2* mutation, which results in disruption of the SA biosynthetic gene isochorismate synthase (*ICS1*), significantly reduces the plant's ability to synthesize SA in response to infection (Wildermuth *et al.* 2001). As previously reported (Dewdney *et al.* 2000; Nawrath and Metraux 1999), *sid2* mutant plants exhibited more severe disease symptoms than wild-type plants following infection with virulent *P. syringae* strains (Fig. 6B). The *jin1 sid2* double mutants also had visibly increased symptoms, as compared to the *jin1-1* parental line, with the double mutants developing extensive chlorosis and some disease lesions (Fig. 6B). Bacterial growth levels correlated well with the increased disease symptoms observed in these plants; *jin1 sid2* double mutants supported bacterial levels nearly identical to those seen in *sid2-2* mutants and well in excess of those observed in *jin1-1* plants (Fig. 6C). These data are consistent with the results obtained from *jin1 nahG* lines and demonstrate that the reduced disease susceptibility of *jin1-1* plants depends on the presence of SA.

Further, although the *jin1 sid2* and *JIN1 sid2* plants supported equivalent levels of bacterial growth, we reproducibly observed that the *jin1 sid2* plants developed fewer disease lesions and less chlorosis than the *sid2-2* parent (Fig. 6B, C, Table 1). These results suggest that, while *sid2-2* suppresses the inability of *jin1-1* plants to support high

levels of pathogen growth, it does not fully restore disease susceptibility. Thus, *JIN1* may also be required for wild-type disease symptom development via an SA-independent mechanism.

DISCUSSION

Our results provide important experimental data in support of the hypothesis that *Pst*DC3000 utilizes the phytotoxin COR to manipulate jasmonate signaling within the host in a manner that promotes both pathogen growth and disease development. Our data are consistent with earlier observations (Kloek *et al.* 2001; Nickstadt *et al.* 2004; Zhao *et al.* 2003) that disease susceptibility requires an intact jasmonate signaling pathway. This requires the activity of the *JIN1* transcription factor but is independent of synthesis of the JA-amino acid conjugates produced through the activity of JAR1 (Fig1). As reduced susceptibility to *Pst*DC3000 is observed in several jasmonate signaling mutants, *jin1*, *coi1*, and *jai1* (Fig. 1; Kloek *et al.* 2001; Zhao *et al.* 2003), it seems likely that manipulation of jasmonate signaling is an important virulence strategy for *P. syringae* on both *A. thaliana* and tomato.

A unified model for jasmonate signaling

Based on several recent studies, it is becoming clear that the jasmonate signaling pathway is more complex than previously described, and we have made an effort to incorporate these findings into a new, more comprehensive model (Fig. 7). The recent discovery that *JAR1* encodes an active JA-amino acid conjugase (Staswick and Tiryaki 2004) reinforces the idea that there are multiple forms of jasmonate acting within the plant, including MeJA and JA-amino acid conjugates, most notably JA-Ile. The main feature of this model is that jasmonate signaling occurs through a branched pathway with different jasmonates controlling distinct processes. For example, *jar1* mutants are fully

fertile and only partially impaired in their responses to exogenous JA treatment (Staswick *et al.* 1992; Staswick *et al.* 2002; Fig. 3), suggesting that pollen development and some aspects of root inhibition do not require JA-Ile. In contrast, *JAR1* is required for resistance to several necrotrophic pathogens and full sensitivity to exogenous JA (Berrocal-Lobo and Molina 2004; Ferrari *et al.* 2003; Mellersh and Heath 2003; Staswick *et al.* 1992). MeJA also appears to be important for defense against *Botrytis cinerea*, and possibly other necrotrophic pathogens, as overexpression of the jasmonate methyl transferase (JMT) enzyme that forms MeJA results in decreased susceptibility to *B. cinerea* (Seo *et al.* 2001). *COI1* appears to mediate signaling through both of these branches as this gene is required for all aspects of jasmonate signaling. As AXR1 is involved in proper activation of the SCF^{COI1} complex, it is likely to act at the same step in the pathway as COI1 and appears to affect all of the same responses, in addition to its independent effects on auxin-related signaling (Lincoln *et al.* 1990). *EDS8* is not included in our model as it is presently unclear what role it plays in jasmonate signaling or what form of jasmonate it may be responding to. The wild-type COR sensitivity of *eds8* mutants suggests that the gene is not involved in JA-Ile signaling.

We place *JIN1* in the *JAR1*-dependent (JA-Ile responsive) signaling pathway downstream of *COI1* for the following reasons. First, *JIN1* and *JAR1* appear to act in the same pathway leading to root sensitivity to exogenous jasmonates (Fig. 3). Second, *JIN1* is required for full susceptibility to infection by *P. syringae* (Fig. 1; Nickstadt *et al.* 2004) and mediates jasmonate responses induced by the phytotoxin COR (Fig. 3). Given that COR is proposed to act as a molecular mimic of the endogenous jasmonate JA-Ile

(Krumm *et al.* 1995; Staswick and Tiryaki 2004) and the recent observation that JAR1 catalyzes the formation of JA-Ile, it is likely that COR stimulates signaling through the *JAR1*-dependent pathway. Placement of *JIN1* downstream of *JAR1* in a pathway leading to *Pst*DC3000 susceptibility may initially appear contradictory, as *jar1* mutants retain full susceptibility to infection (Fig. 1; Kloek *et al.* 2001; Nickstadt *et al.* 2004). However, this observation is consistent with the hypothesis that COR bypasses the requirement for production of JA-Ile to activate *JIN1*-dependent jasmonate responses leading to *P. syringae* growth and disease development. Thus, the presence of COR effectively complements the inability of the *jar1* mutant to produce JA-Ile. Furthermore, our observation that a *JMT* overexpressing line that accumulates elevated levels of MeJA (Seo *et al.* 2001) does not exhibit enhanced susceptibility to *P. syringae* is consistent with the hypothesis that this process does not involve MeJA (N. Laurie-Berry, unpublished data).

To add to the complexity of this developing model, recent studies on *JIN1* have surprisingly demonstrated that the JIN1/AtMYC2 transcription factor negatively regulates several genes regarded as being positively regulated by jasmonates (Boter *et al.* 2004; Lorenzo *et al.* 2004). This finding is further supported by the accumulation of elevated transcript levels of *PDF1.2* in *jin1-1* mutants during *Pst*DC3000 infection (Fig. 5B). Recent findings that *jin1* mutants, unlike *jar1* and *coi1* plants, exhibit reduced susceptibility to some necrotrophic fungal pathogens provides further evidence that *JIN1* negatively regulates some aspects of jasmonate signaling (Lorenzo *et al.* 2004; Nickstadt *et al.* 2004).

The abundance of new data and our appreciation for the ever increasing complexity of jasmonate signaling should be taken into account in future analyses of these processes. For example, *coi1* mutants should be used in combination with other jasmonate-related mutants to fully examine the role of jasmonate in a process. While the *COI1* F-box is required for most traditionally accepted jasmonate-mediated effects, the discrepancies between *coi1* and *jin1* mutants with regard to fungal susceptibility (Lorenzo *et al.* 2004) and regulation of *PDF1.2* (this work and Boter *et al.* 2004; Lorenzo *et al.* 2004) demonstrate that the severe block in jasmonate signaling in *coi1* mutants prevents analysis of different branches within the pathway. Thus *coi1* mutants can mask more subtle and complex regulation within this pathway. Likewise, caution should be used when interpreting the phenotypes of *jar1* mutants. JAR1 is a biosynthetic enzyme that activates JA by conjugating it to isoleucine (Staswick and Tiryaki 2004). While *jar1* plants do not produce JA-Ile, they do exhibit normal sensitivity to JA-Ile and COR, demonstrating that *jar1* mutants are not compromised in their ability to perceive and respond to these signaling molecules. Thus, while *jar1* mutants can be used to determine whether a jasmonate-dependent response requires the formation of JA-amino acid conjugates, data gathered using these mutants alone cannot establish a requirement for intact jasmonate signaling in a process.

JA and SA signaling interactions and their role in *P. syringae* pathogenesis

One of the unanswered questions in the study of *P. syringae* virulence mechanisms is why jasmonate signaling, which is known to be involved in defense, is

required for disease susceptibility to *P. syringae*. In this study, we demonstrate that the reduced disease susceptible phenotype of *jin1-1* mutant plants is dependent on the accumulation of SA (Fig. 6) and is associated with elevated expression of SA-dependent defense responses (Fig. 5). This suggests that *JIN1*-dependent signaling is required for suppression of SA-mediated defenses during *P. syringae* infection. These data are consistent with the recent demonstration that COR is required to overcome SA-mediated defenses during *Pst*DC3000 infection of *A. thaliana* (Brooks *et al.* 2005). The overall picture that emerges from these studies suggests that *Pst*DC3000 uses COR as a jasmonate analog to manipulate host physiology in a manner that inhibits SA-mediated defenses, thereby providing the pathogen with an opportunity to grow to high levels and cause disease. This further supports the developing theory of mutual antagonism between jasmonate and SA pathways as a significant factor in regulating plant defense (Glazebrook *et al.* 2003; Kunkel and Brooks 2002; Reymond and Farmer 1998). It is important to note that this simple model does not account for all jasmonate-dependent events that occur during plant-microbe interactions. For example, we do not incorporate the signaling events that occur during induced systemic resistance (ISR), a plant defense response triggered by non-pathogenic rhizobacteria strains such as *P. fluorescens* WCS417-3 (Pieterse *et al.* 1998). ISR is mediated through a jasmonate-dependent pathway and induces resistance to *P. syrinage* via a process that is independent of SA (Pieterse *et al.* 1998; Ton *et al.* 2002).

In our model for jasmonate signaling (Fig. 7), we have incorporated a possible explanation for how interactions between jasmonate- and SA-dependent signaling could

result in the observed outcomes. It has been shown that SA-dependent signaling down-regulates jasmonate signaling in an *NPR1*-dependent fashion (Spoel *et al.* 2003), while several recent reports suggest that the reciprocal occurs and is dependent upon *JIN1* and *COI1* (Fig. 6; Kloek *et al.* 2001; Nickstadt *et al.* 2004; Zhao *et al.* 2003). The exact points of mutual inhibition between these pathways are not known, but evidence suggests that NPR1-dependent SA signaling leads to the down-regulation of JA synthesis within the plant (Spoel *et al.* 2003). Likewise, the finding that *jin1-1* mutants accumulate elevated levels of SA (Nickstadt *et al.* 2004) suggests that jasmonate signaling may interfere with SA synthesis or accumulation. This effect does not appear to occur at the level of *ICS1* transcript accumulation, as we did not observe elevated *ICS1* transcript levels in infected *jin1-1* plants as compared to wild-type (Laurie-Berry, unpublished data).

SA-dependent defenses are required for the plant to limit virulence of *P. syringae*, as evidenced by the increased disease susceptibility observed in SA-deficient plants (Delaney *et al.* 1994; Dewdney *et al.* 2000; Nawrath and Metraux 1999). Likewise, *JIN1*-dependent jasmonate signaling is required for full *P. syringae* growth *in planta* (Fig. 1; Nickstadt *et al.* 2004). This growth-promoting effect could be accomplished in two ways. The ability of *JIN1* to mediate COR-dependent suppression of SA signaling, as indicated by accumulation of elevated SA levels (Nickstadt *et al.* 2004) and hyperactivation of SA-dependent defenses in *jin1-1* mutants (Fig. 5), raises the possibility that *JIN1* promotes bacterial growth indirectly by decreasing SA-dependent defenses that limit bacterial proliferation. Alternatively, *JIN1* may act more directly to actively promote bacterial growth through currently unknown mechanisms. These two proposed mechanisms are not

mutually exclusive, and it is possible that *JIN1* acts to increase bacterial populations by both limiting SA-dependent defenses and inducing pathogen growth via some other mechanism.

Normally, mutual antagonism between SA and jasmonate signaling should allow a plant to properly regulate activation of defense responses against a given pathogen, allowing the plant to selectively induce effective defenses without stimulating inappropriate and possibly counterproductive responses (Felton and Korth 2000; Feys and Parker 2000; Kunkel and Brooks 2002; Reymond and Farmer 1998; Thomma *et al.* 2001). In the case of *P. syringae* infection the appropriate plant defense response would be activation of SA-mediated defenses and a decrease in jasmonate-dependent signaling that would otherwise increase susceptibility. Activation of *NPR1*-dependent defenses would accomplish both of these goals. We propose *P. syringae* evolved the ability to produce COR as a molecular mimic of JA-Ile to bypass this inhibition, thereby restoring *JIN1*-dependent signaling to downregulate the plant's SA-dependent defenses and increase its susceptibility. To most effectively accomplish this goal, COR would need to stimulate jasmonate signaling downstream of *NPR1*-mediated repression, hence the placement of this inhibition upstream of *COI1* in our model.

Other JIN1-dependent processes may also contribute to *P. syringae* pathogenesis

While COR-activated suppression of SA-mediated defenses appears to be a critical factor for *P. syringae* growth *in planta*, promotion of disease symptom development is likely to occur through an SA-independent mechanism. While *jin1 sid2*

plants permit wild-type levels of bacterial growth (Fig. 6C), these plants do not develop the severe disease symptoms observed on wild-type or *sid2-2* plants. We observed a similar SA-independent reduction in symptoms in *coi1 nahG* plants (Kloek *et al.* 2001). These data suggest that the decreased symptom production observed in *coi1-20* and *jin1-1* plants is not simply due to decreased levels of pathogen growth resulting from hyperactivation of SA-dependent defenses, as impairment of SA synthesis during infection does not fully restore wild-type symptom development. Rather, *JIN1*-mediated signaling may lead to additional, SA-independent processes that promote chlorosis and formation of disease lesions in infected plants. It is likely that this pathway is also stimulated by COR as we have observed a similar decrease in symptom development despite full bacterial growth when examining *sid2-2* plants infected with bacteria unable to synthesize COR (Brooks *et al.* 2005).

It is unlikely that COR is the only *P. syringae* virulence factor manipulating jasmonate signaling during this interaction. Loss of COR is not sufficient to result in elevated *PR-1* expression during *Pst*DC3000 infection of *A. thaliana* (Brooks *et al.* 2005), suggesting that one or more additional virulence factors could be suppressing SA-mediated defenses. The strong elevation of the *PR-1* transcript in *jin1-1* plants indicates that the activity of any such additional factor would require intact jasmonate signaling (Fig. 5). As evidenced by the extremely reduced susceptibility of *coi1* mutants (Feys *et al.* 1994; Kloek *et al.* 2001; Zhao *et al.* 2003), activation of jasmonate signaling is a critical aspect of *P. syringae* virulence. It is not unreasonable to assume that the pathogen might have evolved more than one means to stimulate signaling through this pathway to

insure its ability to colonize its plant hosts. This hypothesis is supported by evidence that some type three secreted effectors may require *COI1* function to activate a marker of susceptibility (He *et al.* 2004). Comparative analysis of gene expression during infection of plant mutants defective in jasmonate signaling and by bacteria unable to synthesize COR might offer insights into jasmonate-dependent processes necessary for proper infection that do not require the presence of COR. Presumably, any genes involved in such processes would show altered expression in *jin1* plants as compared to wild-type but not in plants infected with COR-deficient bacteria.

Overall, our data provide new insight into the physiological changes *P. syringae* fosters in *A. thaliana* in order to create a suitable environment for bacterial growth and disease development. The ability to co-opt the plant's own signaling networks to prevent it from mounting an effective defense suggests a combination of both complexity and subtlety in this interaction. Future studies in this area will doubtless yield more information about signaling interactions within the plant system as well as those between the plant and pathogen.

MATERIALS AND METHODS

Bacterial strains

The bacterial pathogen *Pseudomonas syringae* pv. *tomato* strain DC3000 has been described previously (Cuppels 1986). Bacteria were grown on King's B media (KB; King *et al.* 1954) or NYG (Daniels *et al.* 1988) containing 50ug ml^{-1} rifampicin at 28°C.

Plant materials, growth conditions, and inoculation procedures

Arabidopsis thaliana ecotype Colombia (Col-0) was used in this study. The *jin1-1 gl1* mutant line (Berger *et al.* 1996) was obtained from Susanne Berger and the *jar1-1* mutant line (Staswick *et al.* 1992) from the Arabidopsis Biological Resource Center (ABRC). The male sterile *coi1-20* (Kloek *et al.* 2001) line was maintained as a heterozygous stock. The *nahG* transgenic line (Reuber *et al.* 1998) was obtained from Peter Yorgey and Fred Ausubel, and the *sid2-2* (*eds16*) line (Dewdney *et al.* 2000; Wildermuth *et al.* 2001) was obtained from Mary Wildermuth. The *axr1-12* line was obtained from ABRC. This allele was chosen because it is believed to be a null allele (Lincoln *et al.* 1990). The *JMT* overexpressor line (Seo *et al.* 2001) was obtained from Scigen Harvest Company, Ltd., Seoul, Korea.

Plants were grown from seed in growth chambers with an 8 hr photoperiod at 22°C and 75% relative humidity with light intensity of 140 to 160 μEin s^{-1} m^{-2}. All plants used for virulence studies were approximately four weeks old at the time of infection. All infections were carried out using dip inoculations conducted by immersing whole rosettes

into bacterial suspensions of approximately $5x10^8$ cfu ml^{-1} containing 0.02% (v/v) Silwet L-77 (OSi Specialties Inc., Danbury, CT, USA) and 10mM $MgCl_2$, as described previously (Kunkel *et al.* 1993). To monitor bacterial populations within the plant, individual rosette leaves were removed 0, 2, and 4 days post inoculation. For the day 0 time point, leaf tissue was sampled approximately 2 hr after inoculation. Leaves were weighed, surface-sterilized in 15% (v/v) H_2O_2 for 5 to 10 min, and rinsed three times with sterile water. Leaves were then homogenized, and appropriate dilutions were plated on NYG medium containing rifampicin as described above. Plates were incubated at 28°C for 48 hours before counting colony forming units (cfu).

Methyl jasmonate and coronatine root inhibition assays

The sensitivity of seedlings to MeJA and COR was assayed by germinating sterilized seeds on one-half strength Murashige and Skoog (1/2x MS; Murashige and Skoog 1962) plates (pH 6.0, 1% (w/v) agar, 1% (w/v) sucrose) containing 10 μM MeJA (Sigma Aldrich) or 0.1 μM coronatine (C. Bender, OKSU). Seedlings were grown vertically on square plates. To ensure that the roots remained completely within the agar, an approximately one-inch thick section of agar was removed from the top of each plate, and seeds were placed on the resulting cut surface. After two days of cold treatment in the dark, plates were placed vertically in a growth chamber such that roots grew downwards through the agar. Digital images of the plates were taken after 10 days of growth in continuous light, and roots were measured using NIH Image (Research Services Branch of the National Institute for Mental Health).

Coronatine sensitivity in leaf tissue

Coronatine sensitivity of leaf tissue was measured in leaves of 4 week old plants. Leaves were syringe-infiltrated with either 5 nM COR dissolved in 20% (v/v) methanol or a mock solution containing 20% (v/v) methanol in water. Infiltration was conducted so that approximately half of the leaf area was saturated with the solution. Seven days later, the leaves were harvested and weighed, and areas of anthocyanin production were noted. Pigments were extracted by shaking overnight at 4°C in 500 µl of methanol containing 1% (v/v) HCl (Rabino and Mancinelli 1986). Absorbance of the extracted solution was measured at 530 nm and 657 nm. Anthocyanin levels for each leaf were calculated as A_{530}-(0.25 x A_{657})/(g fresh weight) to correct for absorption by chlorophyll (Rabino and Mancinelli 1986).

Generation of *A. thaliana* lines used in this study

Creation of *jin1 GL1* lines

Homozygous *jin1-1 gl1* plants were crossed to wild-type Col-0 plants. The resulting F1 plants were allowed to self-pollinate and their seed was harvested and planted. From this population, 112 of the resulting F2 plants were examined for the presence of trichomes and assayed for disease susceptibility by dip inoculation as described above. Of these plants, 60 exhibited wild-type levels of susceptibility and trichomes, 20 were susceptible and lacked trichomes, 21 exhibited both reduced disease susceptibility and the presence of trichomes, and six exhibited reduced disease susceptibility while lacking trichomes.

This is as predicted for Mendelian segregation of two independent loci (χ^2 1.524, $p > 0.5$). F3 progeny from plants with trichomes that exhibited reduced susceptibility were then scored for the presence of trichomes and for disease susceptibility and plants that were *jin1 GL1* were identified based on reduced disease susceptibility and presence of trichomes.

To verify cosegregation of reduced disease susceptibility with JA insensitivity, F2 seed from this cross was grown on 1/2x MS media containing MeJA as described above. Seedlings were scored for JA insensitivity and then 50 exhibiting JA insensitivity and 50 with wild-type sensitivity were transplanted to soil for disease susceptibility assays. All (100%) of JA insensitive plants also exhibited reduced susceptibility to *Pst*DC3000, while 100% of those with wild-type sensitivity also developed wild-type disease symptoms. Thus, the JA insensitivity and reduced disease susceptibility phenotypes cosegregate in this population.

Identification of *jin1 jar1* double mutants

Approximately 80 F2 progeny from a cross between homozygous *jin1-1* and *jar1-1* mutants were assayed for disease susceptibility using dip inoculations as described above. Of these, 24 (~25%) F2 plants were homozygous for the *jin1-1* mutation, based on their reduced susceptibility to infection. F3 progeny from several of these homozygous *jin1-1* plants were screened to identify *jar1-1* homozygotes via derived cleaved amplified polymorphic sequence (dCAPS) analysis (Neff *et al.* 1998) using the following PCR primers: jar1For (5' CAA TGG AAA CGC TAC TGA CCC TGA 3') and jar1Rev (5'

ATA AAC TTT GGA CGG CTT TGA CTA GTT CTA 3'). The resulting 250 bp fragment was then cleaved by *Xba*I to reveal a polymorphism present in wild-type *JAR1* and absent in *jar1-1* mutant plants.

Identification of *jin1 nahG* lines

Homozygous *jin1-1 gl1* plants were crossed to Col-0 *nahG* plants in which the *nahG* transgene had been inserted in a T-DNA construct also containing kanamycin resistance. Segregating F2 seed was plated on 1/2x MS agar containing kanamycin (50 μg ml^{-1}) and 10 μM MeJA. Of the 142 seedlings plated, 100 (~75%) individuals remained green on 1/2x MS Kan plates, indicating the presence of the kanamycin resistance gene present in the T-DNA construct carrying the *nahG* gene. Of these green seedlings 22 (~25%) individuals exhibited insensitivity to MeJA and were transplanted and grown for seed. F3 progeny from these plants were assayed on 1/2x MS agar containing Kan to distinguish *nahG* homozygotes from heterozygous lines segregating for this trait. F3 seedlings were also grown on 10μM MeJA to confirm the *jin1-1* phenotype of JA insensitivity.

Identification of *jin1 sid2* double mutants

F2 seedlings of a cross between *jin1-1* and *sid2-2* homozygous plants were grown on 1/2x MS plates containing 0.01 μM COR, as described above. Seedlings that exhibited JA insensitivity were transplanted to soil and allowed to self-fertilize. F3 populations derived from each of these individuals were grown on 10μM MeJA, as described above, to confirm the presence of the *jin1-1* mutation. *sid2-2* homozygous plants were identified

from these lines by PCR designed to amplify a region in exon IX of the *ICS1* gene that contains a 50 bp deletion in *sid2-2* mutant plants (Wildermuth *et al.* 2001). This was done using primers ICS1F (5' GCT CTG CAG CTT CAA TGC TT 3') and ICS1R (5' CGA AGA AAT GAA GAG CTT GGA AAT G 3'). PCR products were resolved on a 3% (w/v) agarose gel using Tris-Borate-EDTA (TBE) running buffer. Wild-type plants yielded a product of approximately 250 bp, while *sid2-2* mutants yielded a product of approximately 200 bp. Plants heterozygous for the *sid2-2* mutation were identified by the presence of both bands.

RNA isolation and Northern analysis

Leaf tissue harvested from approximately six individual inoculated *A. thaliana* plants was pooled for each time point and stored at -80°C until all samples were obtained. Total RNA was isolated using RNAWiz (Ambion). RNA gel-blot analysis was carried out according to Sambrook *et al* (Sambrook *et al.* 1989). Total RNA (7 μg in 5A, 10 μg in 5B) was loaded in each lane. Hybridization probes were prepared using the Prime-it II kit (Stratagene, LA Jolla, CA, USA). The *A. thaliana* cDNAs corresponding to the *LOX2*, *CORI1*, *PDF1.2*, and *PR-1* genes were used as probes (Bell and Mullet 1993; Benedetti *et al.* 1998; Penninckx *et al.* 1996). The RNA blots were analyzed using a phosphorimager (BioRad Personal Molecular Imager FX).

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Zhao, Y., Thilmony, R., Bender, C. L., Schaller, A., He, S. Y. and Howe, G. A. 2003. Virulence systems of *Pseudomonas syringae* pv. *tomato* promote bacterial speck disease in tomato by targeting the jasmonate signaling pathway. Plant J. 36: 485-499.

Table 1: Quantification of disease symptoms on wild-type, *jin1*, *sid2*, and *jin1 sid2* plants 4 days after infection

	Arabidopsis genotype			
Disease symptoms[a]	wild-type	*jin1-1*	*sid2-2*	*jin1 sid2*
No disease	0 (0%)	8 (33%)	0 (0%)	0 (0%)
Chlorosis only	0 (0%)	16 (67%)	0 (0%)	3 (13%)
Few individual lesions	1 (6%)	0 (0%)	0 (0%)	18 (78%)
Many lesions	17 (94%)	0 (0%)	32 (100%)	2 (9%)
Total plants examined	18	24	32	23

[a]Plants were dip inoculated with *Pst*DC3000 and symptoms were examined 4 dpi. "Lesions" refers to the individual water-soaked lesions that typically develop following dip inoculation. Data presented reflect the number of plants of each genotype exhibiting each type of disease symptom. The percentage of the total plants of each genotype that this number respresents is provided in parentheses. Similar results were seen in a second independent experiment. These data correspond to the experiment shown in Fig. 6B.

Figure 1

Growth of *Pseudomonas syringae* pv. *tomato* strain DC3000 in *Arabidopsis thaliana* jasmonate insensitive mutants following dip inoculation.

A. Growth in Col-0 wild-type (circle), *coi1-20* (open box), *jar1-1* (closed box), and *jin1-1 gl1* (triangle) plants. **B.** Growth in wild-type, *jin1-1 GL1*, *jar1-1* (all symbols as above), and *jin1-1 jar1-1* double mutants (open circle). Data points represent the average of three (panel A) or four (panel B) replicates +/- SEM. Statistical analysis of day 4 growth data using ANOVA followed by Tukey's method for paired comparisons indicated that *jin1 gl1*, *coi1* (panel A), *jin1 GL1* and *jin1 jar1* (panel B) are significantly different from wild-type ($p<0.05$). Similar results were obtained in at least three additional independent experiments.

Figure 1
Laurie-Berry MPMI

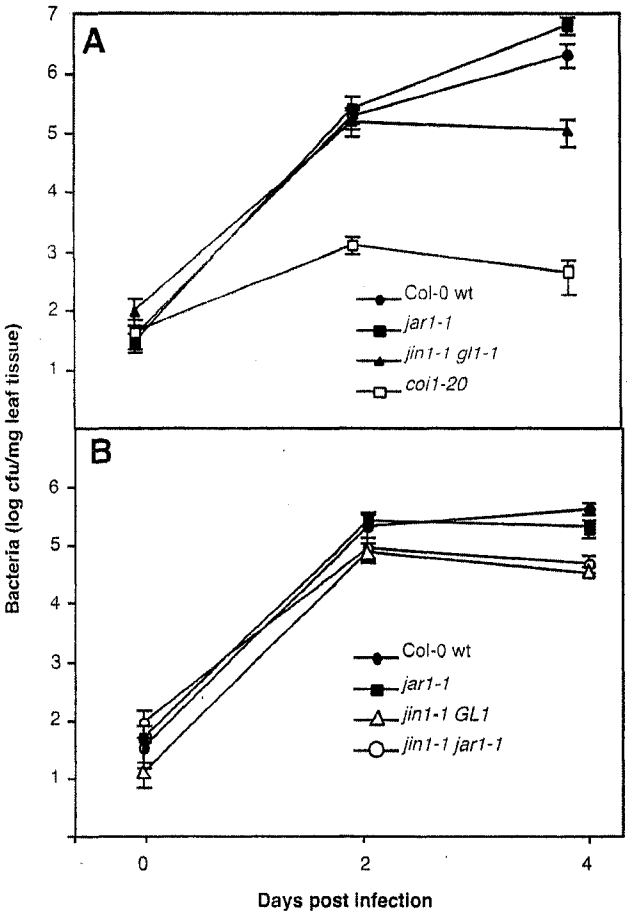


Figure 2

Root growth inhibition of jasmonate insensitive mutants by methyl jasmonate and coronatine.

Root lengths of Col-0 wild-type, *coi1-20*, *jar1-1*, *jin1-1*, and *jin1 jar1* double mutants grown on 1/2x MS media (black bars) or 1/2x MS containing 10 μM MeJA (hatched bars) or 0.1 μM coronatine (white bars). Roots were measured after 10 days of growth. Each value is the average of a minimum of 40 seedlings per treatment, except in the case of *coi1-20* where approximately 10 seedlings were used. Vertical bars represent SEM. The number over each bar represents the length of seedlings on MeJA or COR as a percent of root length when grown on MS. Similar results were obtained in a second independent experiment.

Figure 2
Laurie-Berry MPMI

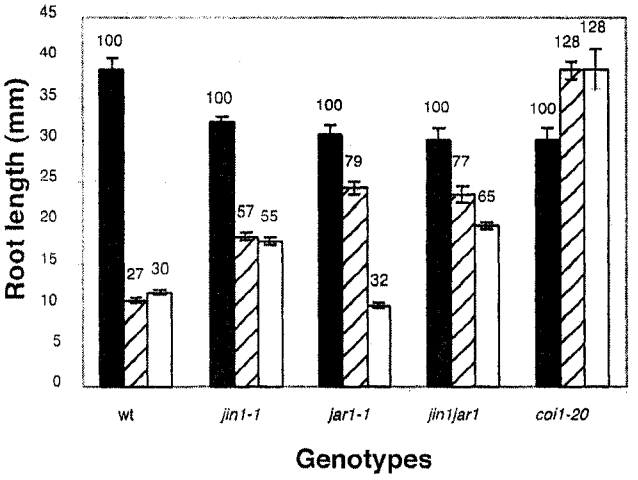


Figure 3

Coronatine-induced anthocyanin accumulation in jasmonate insensitive mutants. Anthocyanin levels in leaves from Col-0 wild-type, *coi1-20*, *jin1-1*, and *jar1-1* plants following infiltration with a 20% (v/v) methanol solution (open bars) or infiltration with 5nM COR in 20% (v/v) methanol (black bars). Samples with absorption values at or below background are represented as 0. Values represent an average of A_{530} readings of six replicates corrected for chlorophyll absorption (see methods). Vertical bars represent SEM. Similar results were obtained in three independent experiments.

Figure 3
Laurie-Berry MPMI

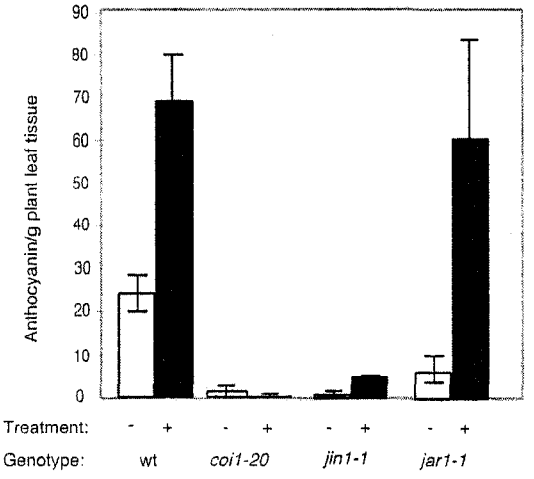


Figure 4

Inhibition of root growth by coronatine on *axr1-12* mutants.

Root lengths of Col-0 wild-type, *coi1-20*, *jin1-1*, and *axr1-12* mutants grown on 1/2x MS plates (black bars) or 1/2x MS plates containing 0.1 μM COR (white bars). Roots were measured after 10 days of growth. Each value is the average of at least 17 seedlings with the exception of *coi1-20* where eight seedlings were used. Vertical bars represent SEM. The number over each bar represents the percent of untreated root length. Similar results were obtained in two independent experiments.

Figure 4
Laurie-Berry MPMI

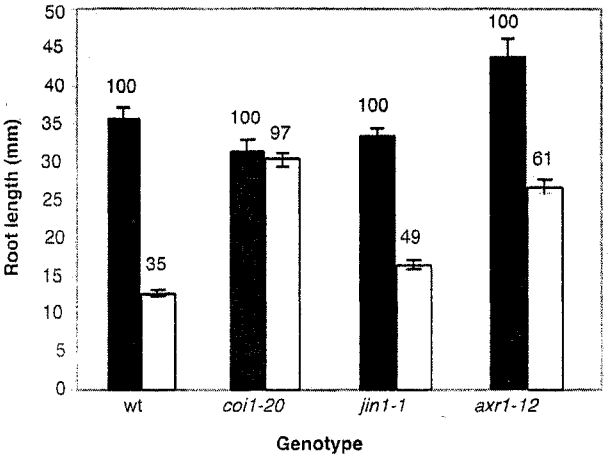


Figure 5

Expression of JA-dependent (*LOX2*, *CORI1*, and *PDF1.2*) and SA-dependent (*PR-1*) defense response genes in wild-type Col-0 and *jin1-1* plants after dip inoculation with *Pst*DC3000.

Data shown in A and B are from two independent experiments. Total RNA was prepared from tissue harvested on the indicated days post inoculation (dpi). Approximately 7 μg of total RNA was loaded for each sample in A and 10 μg in B. Ethidium bromide staining of rRNA is included as a control for equal loading. Similar results were obtained for *PR-1* in a third independent experiment and in a second independent experiment for *PDF1.2*, *LOX2* and *CORI1*.

Figure 5
Laurie-Berry MPMI

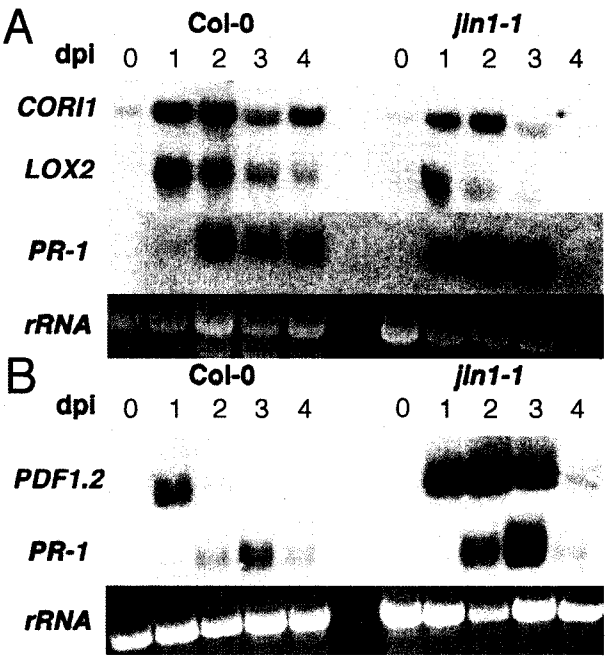
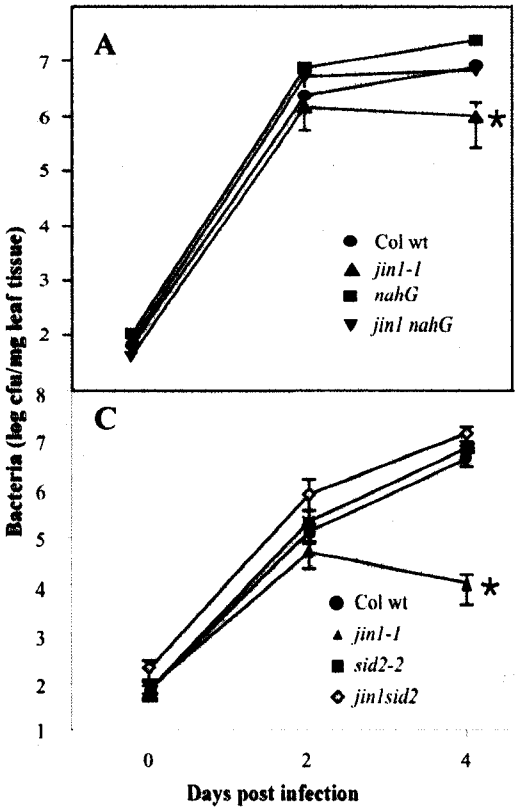


Figure 6

Growth and symptom production in SA-deficient *A. thaliana* plants.

A. Growth of *Pst*DC3000 in Col-0 wild-type (circle), *nahG* (square), *jin1-1* (upright triangle), and *jin1 nahG* (upside down triangle). **B.** Disease symptoms exhibited by Col-0 wild-type, *nahG*, *jin1-1*, *jin1 nahG*, *sid2-2*, and *jin1 sid2* plants four days after dip inoculation with *Pst*DC3000. C. Growth in Col-0 wild-type (circle), *sid2-2* (square), *jin1-1* (triangle), and *jin1sid2* (open diamond). Data points in A and C represent the average of three replicates +/- SEM. Asterisks indicate that day 4 growth is significantly different from wild-type ($p<0.05$) as determined using ANOVA followed by Tukey's method for paired comparisons. In each case, similar results were obtained in an additional independent experiment.


A
Bacteria (log cfu/mg leaf tissue)
Col wt
jin1-1
nahG
jin1 nahG
*
C
Col wt
jin1-1
sid2-2
jin1sid2
*
Days post infection

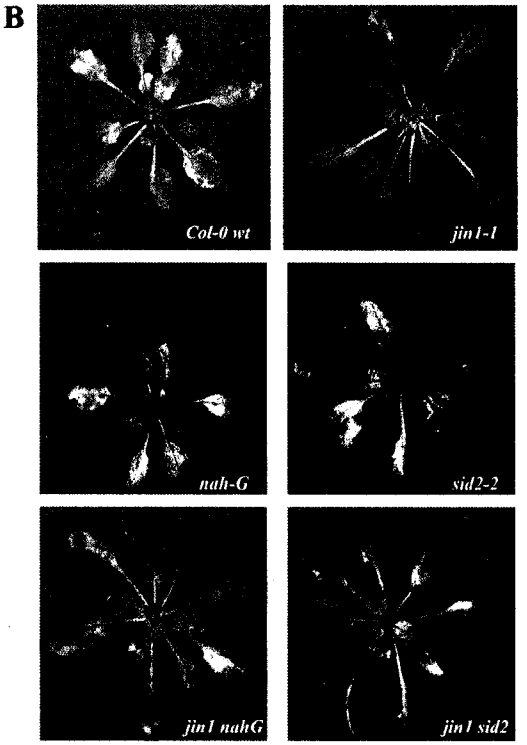

B
Col-0 wt
jin1-1
nah-G
sid2-2
jin1 nahG
jin1 sid2

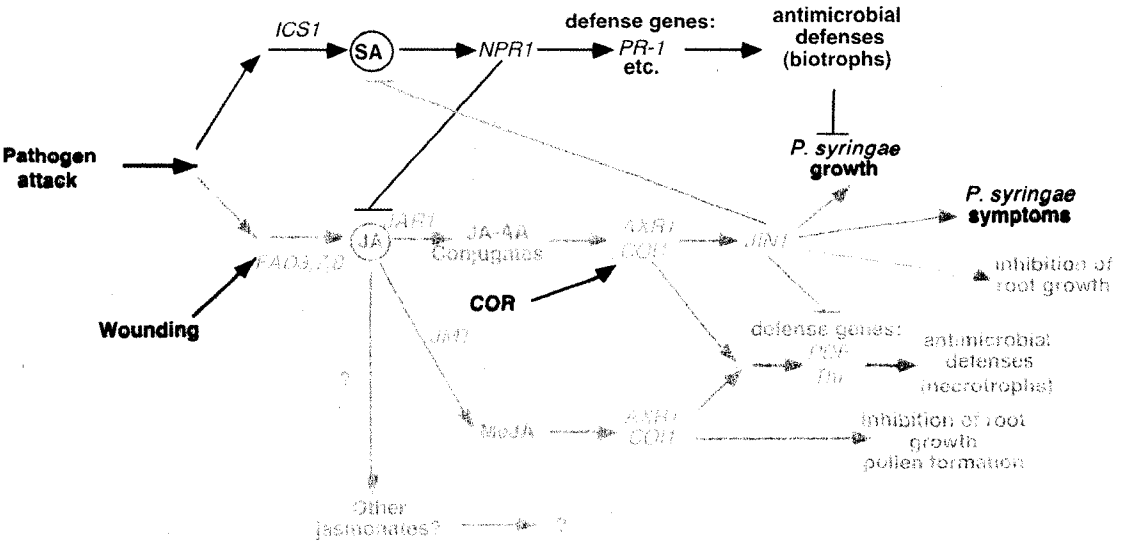
Figure 7

A revised model for the jasmonate and SA defense signaling pathways and their interactions during *P. syringae* infection.

The SA defense signaling pathway is shown in green. *ICS1* is directly involved in pathogen-induced synthesis of SA (Wildermuth *et al.* 2001). *NPR1* is required for most SA-dependent defenses, including expression of *PR-1* and induction of antimicrobial defense responses that limit growth and spread of biotrophic pathogens such as *P. syringae* (Cao *et al.* 1997). The jasmonate signaling pathway is shown in orange. Synthesis of JA is dependent on *FAD3*, *7*, and *8* (Browse *et al.* 1985). *JAR1* and *JMT* encode enzymes that catalyze the formation of modified forms of JA (e.g. JA-amino acid (JA-AA) conjugates and MeJA) that mediate different responses (Seo *et al.* 2001; Staswick and Tiryaki 2004; Staswick *et al.* 2002). The possibility of additional active jasmonates is also indicated. *COI1* and *AXR1* are placed downstream of both MeJA and JA-AA conjugates as *COI1* is required for all known JA responses and *AXR1* is required for proper activity of the SCF^{COI1} complex (Benedetti *et al.* 1998; Benedetti *et al.* 1995; del Pozo *et al.* 2002; Feys *et al.* 1994; Kloek *et al.* 2001; Penninckx *et al.* 1996; Thomma *et al.* 1998; Tiryaki and Staswick 2002). *JIN1* is placed downstream of *COI1* as *jin1* mutants are only impaired in a subset of jasmonate-dependent responses (this work and Berger *et al.* 1996; Boter *et al.* 2004; Lorenzo *et al.* 2004; Nickstadt *et al.* 2004). *JIN1* is required for full susceptibility to *P. syringae* (this study and Nickstadt *et al.* 2004). *JAR1*, but not *JIN1*, is required for defense against the necrotrophic pathogens *B. cinerea* and

Pythium sp. (Ferrari *et al.* 2003; Staswick *et al.* 1998). *JIN1* appears to inhibit these defenses, based on decreased susceptibility of *jin1* mutants to *B. cinerea* (Lorenzo *et al.* 2004; Nickstadt *et al.* 2004) and elevated expression of *PDF1.2* in *jin1* mutants following JA treatment or *P. syringae* infection (this work and Boter *et al.* 2004; Lorenzo *et al.* 2004). MeJA appears to contribute to defense against necrotrophic pathogens as overexpression of *JMT* leads to resistance against *B. cinerea* (Seo *et al.* 2001). Inhibition of jasmonate signaling during SA-mediated defense responses is dependent on *NPR1* and appears to occur through inhibition of JA synthesis (Spoel *et al.* 2003). Inhibition of SA defenses by the jasmonate signaling pathway is dependent on *JIN1* and may occur at the level of SA synthesis or accumulation as *jin1* mutants exhibit increased SA levels (Nickstadt *et al.* 2004). The jasmonate signaling pathway can also be stimulated by wounding or the *P. syringae* phytotoxin COR, which is proposed to be a functional mimic of JA-Ile (Bender *et al.* 1999; Feys *et al.* 1994; Staswick and Tiryaki 2004; Weiler *et al.* 1994).

Figure 7
Laurie-Berry MPMI



Chapter 3

Identification of new JA signaling components involved in DC3000 infection

INTRODUCTION

The jasmonate signaling pathway is a critical virulence target during infection of *Arabidopsis thaliana* by *Pseudomonas syringae* (Kloek *et al.* 2001; Feys *et al.* 1994; Laurie-Berry *et al.* 2006). Despite widespread acceptance of the importance of manipulation of this pathway by the phytotoxin coronatine (COR) during infection (Brooks *et al.* 2004; Laurie-Berry *et al.* 2006; Staswick and Tiryaki 2004), little is known about the actual signaling components involved. Infection is dependent on the presence of a wild-type *COI1* gene in the host plant, as bacterial growth and symptoms are severely decreased in *coi1* mutant plants (Feys *et al.* 1994; Kloek *et al.* 2001). Because *COI1* is responsible for multiple jasmonate-mediated aspects of plant growth and development, in addition to responses to biotic and abiotic stresses (Feys *et al.* 1994; Lorenzo and Solano 2005), *coi1* mutants are not ideal candidates for investigating the specific processes required for successful *P. syringae* infection. The work presented in the previous chapter demonstrates that *JIN1* is also required for successful infection, and this gene is involved in a specific subset of jasmonate responses (Laurie-Berry *et al.* 2006; Lorenzo *et al.* 2004; Nickstadt *et al.* 2004; Berger *et al.* 1996), making it a more useful tool for studying aspects of jasmonate signaling manipulated by COR during infection. However, the partial reduction in susceptibility exhibited by null *jin1* mutants suggests that there are additional unidentified genes with significant roles in this process.

In order to better understand the changes induced by COR in the host plant that may contribute to susceptibility, we felt it was important to identify additional members of the signaling pathway that, like *JIN1*, are manipulated by the toxin during infection.

Our data from the *jin1 NahG* and *jin1 sid2* plants (Chapter 2; Laurie-Berry *et al.* 2006) suggest that jasmonate signaling is critical for disease symptom development through some mechanism independent of inhibition of SA-mediated defenses. I hypothesized that further analysis of the normal physiological role of jasmonate signaling components that respond to COR might provide insight into the effects of COR during the infection process. This chapter provides information on studies I carried out using existing jasmonate signaling and synthesis mutants. It also includes a summary of the results of a screen I initiated to identify new mutants with specific defects in COR sensitivity and preliminary characterization of the resulting mutants.

RESULTS

Analysis of existing jasmonate-related mutants

Many Arabidopsis mutants and transgenic lines with altered production of or responses to jasmonates are reported in the literature, but few of them have been examined with respect to *P. sryingae* infection and none with respect to COR sensitivity. We obtained several of these mutants to test them with respect to these phenotypes. The list of mutants obtained and examined is provided in Table 1, along with their responses to MeJA, COR, and *P. syringae* infection.

As indicated in Table 1, the majority of the jasmonate-related mutants and transgenic lines we studied exhibited levels of susceptibility to P. syringae infection or sensitivity to COR and jasmonate treatment similar to that observed in wild-type plants. The exceptions to this were *cev1*, which was less susceptible to DC3000 infection, 35S::*JIN1*, which was hypersensitive to jasmonate treatment, and *axr1*, which was less susceptible to DC3000 infection and insensitive to jasmonate treatment. The responses of *axr1* plants are described in the previous chapter (Laurie-Berry *et al.* 2006) and are consistent with AXR1 being required for the function of SCF complexes, including the one containing COI1 (del Pozo *et al.* 2002). On the other hand, *cev1* mutants exhibit wild-type responses to COR, which is not surprising given published data indicating that these mutants have a defect in cell wall formation that leads to activation of defenses against a wide variety of pathogens (Ellis *et al.* 2002). As this plant's mutant phenotypes and disease resistance are not specific to the jasmonate signaling pathway, a wild-type response to COR would be expected. The 35S::*JIN1* plants, a transgenic line

constitutively expressing high levels of JIN1, exhibited wild-type levels of susceptibility to DC3000 infection, despite slight hypersensitivity to jasmonate treatment. This is consistent with evidence that JIN1 activity is not regulated solely via transcription, but rather via interaction with negative regulators, such as members of the JAZ family (Chini *et al.* 2007; Thines *et al.* 2007), and potentially additional levels of post-transcriptional regulation (Lorenzo *et al.* 2004; Boter *et al.* 2004). As a result of this more complex regulation, the increased levels of transcript and/or protein in the 35S::*JIN1* line would be insufficient to strongly alter plant phenotypes.

Rationale and design for a screen for mutants with altered COR sensitivity

Ultimately, none of the available jasmonate-related mutants appeared likely to provide any significant new insights into COR-mediated jasmonate signaling or disease susceptibility. Based on this, we decided to conduct a screen specifically looking for mutants with altered responses to COR. Very few screens have been conducted using COR because of the time and expense involved in obtaining the purified toxin which can only be extracted from bacterial exudates and was not commercially available until very recently. The only previous screen using purified COR (Feys *et al.* 1994) did so using relatively high concentrations (1uM), levels toxic to wild-type plants and at which only the insensitive *coi1* mutants survived. We obtained COR from our collaborator, Dr. Carol Bender at Oklahoma State University, making this screen feasible. To avoid the potential limitation of toxicity, we conducted our screen using a relatively low concentration of COR (50 nM) which was inhibitory, but not toxic, to wild-type plants. This allowed us to

identify mutants that were only slightly insensitive to the toxin, similar to *jin1* plants, and also provided the possibility of obtaining mutants that were more sensitive to COR than wild-type plants. The latter consideration was particularly important because mutants with hypersensitivity to COR had not previously been observed and had the potential to represent genes with a negative regulatory role in jasmonate signaling. A flowchart indicating the basic screen design is provided as Figure 1.

The individuals screened consisted of Arabidopsis ecotype Col-0 seeds randomly mutagenized by either EMS treatment or T-DNA insertion. The EMS-mutagenized seeds were divided into 11 lots, each of which contained seeds pooled from approximately 1200 M1 plants with an M1:M2 ratio of 1:8 (Lehle seeds). The T-DNA insertion mutants were obtained as 100 pools, each containing seed from 100 T-DNA lines. At least 1000 M2 seeds were screened from each EMS pool, and at least 100 T3 from each T-DNA pool. In total, over 23,500 mutagenized seedlings were screened for abnormally long or short root growth on media containing 50 nM COR, and 83 putative mutants were identified and transplanted following the primary screen. Of these, 9 did not survive the transplantation process, did not reach maturity, or failed to set viable seed.

The remaining 74 plants were allowed to set self seed which was harvested and subjected to a secondary screen to confirm the abnormal COR response observed in the initial screen. The secondary screen was conducted by growing M3 seed on both media with and without COR. Root lengths in the two conditions were compared to gain a more accurate assessment of each mutant's response to COR. There were multiple factors that necessitated this additional step in the screening process. First, root length can vary

widely even among wild-type plants. In order to ensure that plants with subtle phenotypes would not be overlooked, many plants with small differences from the wild-type were selected in the primary screen. Based on this, we expected a high rate of false positives that would be removed from consideration by the secondary screen. Additionally, the secondary screen allowed us to rule out mutants that had abnormal root growth unrelated to COR sensitivity. For example, several mutants that were tentatively identified as insensitive based on longer root growth in the primary screen were rejected from the secondary screen because they developed abnormally long roots in the absence of COR treatment. Thus, the secondary screen allowed us to identify and eliminate mutants with defects in root growth or germination, unrelated to COR sensitivity. This then allowed us to focus only on the mutants with altered responses that were specific to COR.

Identification of mutants in several known genes and three novel mutants

Following secondary screening, 7 putants remained which exhibited altered root sensitivity. Of these, 4 were insensitive to COR and MeJA and were designated coronatine-insensitive (coi) mutants (Table 2). The remaining 3 were hypersensitive to treatment and designated coronatine-hypersensitive (coh) mutants (Table 2). Of these, 2 of the insensitive mutants, *coi-5* and *coi-27*, were determined to be alleles of *axr1* based on overall morphology, insensitivity to the auxin analog NAA, and complementation tests with the null allele *axr1-12* (Lincoln *et al.* 1990; Tables 2, 3). The remaining insensitive mutants (*coi-11* and *coi-32*) were determined to be alleles of *coi1* and *jin1*, respectively, via complementation tests with known alleles (Tables 2, 3). The 3

hypersensitive mutants (*coh-16*, *coh-23*, and *coh-36*) all exhibit the novel phenotype of coronatine hypersensitivity and are thus likely to represent mutations in new genes not previously recognized as components of jasmonate or COR signaling (Table 2). Two of the *coh* mutants were determined to carry a recessive single gene trait based on Mendelian segregation ratios of the coh phenotype in the F2 generation resulting from back-crossing with wild-type Col-0 plants (Table 3). The *coh* mutants are believed to represent 3 separate genes (Table 3), suggesting that the screen did not reach saturation as most mutants identified represented separate genes. It seems likely that continuation of this screen could yield additional mutants with altered COR responses.

Additional characterization was performed upon the hypsersensitive mutants to further investigate our models and hypotheses about the relationship between COR, jasmonates, and *P. syringae* susceptibility. The *coh-16* mutant was largely excluded from these studies as it exhibited dwarfing and anthocyanin accumulation in the absence of infection or jasmonate treatment, suggesting constitutive activation of jasmonate signaling, something we were not interested in pursuing at this time. Therefore, our characterization focused on *coh-23* and *coh-36*.

Initial characterization of two novel mutants with COR hypersensitivity

Our screen was designed to identify mutants in components of the jasmonate signaling pathway with altered sensitivity to COR. This is based on the hypothesis that COR is acting as a jasmonate mimic, an assumption made because all previous mutants with altered sensitivity to COR have exhibited similar responses to jasmonates, taking

into consideration the relative potencies of the two chemicals (i.e. *coi1*, *jin1*, *axr1*) (Laurie-Berry *et al.* 2006; Uppalapati *et al.* 2005; Weiler *et al.* 1994). However, we felt that it was possible that our hypothesis was too simplistic and that a mutant could exist with an altered response to COR that was not correlated with its jasmonate sensitivity. To examine this possibility, we tested *coh-23* and *coh-36* mutants for sensitivity to MeJA across a range of concentrations. As expected based on the results of experiments with the *coi1*, *jin1*, and *axr1* mutants presented in the previous chapter, both *coh* mutants also exhibited hypersensitivity to MeJA (Fig 2). Interestingly, the differences between wild-type and *coh-23* were more pronounced at lower concentrations of MeJA, with barely 5% difference at 10uM MeJA (data not shown) and differences increasing to 8% at 5uM MeJA and nearly 13% at 10uM MeJA (Fig 2A). In contrast, *coh-36* exhibited a stronger degree of hypersensitivity, 10-15% different from wild-type at all concentrations tested (Fig 2B).

Based on this increased sensitivity to jasmonate treatment, we are proposing that these mutants be renamed to jasmonate hypersensitive (jah) rather than coh. We believe that this change reflects the greater physiological relevance of altered response to an endogenous plant hormone rather than a bacterial phytotoxin. Thus, we will now refer to mutants *coh-36* and *coh-23* and *jah1* and *jah2*, respectively.

We also carried out preliminary experiments to assay the responses of these mutants to infection with DC3000. Initial results were not conclusive for either mutant (data not shown), presumably due to the presence of unlinked mutations that impacted the plants' responses to infection. *jah2* mutant plants appeared to exhibit slightly more severe

symptoms than wild-type plants, but no corresponding increase in bacterial growth was observed. The *jah1* mutant plants also appeared to exhibit increased disease symptoms compared to wild-type plants, but they spontaneously developed increased chlorosis under mock treatment and at five weeks of age when untreated, indicating that the plants developed spontaneous age-related yellowing independent of infection. These plants did appear to support slightly higher levels of bacterial growth, but it was uncertain what effect the spontaneous chlorotic phenotype might have on conditions for bacterial growth *in planta*. It was decided that further characterization of infection responses in both *jah1* and *jah2* would be conducted only after the lines were backcrossed to remove any unlinked mutations that might be complicating analysis.

Further characterization of *jah2*

A single backcross to wild-type Col-0 was conducted with line *jah2*, and F2 seed from this cross was tested for hypersensitivity to JA to identify individuals retaining the hypersensitive phenotype. As expected for a single, recessive mutation, the trait segregated in a ratio of 3 wild-type plants to 1 hypersensitive (Table 3). Several of the hypersensitive individuals were retained and allowed to set self seed to produce lines homozygous for the *jah2* mutation.

In order to examine the jasmonate sensitivity of the *jah2* mutant plants following backcrossing, several of the mutant lines were plated on media containing 0 or 10 uM MeJA (data not shown). The line with the most pronounced jasmonate hypersensitive phenotype (line 5A) was then plated on multiple concentrations of MeJA. As expected,

the wild-type plants exhibit typical levels of inhibition (Fig 3). Plants from an F3 line from the backcross which is believed to not carry the *jah2* mutation (line 4D) behaves similarly to wild-type plants at all concentrations tested (Fig 3). Surprisingly, the back-crossed *jah2* mutant line 5A does not entirely resemble the original *coh-23* mutant. While the original mutant shows the greatest deviation from wild-type at low concentrations of MeJA (<1uM), this back-crossed line is very similar to wild-type at low concentrations (<1 uM) and increasingly inhibited at higher concentrations (5-10 uM) (Fig 3). The growth of these plants is severely impaired on 10 uM MeJA, producing very short roots (an average of 5.5 mm compared to 20 mm for wild-type) and stunted shoots and leaves that accumulate high levels of anthocyanin (data not shown). This hypersensitive response is significantly more pronounced than that observed in the original *coh-23* mutant plants, suggesting that back-crossing may have removed an unlinked mutation that was partially masking this phenotype.

This F3 line was then infected with DC3000 using hand inoculation (Fig 4). As expected, wild-type Col-0 plants support high levels of bacterial growth, around 10^8 cfu/g fresh tissue. Contrary to the increased susceptibility expected based on preliminary experiments, the *jah2* mutant line exhibits either reduced susceptibility (Fig 4A) or wild-type responses (Fig 4B), supporting bacterial levels between 10^7 and 10^8 cfu/g fresh tissue. Although there is variation, two other lines (1A and 5B) were also observed to support lower levels of bacterial growth than wild-type plants four days after infection (Fig 4A). However, a backcrossed line which does not exhibit jasmonate hypersensitivity (line 4D) also exhibits reduced susceptibility, supporting around 10^7 cfu/g fresh tissue

(Fig 4). This suggests that the reduced susceptibility to infection may result from a second mutation that is unlinked to the *jah2* mutation causing jasmonate hypersensitivity. This observation, along with the presence of enhanced susceptibility in the original mutant, underscores the importance of carrying out multiple rounds of back-crossing with a newly-obtained mutant line to remove additional mutations that may be present. I believe that the initial eds phenotype was a result of an additional mutant rather than experimental variation as siblings from the F2 population that were not homozygous for *jah2* appeared to be segregating a trait for enhanced symptom production (data not shown). Further back-crossing is required to determine whether the *jah2* mutation has an impact on DC3000 disease susceptibility and, if so, what that effect is.

DISCUSSION

Survey of existing jasmonate mutants

The results of our analysis of various previously described jasmonate signaling mutants (Table 1) support the hypothesis drawn from previous mutant studies. As expected based on the wild-type response of *jar1* muants to infection and COR treatment (Chapter 2; Laurie-Berry *et al.* 2006; Nickstadt *et al.* 2004; Kloek *et al.* 2001), mutation of genes involved in jasmonate synthesis, such as *OPR3*, had no effect on the plant's susceptibility to DC3000 infection or sensitivity to COR. This agrees with the theory that COR acts as a molecular mimic of JA-Ile to manipulate jasmonate signaling in a process that does not require JA synthesis (Laurie-Berry *et al.* 2006). Also not surprising was the wild-type response of *cev1* mutant plants to COR; this mutation upregulates jasmonate synthesis (Ellis and Turner 2001) and thus does not directly affect the signaling pathway manipulated by the phytotoxin. The reduced susceptibility of this mutant to infection is likely due to the constitutive elevation of both jasmonate- and salicylic acid-mediated defenses as a result of altered cell wall development (Ellis *et al.* 2002).

An additional conclusion that can be drawn from this survey is that previous approaches for identifying mutants involved in jasmonate signaling have failed to identify new signaling components. Several of the mutants included in this survey (e.g. *cev1, joe1&2, jue1&2*) were identified based on altered expression of a single jasmonate-dependent gene. Our results, along with the identification of *CEV1* as a gene involved in cell wall structure (Ellis *et al.* 2002), suggest that this molecular approach may not be the best method to identify novel genes specific to jasmonate signaling. This is not

completely surprising given our limited understanding of the pathway and the specific control of downstream genes; because it is not entirely certain how the marker genes are regulated, it is difficult to predict the sort of mutations that will alter their expression. Based on this, we chose to conduct a traditional screen using a physiological response that we hoped would be more directly related to jasmonate signaling.

Results of mutant screen

As mentioned in the rationale for the screen design, COR hypersensitivity is a phenotype that had not been described prior to this work. Thus, the two non-allelic mutants identified with this phenotype (*jah1* and *jah2*) are very likely to represent genes not previously appreciated to have a role in jasmonate signaling. This identification of at least one completely novel gene (see Chapter 4) involved in jasmonate signaling demonstrates that our screen is a viable method to investigate this hormone signaling pathway. We obtained both insensitive and hypersensitive mutants, demonstrating that intermediate hormone concentrations can be used to screen for both phenotypes simultaneously. Also, the identification of multiple *jah* mutants supports previous studies (Chini *et al.* 2007; Thines *et al.* 2007) that indicate that negative regulation is an important means of controlling jasmonate signaling. It is likely that further screening could identify additional genes involved in jasmonate signaling, as we did not attempt to saturate the mutant populations being examined. The fact that the seven mutants identified represented six separate genes supports this idea and suggests that continuing this screen would likely identify additional mutants with altered responses to COR and/or

jasmonic acid. However, as all four *coi* mutants represent known genes, it is possible that there are few unknown genes with a positive role in jasmonate signaling that can be identified using this approach.

jah2 may represent a gene involved in JIN1-independent signaling

The identification of a mutant with hypersensitivity to jasmonates and possibly reduced susceptibility to DC3000 was largely unexpected. We had hypothesized that hypersensitivity would correlate with increased disease susceptibility, as the inverse of the correlation between insensitivity and reduced susceptibility observed in mutants like *jin1* and *coi1* (Laurie-Berry *et al.* 2006; Nickstadt *et al.* 2004; Kloek *et al.* 2001; Feys *et al.* 1994). This expectation was also supported by the critical role of COR in DC3000 virulence, which would suggest that increased responsiveness to COR would result in greater susceptibility to the pathogen. The observation that *jah2* mutant plants may deviate from this expectation raises the possibility that the gene affected in these mutants may regulate a different branch of jasmonate signaling than we had previously anticipated. Given that our screen relied on root sensitivity, a response which is not completely dependent on *JIN1* (Laurie-Berry *et al.* 2006; Berger *et al.* 1996; Lorenzo *et al.* 2004), this is not entirely surprising.

Figure 5 shows a simplified model of the jasmonic acid signaling pathway, focusing particularly on the processes governed by the jasmonic acid conjugate JA-Ile that COR is believed to mimic (Staswick and Tiryaki 2004). The most significant feature of this pathway is that it is branched, possibly to allow complex regulation or integration

of signals from other hormone pathways. Signaling through the *JIN1*-dependent branch results in susceptibility to DC3000 infection and expression of some downstream genes, such as *CORI1/CHL1* and *VSP1* (Laurie-Berry *et al.* 2006; Lorenzo *et al.* 2004; Boter *et al.* 2004). In contrast, *JIN1*-independent signaling is responsible for defenses against several necrotrophic fungal pathogens and expression of genes correlated with those defenses, such as *PDF1.2* (Laurie-Berry *et al.* 2006; Lorenzo *et al.* 2004; Boter *et al.* 2004; Nickstack *et al.* 2004). *JIN1*-dependent signaling is also able to suppress signaling through the *JIN1*-independent branch, as demonstrated by *jin1* mutants that exhibit resistance to some fungal pathogens (Lorenzo *et al.* 2004; Boter *et al.* 2004; Nickstadt *et al.* 2004) and increased expression of *PDF1.2* and similar genes (Laurie-Berry *et al.* 2006; Lorenzo *et al.* 2004; Boter *et al.* 2004; Nickstadt *et al.* 2004).

We propose that the *JAH2* gene may encode a negative regulator located on or otherwise impacting the *JIN1*-independent branch. The coh phenotype suggests that the gene must be a negative regulator located downstream of JA-Ile perception. However, the mutant's potential reduced susceptibility to DC3000 infection makes it unlikely that the gene is directly involved in *JIN1*-dependent signaling. This phenotype would be more readily explained if we postulate that there is mutual inhibition between the two branches and that *JAH2* is a negative regulator that represses the inhibition of *JIN1*-dependent signaling. This would result in the observed phenotype of reduced susceptibility in the *jah2* mutants as the repression of *JIN1*-independent signaling would be lost, allowing further inhibition of *JIN1*-dependent signaling and resulting in a response to infection similar to that observed in *jin1* mutant plants (fig 5).

If the reduced susceptible phenotype is retained following additional backcrossing and this hypothesis is correct, this would make the *JAH2* gene very interesting for further study as there are currently no genes known to be involved in *JIN1*-independent signaling. Indeed, the existence of this branch has not been conclusively proven, only inferred (Laurie-Berry *et al.* 2006; Lorenzo *et al.* 2004; Boter *et al.* 2004). Identification of a gene that plays a role in *JIN1*-independent signaling would be very valuable in understanding the complexities of jasmonate signaling as a whole.

MATERIALS AND METHODS

Bacterial strains

The bacterial pathogen *Pseudomonas syringae* pv. *tomato* strain DC3000 has been described previously (Cuppels 1986). Bacteria were grown on King's B media (KB; King *et al.* 1954) or NYG (Daniels *et al.* 1988) containing 50ug ml^{-1} rifampicin at 28°C.

Plant materials, growth conditions, and inoculation procedures

Arabidopsis thaliana ecotype Colombia (Col-0) was used in this study, except where indicated otherwise. The *joe1*, *joe2*, *jue1*, and *jue2* mutants were obtained from Anders B. Jensen (Jensen *et al.* 2002). *opr3* seed was obtained from John Browse (Stintzi and Browse 2000). The *cev1* mutant and its parental line were obtained from John Turner (Ellis and Turner 2001). The 35S::*JMT* line (Seo *et al.* 2001) was obtained from Scigen Harvest Company, Ltd., Seoul, Korea. We received *eds8* mutant seed and its parental line from Fred Ausubel (Glazebrook *et al.* 1996). The *sgt1* mutant was received from Roger Innes (Tor *et al.* 2002). We obtained *ein3-3* and 35S::*JIN1* from Roberto Solano (Solano *et al.* 1998; Lorenzo *et al.* 2004) and *erf4* from Kemal Kazan (McGrath *et al.* 2005). We obtained *wrky70-1* and *-2* seed from Zhixiang Chen (Li *et al.* 2004). The *axr1-12* line was obtained from ABRC. This allele was chosen because it is believed to be a null allele (Lincoln *et al.* 1990). The EMS-mutagenized seed used in the screen was purchased from Lehle Seed Company (Round Rock, TX). The random T-DNA insertion lines were obtained from ABRC (Ohio State University, Columbus, OH) as pools of T3 seed.

Plants were grown from seed in growth chambers with an 8 hr photoperiod at 22°C and 75% relative humidity with light intensity of 140 to 160 μEin s^{-1} m^{-2}. All plants used for virulence studies were approximately four weeks old at the time of infection.

Infections were carried out using either dip inoculations or hand infiltration, as indicated. Dip inoculations were conducted by immersing whole rosettes into bacterial suspensions of approximately $5x10^8$ cfu ml^{-1} containing 0.02% (v/v) Silwet L-77 (OSi Specialties Inc., Danbury, CT, USA) and 10mM $MgCl_2$, as described previously (Kunkel *et al.* 1993). Hand infiltrations were carried out by using a 1ml needleless syringe to flood half of the leaf with a bacterial suspension of approximately $5x10^6$ cfu ml^{-1} in 10mM $MgCl_2$. To monitor bacterial populations within the plant, individual rosette leaves were removed 0, 2, and 4 days post inoculation. For the day 0 time point, leaf tissue was sampled approximately 2 hr after inoculation. In the case of dip inoculations, these leaves were surface sterilized in 15% (v/v) H_2O_2 for 5 to 10 min, and rinsed three times with sterile water. Leaves were weighed and homogenized, and appropriate dilutions were plated on NYG medium containing rifampicin. Plates were incubated at 28°C for 48 hours before counting colony forming units (cfu).

Methyl jasmonate and coronatine root inhibition assays

The sensitivity of seedlings to MeJA and COR was assayed by germinating sterilized seeds on one-half strength Murashige and Skoog (1/2x MS; Murashige and Skoog 1962) plates (pH 6.0, 1% (w/v) agar, 1% (w/v) sucrose) containing concentrations of MeJA (Sigma Aldrich) or coronatine (C. Bender, OKSU) as indicated. Seeds were sterilized by

immersion for 5 minutes in 70% (v/v) ethanol containing 0.05% (v/v) Triton X100, followed by immersion for 5 minutes in 95% (v/v) ethanol containing 0.05% (v/v) Triton X100, and then a final immersion for 3 minutes in 95% (v/v) ethanol. Seedlings were grown vertically on square plates. To ensure that the roots remained completely within the agar, an approximately one-inch thick section of agar was removed from the top of each plate, and seeds were placed on the resulting cut surface. After two days of cold treatment in the dark, plates were placed vertically in a growth chamber such that roots grew downwards through the agar. Digital images of the plates were taken after 10 days of growth in continuous light, and roots were measured using NIH Image (Research Services Branch of the National Institute for Mental Health).

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Table 1: Jasmonate signaling mutants and transgenic lines analyzed in this study

Genotype	COR sensitivity[a]	MeJA sensitivity[a]	Response to DC3000	Reference
Col-0 wt	sensitive	sensitive	susceptible	
joe1	nt	nt	susceptible	Jensen *et al.* 2002
joe2	nt	nt	susceptible	Jensen *et al.* 2002
jue1	nt	nt	susceptible	Jensen *et al.* 2002
jue2	nt	nt	susceptible	Jensen *et al.* 2002
opr3	nt	nt	susceptible	Stintzi and Browse 2000
cev1	nt	sensitive	resistant	Ellis and Turner 2001
35S::*JMT*[b]	nt	nt	susceptible	Seo *et al.* 2001
wrky70-1, -2	nt	sensitive	nt	Li *et al.* 2004
erf4-1	nt	sensitive	susceptible	McGrath *et al.* 2005
ein3-3	nt	sensitive	nt	Solano *et al.* 1998
sgt1	nt	nt	susceptible	Lorenzo and Solano 2005
35S::*JIN1*[b]	nt	hypersensitive	susceptible	Lorenzo *et al.* 2004
axr1-12	insensitive	insensitive	resistant	Lincoln *et al.* 1990
eds8	sensitive	sensitive	enhanced symptoms	Glazebrook *et al.* 1996

nt = not tested
a = based on response in seedling root length assay
b = transgenic plants constitutively over-expressing the indicated genes under control of the CaMV 35S enhancer

Table 2: Mutants identified through screening for altered COR response

Mutant	COR response	JA response	Notes
coi-5	insensitive	nt	allelic to *axr1*
coi-11	insensitive	nt	allelic to *coi1*
coi-27	insensitive	nt	allelic to *axr1*[a]
coi-32	insensitive	nt	allelic to *jin1*
coh-16	hypersensitive	nt	dwarfed, constitutive anthocyanin
coh-23	hypersensitive	hypersensitive	recessive single gene
coh-36	hypersensitive	hypersensitive	recessive single gene

nt = not tested
a = allelism assigned based on phenotypes only

Table 3: Phenotypic segregation data for crosses of *coi* and *coh* mutants

Cross		Phenotype[a]		Chi square	p value
		wt	mutant		
coi-5 x *axr1-12*	F1	0 (0%)	4 (100%)		
coi-11 x *coi1-20*	F1	0 (0%)	6 (100%)		
	F2	2? (4%)	42 (96%)	47.8[c]	<0.001
coi-32 x *jin1-1*	F1	0 (0%)	4 (100%)		
	F2	3? (4%)	77 (96%)	89.6[c]	<0.001
coh-23 x wt	F2	41 (82%)	9 (18%)	1.307[b]	.25
coh-23 x *coh-36*	F1	5 (100%)	0 (0%)		

a = jasmonate sensitivity determined via root growth assay
b = Chi-square test using 3:1 wt:mutant ratio expected for segregation of a recessive single gene trait
c = Chi-square test using 9:7 wt:mutant ratio expected for segregation of two independent mutations

Figure 1

Screen design to identify mutants with altered coronatine sensitivity.

In the primary screen, M2 seed was grown on 1/2x MS media containing 50 nM COR. Seedlings with roots that appeared longer or shorter than wild-type control seedlings were transplanted to soil; all others were discarded. M3 seed from transplanted seedlings was then plated on 1/2x MS media with or without COR for the secondary screen to remove false positives. M3 populations that exhibited a degree of inhibition similar to wild-type were discarded. Populations that were proportionally less inhibited than wild-type were classified as *COR insensitive* (*coi*), and those that were proportionally more inhibited than wild-type were classified as *COR hypersensitive* (*coh*). Both groups were retained. The *coi* mutants were then tested for allelism to known coi mutants: *coi1*, *jin1*, and *axr1*, in order to identify any novel *coi* mutants resulting from the screen. The two categories of interest, *coh* mutants and novel *coi* mutants, are indicated by stars.

Screen and Characterization

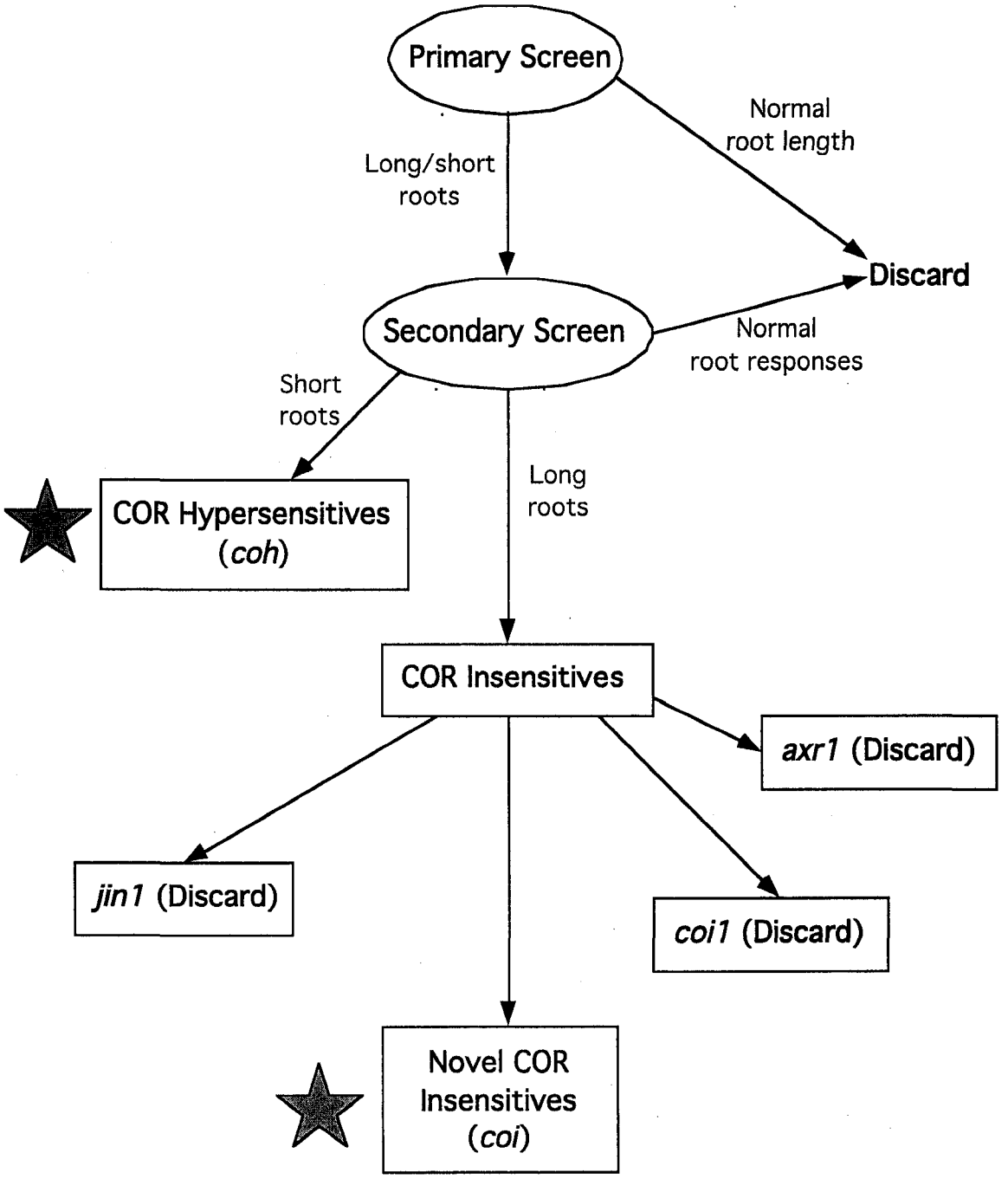
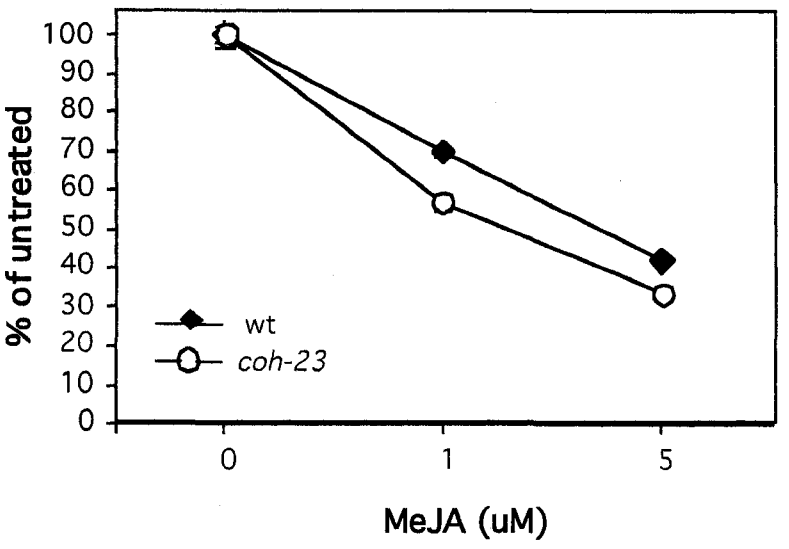


Figure 2

Root growth inhibition of *coh-23* and *coh-36* mutants by methyl jasmonate.

A. Root lengths of Col-0 wild-type (diamond) and original *coh-23* mutant (open circle) grown on 1/2x MS media or 1/2x MS containing 1 or 5 μM MeJA. **B.** Root length of Col-0 wild-type (diamond) and original *coh-36* mutant (open circle) grown on 1/2x MS media or 1/2x MS containing 5 or 10 μM MeJA. Root lengths are presented as a percentage of untreated length for each genotype. Roots were measured after 10 days of growth. Each value is the average of a minimum of 40 seedlings per treatment. Vertical bars representing +/- SEM are obscured by symbols in most cases; these are calculated using normalized percentages rather than raw root length data. Similar results were obtained in two independent experiments.

A


100
90
80
70
60
50
40
30
20
10
0
% of untreated
wt
coh-23
0
1
5
MeJA (uM)

B

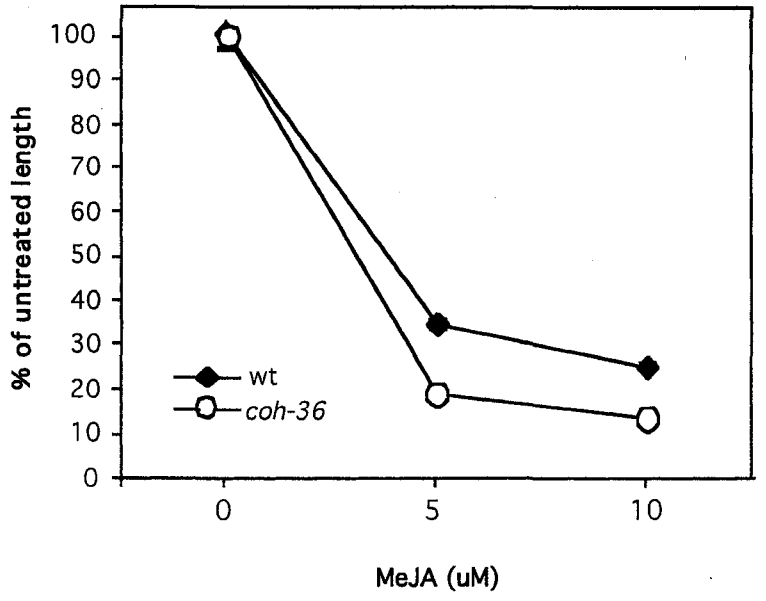

100
90
80
70
60
50
40
30
20
10
0
% of untreated length
wt
coh-36
0
5
10
MeJA (uM)

Figure 3

Root growth inhibition of backcrossed *jah2* mutants by methyl jasmonate.

Root length of Col-0 wild-type (diamond), *jah2* mutants (line 5A, open circle), and a nonmutant sibling line (line 4D, triangle) grown on 1/2x MS media or 1/2x MS containing 0.1, 1, 5, or 10 uM MeJA. Root lengths are presented as a percentage of untreated length for each genotype. Roots were measured after 10 days of growth. Each value is the average of a minimum of 40 seedlings per treatment. Vertical bars representing +/- SEM are obscured by symbols in most cases; these are calculated using normalized percentages rather than raw root length data. Similar results were obtained in two independent experiments.

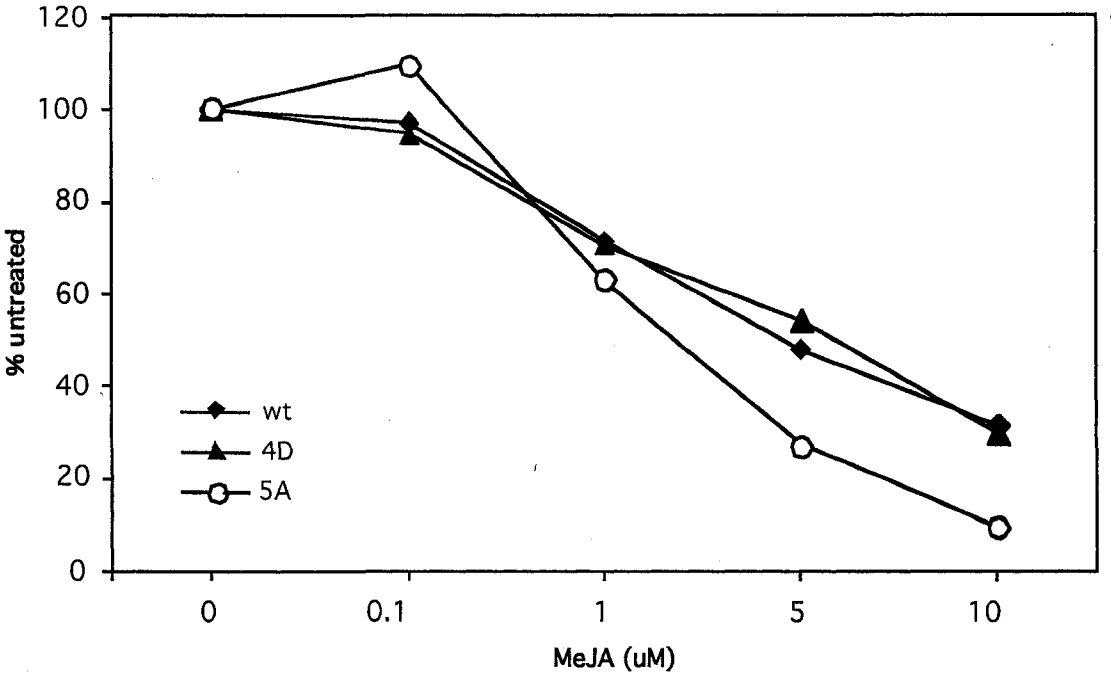
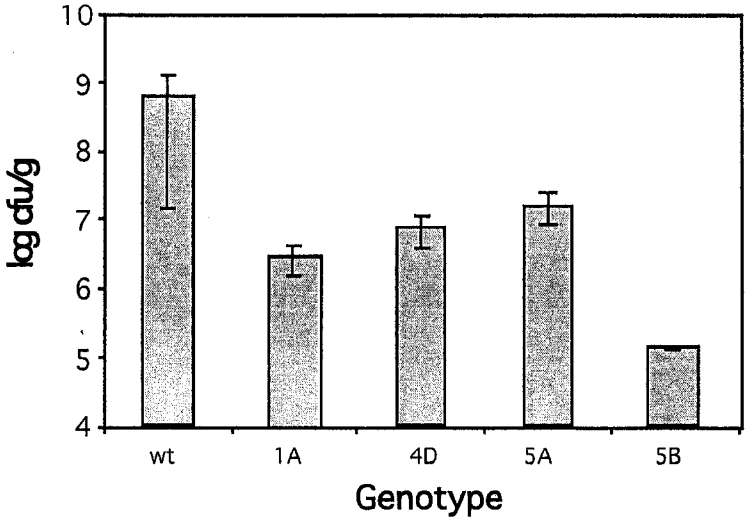

120
100
80
60
40
20
0
% untreated
wt
4D
5A
0
0.1
1
5
10
MeJA (uM)

Figure 4. Growth of *Pseudomonas syringae* pv. *tomato* strain DC3000 in *jah2* mutants following hand infiltration. A. Day 4 growth in wild-type Col-0, three backcrossed *jah2* lines (1A, 5A, 5B) and a nonmutant sibling line (4D). Data points represent the average of three samples, and vertical bars indicate SEM. B. Growth in Col-0 wild-type (diamond), *jah2* (5A, triangle), and nonmutant sibling (4D, open circle) plants over the course of infection. Data points represent the average of three samples +/- SEM. Panels A and B represent two independent experiments.

A


10
9
8
7
6
5
4
log cfu/g
wt
1A
4D
5A
5B
Genotype

B

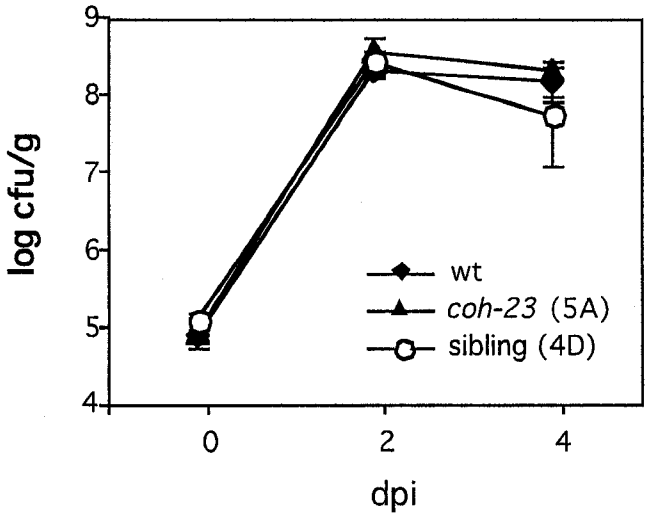
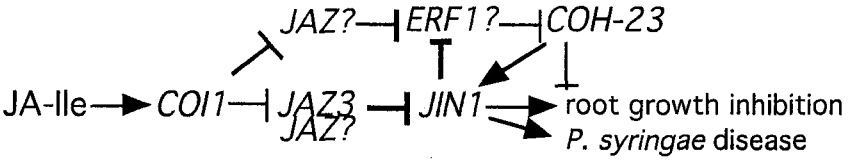

9
8
7
6
5
4
log cfu/g
wt
coh-23 (5A)
sibling (4D)
0
2
4
dpi

Figure 5

Model for jasmonate signaling pathway, including possible placement for *JAH2*. This model focuses on the section of jasmonate signaling controlled by JA-Ile and manipulated by COR during infection by *P. syringae*. In the presence of JA-Ile, COI1 is proposed to ubiquitinate JAZ proteins, resulting in their degradation (Thines *et al.* 2006; Chini *et al.* 2006). This relieves inhibition of transcription factors including JIN1 and possibly ERF1, allowing activation of downstream responses. We propose that *JAH2* may be negatively regulated by *ERF1* in order to mediate interactions between *JIN1*-dependent and *JIN1*-independent signaling processes.


JAZ?
ERF1?
COH-23
JA-Ile
COI1
JAZ3
JAZ?
JIN1
root growth inhibition
P. syringae disease

Chapter 4

Further characterization of *coh-36* mutants

INTRODUCTION

In order to be successful as a pathogen and cause disease on a host plant, an organism must create a suitable environment for growth within the plant. Important components of this process are suppression or evasion of plant defenses (da Cunha *et al.* 2007; Nomura *et al.* 2005) and stimulation of water and nutrient release into the apoplast (Ponciano *et al.* 2003; Alfano and Collmer 1996). One virulence factor that has been shown to be involved in causing these some of these effects during *Pseudomonas syringae* infection is the phytotoxin coronatine (COR; Bender *et al.* 1999).

P. syringae strains unable to produce COR are less able to grow and cause disease on Arabidopsis plants (Brooks *et al.* 2004; Brooks *et al.* 2005; Mittal and Davis 1995). While the entire chain of events by which COR produces these effects is not clear, it is believed to exert its effects by acting as a mimic of the jasmonate family of hormones (Feys *et al.* 1994; Laurie-Berry *et al.* 2006; Chapter 2; Katsir *et al.* 2008; Weiler *et al.* 2004). This hypothesis is supported by structural similarity (Bender *et al.* 1999; Staswick and Tiryaki 2004; Katsir *et al.* 2008) and similar effects of exogenous application of both compounds, such as inhibition of root elongation and production of the stress pigment anthocyanin (Laurie-Berry *et al.* 2006; Chapter 2; Staswick and Tiryaki 2004; Bender *et al.* 1999; Weiler *et al.* 1994; Feys *et al.* 1994). Additionally, the idea that COR is acting as a jasmonate mimic is supported by several Arabidopsis mutants that exhibit altered responses to both COR and jasmonates, including *coi1* (Feys *et al.* 1994; Laurie-Berry *et al.* 2006; Chapter 2) and *jin1* (Laurie-Berry *et al.* 2006; Chapter 2).

The *coi1* and *jin1* mutants are also less susceptible to *P. syringae* infection (Feys *et al.* 1994; Kloek *et al.* 2001; Laurie-Berry *et al.* 2006; Chapter 2; Nickstadt *et al.* 2004), reinforcing the importance of COR for virulence of this bacterium. These data also highlight the importance of the jasmonate signaling pathway for susceptibility to *P. syringae* infection. One hypothesis to explain the role of COR in virulence is based on the well-documented antagonism between jasmonate and salicylic acid (SA) signaling (Spoel *et al.* 2003; Beckers and Spoel 2006; Thaler *et al.* 2002) and proposes that increased jasmonate signaling stimulated by COR results in inhibition of SA-mediated defenses that are protective against *P. syringae* infection (Brooks *et al.* 2005; Kloek *et al.* 2001; Laurie-Berry *et al.* 2006; Chapter 2; Nickstadt *et al.* 2004). This hypothesis is supported by increased expression of SA-mediated defense genes in jasmonate signaling mutants with reduced sensitivity to COR (Kloek *et al.* 2001; Laurie-Berry *et al.* 2006; Chapter 2; Nickstadt *et al.* 2004).

However, inhibition of SA-mediated defenses is not likely to be the only effect of COR that promotes virulence. While plants lacking proper expression of both *JIN1* and the *ICS1* gene required for SA synthesis during infection exhibit normal levels of bacterial growth during infection, they do not develop disease symptoms to the same degree of severity as wild-type plants (Laurie-Berry *et al.* 2006; Chapter 2). This suggests that the *jin1* mutation disrupts some aspect of jasmonate signaling required for production of disease symptoms, in addition to their inability to suppress SA-mediated defenses.

To further explore aspects of jasmonate signaling affected by COR, I conducted a screen for Arabidopsis mutants with altered sensitivity to exogenous COR treatment

(Chapter 3). This screen identified at least two distinct mutants with increased sensitivity to COR and JA treatment (Chapter 3). In this chapter, I further characterize one of these mutants, *jah1* and demonstrate that these mutant plants exhibit increased susceptibility to *P. syringae* infection and altered expression of some JA- and SA-induced genes during infection. I identify a defined interval containing the mutation and propose a candidate gene within this interval that may be disrupted in *jah1* mutants. Finally, I discuss the potential role of the gene represented by this mutation in jasmonate signaling during *P. syringae* infection.

RESULTS

jah1 mutants exhibit hypersensitivity to MeJA

Following two generations of back-crossing to wild-type Col-0 plants, homozygous *jah1* mutants were identified based on seedling root hypersensitivity to MeJA. These plants were allowed to self-fertilize, and the resulting F3 seed was used to characterize the backcrossed *jah1* line.

In order to examine the jasmonate hypersensitivity in the *jah1* mutant plants following backcrossing, these plants were then assayed on two different concentrations of MeJA (10uM and 50 uM; Fig 1). The roots of wild-type plants exhibit a typical response curve, growing to approximately 35% of their untreated length after 10 days of growth on 10uM MeJA and reaching approximately 25% of their untreated length on 50uM MeJA. As expected, the *jah1* mutant plants have increased sensitivity at both 10uM and 50uM MeJA. Two different F3 families were assayed, and they exhibit extremely similar levels of inhibition, both growing to approximately 22% of their untreated length on 10uM MeJA and approximately 15% on 50uM MeJA (Fig 1). These results confirm the preliminary analysis and support the identification of the *jah1* mutant as hypersensitive to jasmonate treatment. Because the two families examined responded nearly identically to jasmonate treatment, only one of these (*jah1*, KAT accession #2707) was used for further characterization.

jah1 mutants have enhanced susceptibility to DC3000 infection

Plants with reduced sensitivity to jasmonates and COR, such as *coi1* and *jin1*, have correspondingly decreased susceptibility to DC3000 (Laurie-Berry *et al.* 2006; Kloek *et al.* 2001; and Chapter 2). Given the importance of COR in pathogenesis and the similarities between COR and JA-Ile (Katsir *et al.* 2008), we would predict that plants with increased jasmonate sensitivity would exhibit enhanced susceptibility to infection. However, this may not always be the case, as suggested by the *jah2* mutant (Chapter 3). Thus, we decided to use two different infection methods to examine *jah1* mutants that would allow us to observe either increased or decreased susceptibility to DC3000: a dip infection and hand infiltration using a relatively low concentration of bacteria. In a dip infection, *jah1* mutants exhibit a very slight increase in symptom severity but supported similar levels of pathogen growth to wild-type plants (data not shown). In contrast, the infiltration protocol produced more definitive results that support the prediction that *jah1* mutants have increased susceptibility to infection (Fig 2A). Wild-type plants supported typical levels of growth over four days of infection, reaching a final concentration of approximately 10^9 cfu/g. The *jah1* mutants supported similar levels of growth to wild-type following 4 days of infection. Interestingly, these mutants supported slightly higher levels of bacteria at the day 2 time point, typically less than a full log more than wild-type. This slight increase in bacterial levels at day 2 was reproducible in three independent experiments, although not statistically significant in all experiments.

Even more strikingly, the *jah1* plants exhibit significantly more severe disease symptoms following hand infiltration. The wild-type plants develop mild disease symptoms, consisting of small amounts of chlorosis around the infiltration site and the

occasional appearance of individual disease lesions (Fig 2B and Table 1). In contrast, *jah1* plants reproducibly exhibit extensive disease symptoms, including large regions of chlorosis and small grey patches of coalesced disease lesions immediately adjacent to the infection site (Fig 2B and Table 1).

jah1 mutants have altered expression of some jasmonate-responsive genes

To further examine the altered jasmonate signaling in the *jah1* mutants, we examined expression of jasmonate-responsive genes during infection with DC3000 using reverse transcription PCR (RT-PCR). The genes chosen were *JIN1* and *coronatine-induced 1/chlorophyllase 1* (*CORI1/CLH1*) each of which represents a specific aspect of jasmonate signaling. *JIN1* encodes MYC2, a transcription factor involved in some of the rapid responses to jasmonate treatment (Lorenzo *et al.* 2004; Chapter 2; Berger *et al.* 1996) and required for cross-talk suppression of salicylic acid defenses during infection (Chapter 2; Laurie-Berry *et al.* 2006; Nickstadt *et al.* 2004). Expression of *CLH1*, a chlorophyllase, is stimulated by jasmonate or COR treatment and induced during DC3000 infection, and may have a role in symptom development (Benedetti *et al.* 1998; Brooks *et al.* 2005; Tsuchiya *et al.* 1999).

Figure 4 shows expression of these genes in Col-0 wt and *jah1* mutant plants over the first 48 hours of infection with DC3000. Because the infection was conducted using hand infiltration, a mock treatment consisting of leaves infiltrated with 10mM $MgCl_2$ is included at 24 hours to account for potential wounding and other stress responses resulting from the infiltration process. Little difference is observed in *JIN1* transcript

levels between the wild-type and *jah1* mutant plants (Fig 3), suggesting that the *jah1* mutation does not alter expression of this transcription factor. Levels of *CLH1*, on the other hand, do appear different. As expected for a jasmonate-responsive gene, wild-type plants show a slight increase in *CLH1* transcript levels in response to the wounding or mock treatment and during infection with DC3000 (Fig 3). The *jah1* mutant plants exhibit a further increase in expression of this gene during infection (Fig 3), suggesting that the signaling pathway responsible for its induction may be hyper-activated in these plants. It is worth noting that neither *JIN1* nor *CLH1* appears strongly induced by infection in this experiment (Fig 3). This is likely due to the limited sensitivity of the semi-quantitative RT-PCR used in these experiments. A more sensitive technique, such as quantitative RT-PCR, might be needed to observe more subtle differences than can be resolved here.

jah1 mutant plants have altered expression of a SA-induced marker gene

Because the *jah1* mutants exhibit increased susceptibility to DC3000 infection and altered jasmonate sensitivity, we wanted to investigate the status of SA-mediated signaling in these plants during infection. A major component of jasmonate-mediated susceptibility to DC3000 involves suppression of SA-mediated defenses, as demonstrated by the elevation of SA signaling in the less susceptible *jin1* mutant plants (Laurie-Berry *et al.* 2006; Chapter 2; Nickstadt *et al.* 2004). Thus, we hypothesized that the increased susceptibility observed in *jah1* mutants might be correlated with a decrease in SA-mediated defenses. To test this hypothesis, we examined expression of *PR-1*, a

pathogenesis-related gene induced in a SA-dependent manner (Chen *et al.* 2004), in wild-type and *jah1* mutant plants during infection with DC3000. As expected, transcription of this gene is induced during infection of wild-type plants, with transcript becoming detectable 24 hours post infection and accumulating to higher levels by 48 hours (Fig 4). In contrast, expression of this gene is reduced in *jah1* mutant plants; transcript is not visible 24 hours after infection and only weakly induced by 48 hours (Fig 4). This observation supports the hypothesis that the increased susceptibility observed in these mutants results, at least in part, from a decrease in SA-mediated defenses.

Mapping of the *JAH1* gene

In order to establish a population for mapping the affected gene, *jah1* was crossed to ecotype Landsberg *erecta*. F1 plants were allowed to self pollinate, and F2 seeds were collected. Approximately 60 F2 offspring homozygous for the *jah1* mutation were identified based on hypersensitivity when grown on MeJA. These plants were used to roughly map the gene to a location linked to the marker CIW2 on the northern half of chromosome 2. An expanded population of 238 F2 seedlings was then used to more closely define the interval containing the gene defined by *jah1*. These plants were used to localize the affected gene to a 3.6 Mb region between markers CIW2 and T10J7-T7. Then 64 individuals with recombination events between these two markers were tested using markers J2-3833298-EcoRI and J2-4449608-EcoRI to localize the gene to the 600 kb region between these markers (Fig 4). A list of the informative recombinants used to obtain this map position are presented in Appendix 2.

Having isolated the gene to this 600 kb region, we began looking at the known and predicted genes in the interval to see if we could identify candidates likely to be affected in the *jah1* mutant. This 600 kb region is annotated to contain 29 genes (TAIR). We obtained a total of 17 T-DNA insertion lines in 8 of these genes from the Arabidopsis Biological Resource Center (ABRC). We received T3 seed obtained from T1 plants heterozygous for the T-DNA insertions. These segregating populations were assayed for abnormal root sensitivity to MeJA. Of these lines, two exhibited increased sensitivity to MeJA treatment: SALK_044479 and SALK_016776 (data not shown), while the rest appeared wild-type. Both of these insertions are localized to the same gene, At2g10940 (Fig 4), making this a likely candidate gene for *JAH1*. Further work must be done to determine whether the jasmonate hypersensitivity phenotype in these lines is reproducible, heritable, and correlated with the presence of the T-DNA. However, the fact that the phenotype was observed in two different T-DNA lines with insertions in the same gene lends strength to the hypothesis that this gene is disrupted in the *jah1* mutants.

To investigate this possibility, we examined the expression of At2g10940 in wild-type and *jah1* mutant plants using RT-PCR with primers designed to amplify the 3' end of the transcript. Consistent with previous published work (Thilmony *et al.* 2006), this transcript is constitutively expressed in wild-type leaves and down-regulated by 48 hours following infection with DC3000 (Fig 3). In *jah1* mutant plants, expression appears very similar to wild-type, indicating that the mutation does not significantly affect transcript levels of At2g10940 (Fig 3). This does not eliminate At2g10940 from consideration as a candidate gene for *JAH1*. This mutant was created via chemical mutagenesis using EMS,

a treatment that induces point mutations. Thus, it is not necessarily expected that a mutation of this type will disrupt the production of a normal transcript. Instead, the mutation may disrupt gene function by altering a splice site, resulting in a premature stop codon, or changing an amino acid residue critical to protein function or structure. Thus, it is quite possible that the *jah1* mutation may disrupt function of At2g10940 in a manner that is not visible at the level of transcript accumulation, at least not with the primers used in this experiment.

DISCUSSION

jah1 may represent a gene that negatively regulates JIN1-dependent signaling

The *jah1* mutant exhibits phenotypes we had originally expected to observe in *coh* mutants: hypersensitivity to both COR and jasmonate treatment and increased susceptibility to DC3000 infection. This combination of responses suggests that the *jah1* mutation disrupts a component of jasmonate signaling that is manipulated by COR to promote susceptibility to DC3000. Both root inhibition and susceptibility to DC3000 are positively regulated by *JIN1* (Laurie-Berry *et al.* 2006; Chapter 2; Berger *et al.* 1996; Nickstadt *et al.* 2004; Lorenzo *et al.* 2004), so we hypothesize that the *JAH1* gene acts as a negative regulator of the pathway controlled by *JIN1*. Because *JIN1* transcript levels appear to be unaffected in the *jah1* mutant as compared to wild-type (Fig 3), we propose that *JAH1* is either downstream or independent of *JIN1* (Fig 5A and B).

We can explore these alternative hypotheses by examining expression of *JIN1*-dependent genes in the *jah1* mutants. Induction of *CORI1/CLH1* during DC3000 infection is partially dependent on *JIN1*, as evidenced by reduced expression of this gene in infected *jin1* mutant plants (Fig 5; Laurie-Berry *et al.* 2006; Chapter 2). Similarly, inhibition of the SA-mediated *PR-1* gene during infection is also controlled by *JIN1*, as demonstrated by increased levels of this gene in infected *jin1* mutant plants (Laurie-Berry *et al.* 2006; Chapter 2; Nickstadt *et al.* 2004). We have shown that *jah1* mutant plants exhibit inverse expression patterns of both of these genes during infection: elevated levels of *CLH1* and decreased expression of *PR-1* (Fig 3). While it does not negate the possibility of *JAH1* acting independently of *JIN1* (Fig 5B), the observation that *jah1*

mutants exhibit opposite phenotypes to *jin1* mutants with respect to jasmonate/COR sensitivity, DC3000 susceptibility, and expression of downstream genes strongly suggests that *JAH1* acts as a negative regulator in the jasmonate signaling pathway controlled by *JIN1*. Based on these data, we propose a model in which *JIN1* exerts some of its activity via repression of the negative regulator *JAH1* (Fig 5A). Analysis of *jah1 jin1* double mutants is needed to test this hypothesis.

jah1 mutants may separate bacterial growth and symptom development

The observation that *jah1* mutants exhibit significantly more severe disease symptoms that wild-type plants 4 days after infection, despite supporting similar levels of bacteria at this stage (Fig 2 and Table 1), suggests that this mutation may impact disease symptom development and bacterial growth differently. Evidence that these two measurements of disease severity are not always correlated comes from studies of *jin1 sid2* mutants that develop less severe disease symptoms despite supporting full levels of bacterial growth (Laurie-Berry *et al.* 2006; Chapter 2). It is tempting to speculate that the increased symptoms observed in *jah1* mutants may be related to elevated expression of the chlorophyllase *CLH1* in these plants (Fig 3), which could be responsible for the more extensive chlorosis observed in these mutants compared to wild-type (Figure 2 and Table 1).

However, we cannot definitely state that the elevated symptoms observed in *jah1* mutants are unrelated to bacterial growth. These mutants support elevated levels of bacteria compared to wild-type 2 days after infection (Fig 2), and this is correlated with a

decrease and delay in activation of SA-mediated defenses, as measured by *PR-1* expression (Fig 3). It seems likely that this decreased and delayed induction of SA-mediated defenses is insufficient to control bacterial growth, allowing DC3000 to grow more rapidly during the second day of infection, when these defenses normally begin to limit bacterial growth in wild-type plants. It is possible that the ability of bacteria to reach their final concentrations more rapidly in the *jah1* mutant results in the increased symptoms observed in these plants. The earlier accumulation of high concentrations of DC3000 could be recognized by the bacteria through quorum-sensing mechanisms, accumulation of other bacterial-derived molecules, or depletion of required nutrients in the apoplast, triggering premature activation of the processes that produce disease symptoms.

Either of these hypotheses could explain the increased symptoms observed in the *jah1* mutants, and the two are not mutually exclusive. It is possible both that increased expression of jasmonate-dependent genes like *CLH1* and decreased activation of SA-mediated defenses could contribute to the increased disease symptoms that develop in these mutant plants. One approach to investigate this question might be application of SA to *jah1* mutants during infection. Nickstadt *et al.* (2004) showed that *jin1* mutants contained elevated levels of SA during infection, suggesting that the jasmonate-mediated suppression of this pathway is acting at the level of hormone accumulation. If this is the case, then exogenous application of SA during infection might complement the decreased SA-dependent defenses in *jah1* mutants, allowing one to observe the effects of elevated jasmonate signaling alone. It might also be useful to analyze the progression of disease

symptoms more carefully in *jah1* mutant plants as compared to wild-type, as it is likely that microscopic changes are occurring in the leaf before visible symptoms develop. For example, measurements could be made of chlorophyll content and electrolyte leakage in infected leaves to assess early stages of chlorosis and lesion development, respectively.

At2g10940 as a candidate gene for *JAH1*

Mapping of the *jah1* mutation has narrowed the interval containing this gene to a 600 kb region annotated to contain 29 genes (TAIR). Based on this information, I hypothesize that At2g10940 is the *JAH1* gene. Of the 29 genes in the interval, At2g10940 is the only one whose expression is strongly affected by both jasmonate treatment and *P. syringae* infection (Thilmony *et al.* 2006; De Vos *et al.* 2005). Expression of this gene is significantly decreased by these treatments, as we might predict for a negative regulator of jasmonate signaling. The decreased expression of At2g10940 in response to infection is dependent on bacterial production of COR (Thilmony *et al.* 2006), supporting the hypothesis that mutation of this gene might alter the plant's sensitivity to the phytotoxin. Additionally, two independent T3 lines with T-DNA insertion in the promoter or coding sequence of At2g10940 (Fig 4) exhibited segregation of increased sensitivity to MeJA in preliminary experiments. Work is underway to determine whether this phenotype is heritable and correlated with the presence of the T-DNA. If this proves to be the case, further confirmation will be obtained by sequencing At2g10940 in *jah1* mutants and complementing the *jah1* mutant phenotypes with the At2g10940 gene.

MATERIALS AND METHODS

Bacterial strains

The bacterial pathogen *Pseudomonas syringae* pv. *tomato* strain DC3000 has been described previously (Cuppels 1986). Bacteria were grown on King's B media (KB; King *et al.* 1954) or NYG (Daniels *et al.* 1988) containing 50ug ml^{-1} rifampicin at 28°C.

Plant materials, growth conditions, and inoculation procedures

Arabidopsis thaliana ecotypes Colombia (Col-0) and Landsberg *erecta* (La-er) were used in this study. The *coi1-35* allele was obtained from Paul Staswick (Staswick *et al.* 2002), and *sid2-2* mutants from Mary Wildermuth (Dewdney *et al.* 2000). SALK lines were developed by The Salk Institute Genomic Analysis Laboratory and obtained from ABRC (Ohio State University, Columbus, OH). The *coh-36/jah1* mutants were originally identified as described in Chapter 3. The back-crossed *jah1* mutant lines used in these experiments were obtained by crossing the original mutant to wild-type Col-0 through two generations.

Plants were grown from seed in growth chambers with an 8 hr photoperiod at 22°C and 75% relative humidity with light intensity of 140 to 160 μEin s^{-1} m^{-2}. All plants used for virulence studies were approximately four weeks old at the time of infection. Dip infections were conducted by immersing whole rosettes into bacterial suspensions of approximately $5x10^8$ cfu ml^{-1} containing 0.02% (v/v) Silwet L-77 (OSi Specialties Inc., Danbury, CT, USA) and 10mM $MgCl_2$, as described previously (Kunkel *et al.* 1993). Hand infiltrations were carried out by using a 1ml needleless syringe to flood half of the

leaf with a bacterial suspension of approximately $5x10^5$ cfu ml^{-1} in 10mM $MgCl_2$. Mock infections consisted of infiltration with 10mM $MgCl_2$. To monitor bacterial populations within the plant, individual rosette leaves were removed 0, 2, and 4 days post inoculation. For the day 0 time point, leaf tissue was sampled approximately 2 hr after inoculation. In the case of dip inoculations, these leaves were surface sterilized in 15% (v/v) H_2O_2 for 5 to 10 min, and rinsed three times with sterile water. Leaves were weighed and homogenized, and appropriate dilutions were plated on NYG medium containing rifampicin. Plates were incubated at 28°C for 48 hours before counting colony forming units (cfu).

Methyl jasmonate root inhibition assays

The sensitivity of seedlings to MeJA was assayed by germinating sterilized seeds on one-half strength Murashige and Skoog (1/2x MS; Murashige and Skoog 1962) plates (pH 6.0, 1% (w/v) agar, 1% (w/v) sucrose) containing concentrations of MeJA (Sigma Aldrich) as indicated. Seeds were sterilized by immersion for 5 minutes in 70% (v/v) ethanol containing 0.05% (v/v) Triton X100, followed by immersion for 5 minutes in 95% (v/v) ethanol containing 0.05% (v/v) Triton X100, and then a final immersion for 3 minutes in 95% (v/v) ethanol. Seedlings were grown vertically on square plates. After two days of cold treatment in the dark, plates were placed vertically in a growth chamber such that roots grew downwards along the agar. Digital images of the plates were taken after 10 days of growth in continuous light, and roots were measured using NIH Image (Research Services Branch of the National Institute for Mental Health).

Genetic markers

Information regarding the genetic markers CIW2 and T10J7-T7, including primer sequences, was obtained from The Arabidopsis Information Resource (TAIR) at www.arabidopsis.org. Markers J2-3833298-EcoRI and J2-4449608-EcoRI were designed using Marker Tracker at bbc.botany.utoronto.ca/markertracker/, based on datasets presented in Jander *et al.* 2002 and Nordborg *et al.* 2005.

RNA isolation and RT-PCR analysis

Leaf tissue harvested from inoculated *A. thaliana* plants was stored at -80°C until all samples were obtained. Total RNA was isolated using Trizol (Invitrogen). Genomic DNA contamination was removed using two treatments with DNA-free (Ambion), and first strand cDNA synthesis was performed using SuperScript II Reverse Transcriptase (Invitrogen) with an oligo dT primer. cDNA levels were normalized based on levels of the constitutively-expressed *ubiquitin 10* (*UBQ10*) transcript using the primers UBQ10F (5'- GGT ATT CCT CCG GAC CAG CAG C -3') and UBQ10R (5'- CGA CTT GTC ATT AGA AAG AAA GAG ATA ACA GGA ACG G -3'). Transcripts of interest were measured using the following primers, as indicated: JIN1rtF (5'- GGC ACA GGC GGG ATT TAA TCA AGA -3') and JIN1rtR (5'- AAG CGA AGC TCT GCG TCA TCG AAA -3'), CORI1rtF (5'- ACC ACA TCG CTT CGC ATG GTT ACA -3') and CORI1rtR (5'- TTT AAG TCC GTT GGT GCG CAT GGT -3'), PR1rtF (5'- TTC CCT CGA AAG CTC AAG ATA GCC CA -3') and PR1rtR (5'- GGC TTC TCG TTC ACA TAA TTC CCA

CG -3'), and At2g10940rtF (5'- TAA AGG CCT CGT TGA AGT CG -3') and At2g10940rtR (5'- AAG CAT AAG GGA ACG ATA GAG G -3').

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Table 1: Symptoms observed 4 days after hand infiltration with DC3000

Genotype	none	chlorosis only[a]	discrete lesions[b]	coalesced lesions[c]	total
Experiment 1					
Col-0 wt	0	11	1	0	12
coi1-35	8	0	0	0	8
jah1	0	0	1	9	10
Experiment 2					
Col-0 wt	0	8	8	0	16
coi1-35	12	2	0	0	14
jah1	0	0	3	17	20
Experiment 3					
Col-0 wt	1	5	0	0	6
coi1-35	7	0	0	0	7
jah1	0	0	3	6	9

a=number of inoculated leaves that developed chlorosis without lesions
b=number of inoculated leaves that developed few discrete lesions
c=number of inoculated leaves that developed many lesions coalesced into large patches

Figure 1

Root growth inhibition of backcrossed *jah1* mutants by methyl jasmonate.

Root length of Col-0 wild-type (diamond) and *jah1* mutant (triangle and open circle) plants grown on 1/2x MS media or 1/2x MS containing 10 or 50 uM MeJA. Root lengths are presented as a percentage of untreated length for each genotype. Roots were measured after 10 days of growth. Each value is the average of a minimum of 40 seedlings per treatment. Vertical bars representing +/- SEM are obscured by symbols in most cases; these are calculated using normalized percentages rather than raw root length data. Similar results were obtained in two independent experiments.

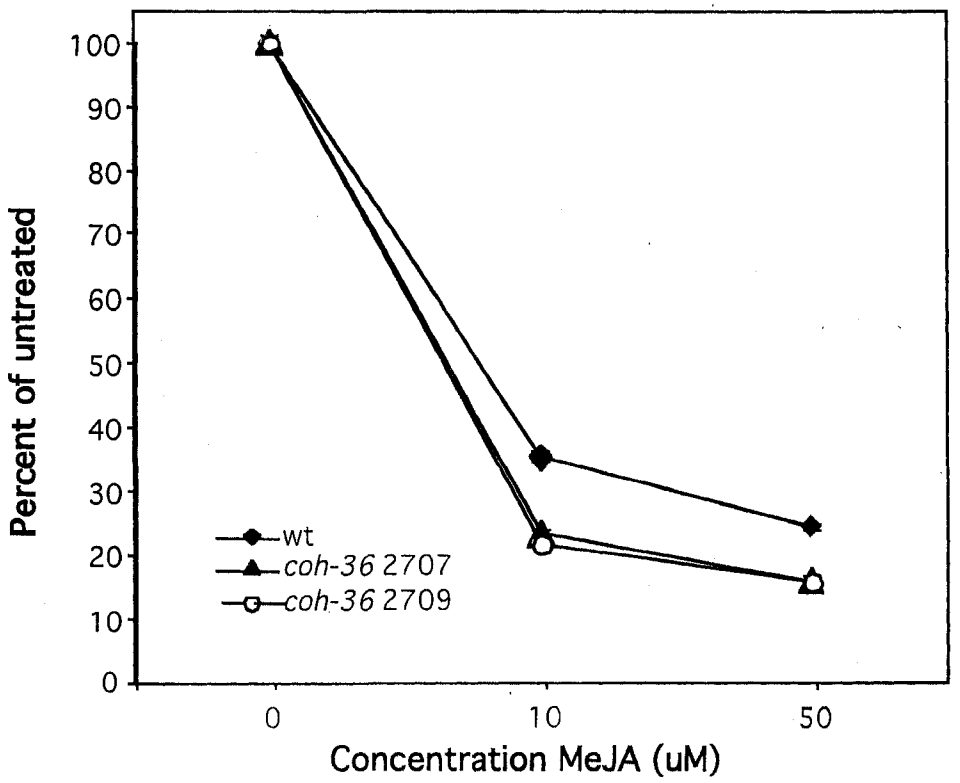
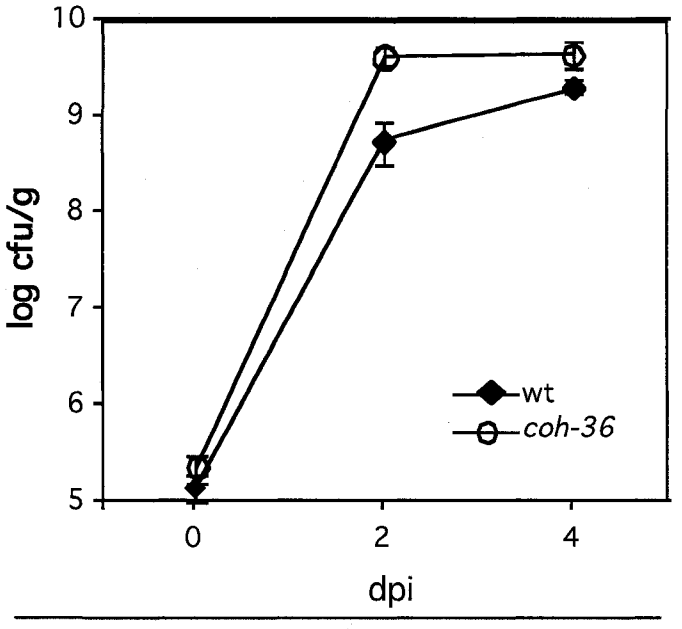

Percent of untreated
100
90
80
70
60
50
40
30
20
10
0
wt
coh-36 2707
coh-36 2709
0
10
50
Concentration MeJA (uM)

Figure 2

Growth of *Pseudomonas syringae* pv. *tomato* strain DC3000 and symptom development on *jah1* mutants following hand infiltration.

A. Growth in wild-type Col-0 (diamond) and *jah1* (open circle) plants over the course of infection. Data points represent the average of three samples, and vertical bars indicate +/- SEM. **B.** Visible symptoms on representative leaves of wild-type and *jah1* mutant plants four days after infection. Similar results were observed in three independent experiments. The data presented in this figure correspond to experiment 3 presented in Table 1.

A



B

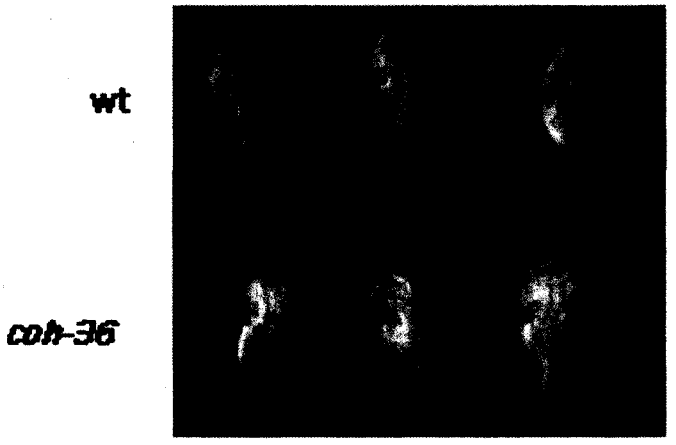


Figure 3

Expression of JA-dependent (*JIN1*, *CLH1*) and SA-dependent (*PR-1*) defense genes in wild-type and *jah1* mutant plants after hand infiltration with DC3000.

Gene expression is examined using RT-PCR. Mock treatment was conducted by infiltration with a 10mM $MgCl_2$ solution. At2g10940 is included as a candidate gene that may be disrupted in *jah1* mutants. *UBQ10* is included as a control for equal loading. Similar results were seen in a second independent experiment.

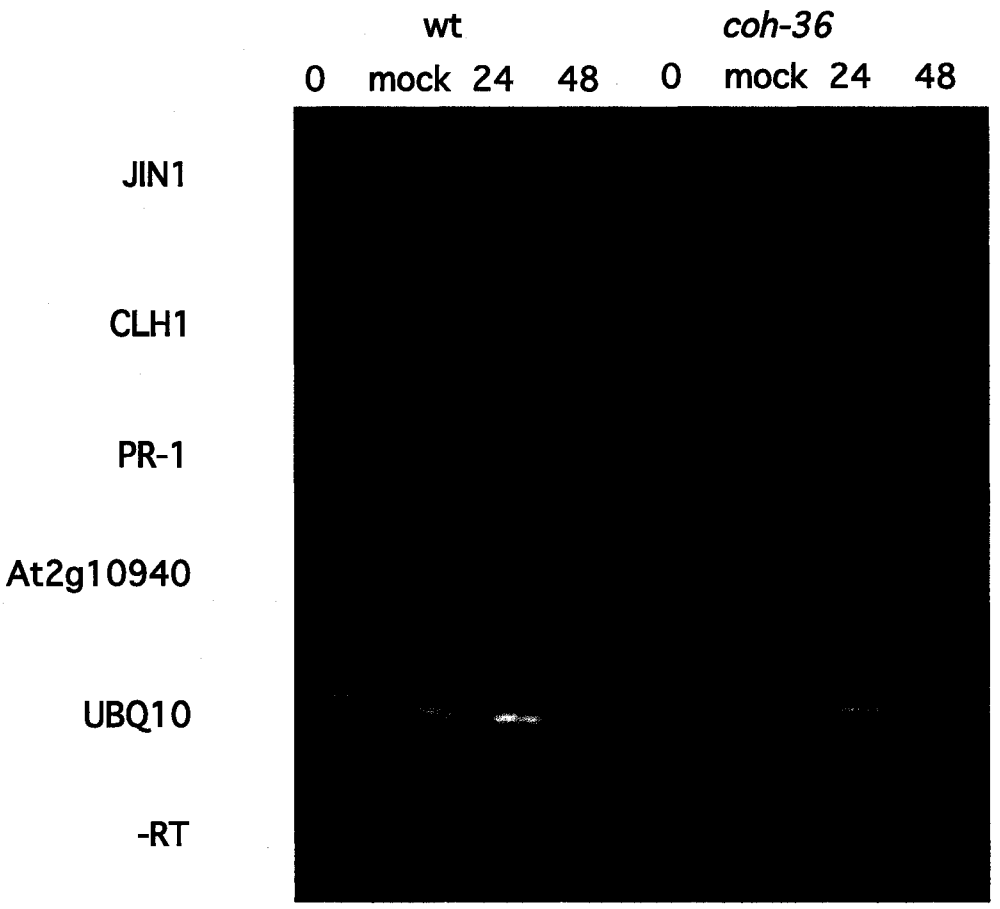

wt
coh-36
0 mock 24 48 0 mock 24 48
JIN1
CLH1
PR-1
At2g10940
UBQ10
-RT

Figure 4

A diagram of the region of chromosome 2 containing the *jah1* mutation.

The *jah1* mutation was initially mapped to a 3.6 Mb region around the centromere (indicated by a circle) of chromosome 2 between markers CIW2 and T10J7-T7. This region is expanded to show the location of these markers, as well as a 1.2 Mb region defined by markers J2-3827242 and T10J7-T7. This region is further expanded to show markers J2-3833298 and J2-4449608 that define a 600 kb interval containing the affected gene. The location of At2g10940, a candidate gene within this region, is indicated and expanded to show the structure of the gene. The grey region of this gene represents its single intron, while the white region represents the promoter region upstream of the gene. Also shown are the locations of T-DNAs in two SALK lines with insertions in this gene and two additional SALK lines with insertions in the promoter region. The number of individual plants with recombinant breakpoints between various markers is also indicated.

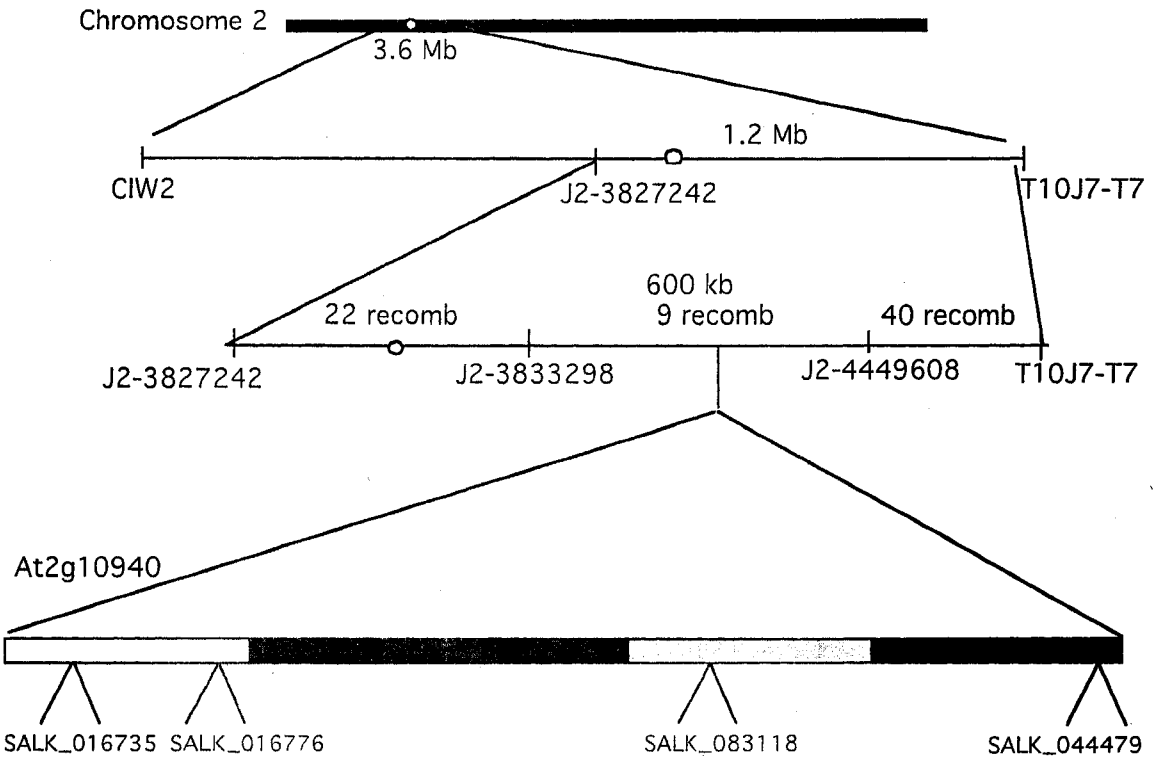
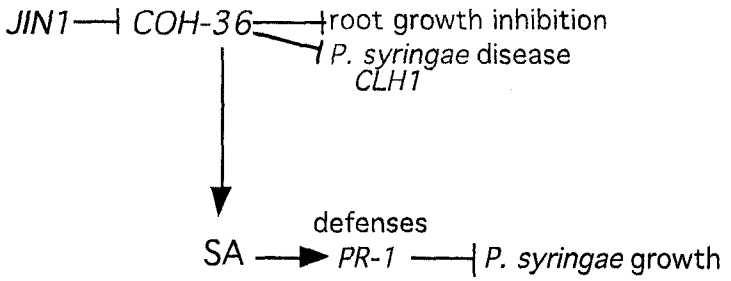

Chromosome 2
3.6 Mb
1.2 Mb
CIW2
J2-3827242
T10J7-T7
600 kb
22 recomb
9 recomb
40 recomb
J2-3827242
J2-3833298
J2-4449608
T10J7-T7
At2g10940
SALK_016735
SALK_016776
SALK_083118
SALK_044479

Figure 5

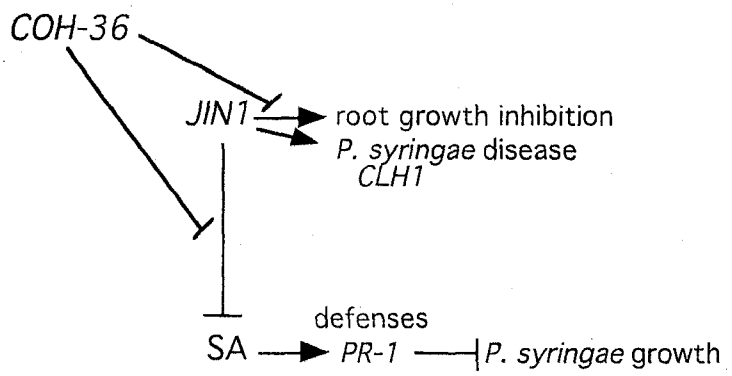
Two models for the role of *JAH1* in jasmonate signaling.

A. We propose that *JAH1* may act downstream of *JIN1* to negatively regulate *JIN1*-dependent responses, including root inhibition, expression of *CLH1*, and *P. syringae* disease development, while simultaneously acting as a positive regulator of SA-mediated signaling leading to defense against *P. syringae*. **B.** An alternative model suggests that *JAH1* may impact jasmonate-mediated responses and SA-dependent defenses independently of *JIN1*.

A


JIN1
COH-36
root growth inhibition
P. syringae disease
CLH1
defenses
SA
PR-1
P. syringae growth

B


COH-36
JIN1
root growth inhibition
P. syringae disease
CLH1
defenses
SA
PR-1
P. syringae growth

Chapter 5

Conclusions and future directions

Overall, my thesis has focused on identifying and characterizing components of jasmonate signaling that are manipulated by coronatine (COR) and may be important in the interaction between Arabidopsis and *Pseudomonas syringae*.

In the work presented in Chapter 2, I collaborated with Vinita Joardar to demonstrate that *JIN1* is required for susceptibility to *P. syringae* pv. *tomato* DC3000. I further showed that this susceptibility is correlated with sensitivity to the phytotoxin COR and is partially due to inhibition of salicylic acid (SA)-mediated defenses. Examination of *jin1 sid2* double mutants revealed that suppression of SA-mediated defenses is responsible for allowing bacterial growth but is not sufficient to permit development of disease symptoms. Based on this work, we developed an integrated model of interactions between jasmonate and SA signaling during infection by DC3000 (Ch. 2, Fig 7).

To identify additional components of jasmonate signaling affected by DC3000 infection, I conducted a screen for Arabidopsis mutants with altered sensitivity to COR (Chapter 3). This resulted in identification of three mutants with hypersensitivity to COR, representing at least two distinct genes involved in this process. Characterization of the *coh-23/jah2* and *coh-36/jah1* mutants revealed that both exhibited increased sensitivity to jasmonates that was similar to their response to COR. I also assayed both of these mutants for their response to infection with DC3000. The *jah2* mutant plants surprisingly exhibit decreased susceptibility to infection, although this phenotype may result from a second independent mutation. In contrast, *jah1* mutants are more susceptible, exhibiting a slight increase in growth early in the infection process and a dramatic increase in symptom development at later time points. Further characterization of *jah1* mutants

during infection reveals altered expression of several genes regulated by *JIN1*, suggesting that the *JAH1* gene may be a negative regulator acting downstream of *JIN1* (Ch. 4, Fig 5).

Through mapping using genetic markers, I identified a 600 kb interval on chromosome 2 that contains the *jah1* mutation. Analysis of available expression data for the 29 genes annotated within this region suggested At2g10940 as a possible candidate gene affected by the *jah1* mutation. Four SALK lines with T-DNA insertion in or near this gene were assayed for jasmonate sensitivity, and two of them exhibited jasmonate hypersensitivity segregating in a T3 population. This supports the hypothesis that At2g10940 is the gene disrupted in *jah1* mutants, and work is underway to confirm this hypothesis.

The role of JIN1 as a transcription factor regulating some jasmonate responses

Our findings that *jin1* mutants exhibit partial insensitivity to jasmonate treatment and decreased expression of several jasmonate-responsive signaling genes support the accepted model that has emerged in the field over the course of this work. JIN1/MYC2 is a transcription factor that is proposed to mediate responses to the active jasmonate JA-Ile (Lorenzo *et al.* 2006; Katsir *et al.* 2008). *JIN1* is required to induce expression of some genes (e.g. *LOX2*, *CORI1/CLH1*), while repressing expression of others (e.g. *PDF1.2*, *PR-1*). It remains to be determined whether JIN1/MYC2 is affecting these genes directly through its activity as a transcription factor or indirectly through unknown components of jasmonate signaling (Ch. 1, Fig 1; Ch. 3, Fig 5).

Regardless of whether it does so directly or indirectly, *JIN1* is one of a number of genes involved in integrating signals from multiple hormone pathways in order to produce the appropriate response to a given set of circumstances. For example, *JIN1* is required for down-regulation of *PDF1.2*, a gene activated by the coordinated activity of jasmonate and ethylene in response to fungal and insect pathogens (Laurie-Berry *et al.* 2006; Lorenzo *et al.* 2004; Boter *et al.* 2004). This suggests that *JIN1* positively regulates responses mediated solely by jasmonate, while negatively regulating responses that require both jasmonate and ethylene signaling. It has also been shown that *jin1* mutants have decreased sensitivity to abcisic acid (ABA; Abe *et al.* 2003; Yadav *et al.* 2005), suggesting that this transcription factor is involved in integrating ABA and jasmonate signaling in a manner that is not yet understood. Further, *JIN1* is required to suppress SA-mediated responses as part of the mutual inhibition between jasmonate and SA signaling (Laurie-Berry *et al.* 2006; Nickstadt *et al.* 2004). Elevated levels of SA have been observed in *jin1* mutants (Nickstadt *et al.* 2004), but transcript of the *ICS1* gene required for SA synthesis appears to be unaffected (Laurie-Berry *et al.* 2006), demonstrating that this interaction may be regulated post-transcriptionally and is certainly more complicated than we currently understand.

Further complexity is added to the picture by the possibility that one or more additional genes may exist with function overlapping that of *JIN1*. The presence of these genes is suggested by the partial reduction in root inhibition and disease susceptibility of *jin1* mutants as compared to *coi1* plants (Berger *et al.* 1996; Laurie-Berry *et al.* 2006). JIN1/MYC2 is part of a family of myc transcription factors, suggesting that other

members of this family might possess some degree of redundancy with *JIN1*. Alternatively, two members of the Arabidopsis NAC family, ANAC019 and ANAC055, have been shown to have a role in jasmonate-dependent signaling (Bu *et al.* 2008). While the authors propose that these genes act downstream of *JIN1*, nothing in their data precludes the possibility of these genes being partically redundant with *JIN1*.

It is unlikely that any redundant genes could be identified through a screen for reduced sensitivity to jasmonates, given that several screens of this type have been conducted and have largely identified the same genes: *COI1*, *JIN1*, *JAR1*, and *AXR1* (Tiryaki and Staswick 2002; Staswick *et al.* 2002; Laurie-Berry *et al.* 2006; Lorenzo *et al.* 2004; Feys *et al.* 1994). An enhancer screen using *jin1* mutants as a sensitized background might be more likely to uncover genes that are functionally redundant with *JIN1*, although the phenotypic effects might be too subtle to distinguish in the insensitive *jin1* background. A more targeted approach could involve creating double or triple mutants between *jin1* and plants with mutations in members of the myc family that are closely related to *JIN1/MYC2*. Additionally, a triple mutant between *jin1*, *anac019*, and *anac055* would be worth examining. Because these double or triple mutants could be assayed at the population level rather than as individual seedlings, this approach would allow greater discriminatory power and the ability to observe a subtle enhancement of the *jin1* phenotype. As an alternative to a genetic approach, genes that may possess redundant function to *JIN1/MYC2* might be identified based on physical interaction with JIN1, either in yeast two-hybrid or immunoprecipitation assays. This approach is

suggested based on the possibility that JIN1/MYC2 may dimerize or otherwise interact with another transcription factor that has overlapping function.

The role of *JIN1* in *P. syringae* disease development

JIN1 is clearly required for full susceptibility to *P. syringae* infection, as demonstrated by the reduced susceptibility of *jin1* mutant plants (Laurie-Berry *et al.* 2006; Nickstadt *et al.* 2004). These mutant plants are not able to support bacterial levels as high as those seen in wild-type plants, and they develop much less severe disease symptoms (Laurie-Berry *et al.* 2006; Nickstadt *et al.* 2004). At least some of this reduction in susceptibility is a result of the *jin1* mutation relieving *JIN1*-mediated inhibition of SA-mediated defenses (Laurie-Berry *et al.* 2006; Nickstadt *et al.* 2004).

However, elevated SA does not account for all of the differences observed in *jin1* mutants, as loss of SA synthesis via a *sid2* mutation does not restore full disease symptoms to *jin1* mutants (Laurie-Berry *et al.* 2006). This demonstrates that *JIN1* is required during DC3000 infection for more than simply suppressing SA-mediated defenses and strengthens the hypothesis that virulence factors like COR can act to promote pathogen virulence in ways that do not always involve suppressing known host defenses (Ch. 2, Fig 7).

It is unclear what changes COR is inducing in the plant in order to directly promote virulence, but we can hypothesize about the type of processes that might be involved. COR has been shown to promote opening of stomata by blocking normal plant defense responses that result in stomatal closure (Melotto *et al.* 2006). This process may

rely on the interactions between jasmonate and ABA signaling mediated by *JIN1* (Abe *et al.* 2003; Yadav *et al.* 2005). COR has been shown to promote opening of stomata from the leaf surface, presumably to facilitate bacterial entry into the plant (Melotto *et al.* 2006). I speculate that COR may also exert this effect later in the infection process to allow *P. syringae* a route of egress from a region of the apoplast that is saturated with bacteria so that it might begin a new infection elsewhere on the same or neighboring plants. Additionally, COR may directly promote disease symptom development through *JIN1*-dependent activation of CLH1, a chlorophyllase that is involved in early stages of chlorophyll degradation and may contribute to the chlorosis observed during disease. It is unclear what advantage the bacteria derive from chlorosis, but it is possible that plant cells undergoing chlorophyll degradation are weakened in a manner that increases nutrient leakage into the apoplast or makes them more susceptible to other effects of the pathogen.

The *jah1* mutant represents a novel component of jasmonate signaling

The *jah1* mutant was identified in my screen based on hypersensitivity to COR. It also exhibits increased sensitivity to jasmonate treatment and increased susceptibility to infection by DC3000. The mapping of the affected gene has defined it to a region that has not been previously implicated in jasmonate signaling. These data suggest that *jah1* represents a novel gene that acts as a negative regulator of jasmonate signaling. Several of the responses observed in *jah1* mutants are opposite to those observed in *jin1* mutants: jasmonate and COR hypersensitivity, increased susceptibility to DC3000, elevated

expression of *CORI1/CLH1*, and decreased expression of *PR-1*. These results suggest that *JIN1* and *JAH1* are likely to act in opposing ways in the same branch of the jasmonate signaling pathway, since they control similar processes (Ch. 4, Fig 5A). Of course, it is possible that *JAH1* exerts its influence independently of *JIN1* (Ch. 4, Fig 5B). Analysis of double mutants is needed to examine this question, and experiments to do this are described in the Future Directions below.

FUTURE DIRECTIONS

The work presented in this thesis leaves many open questions and suggests several avenues for further research. I will describe some of them here and discuss what we can hope to learn from them.

Further characterization of *jah2* mutants

As things currently stand, it is not clear what effect the *jah2* mutation has on susceptibility to *P. syringae* infection. The observation that a sibling line not carrying the *jah2* mutation exhibits reduced susceptibility to DC3000 infection similar to that seen in the *jah2* mutants indicates that a second, unlinked mutation is likely to be present and complicating analysis of the *jah2* mutants. This is not that surprising given that this mutant was generated through chemical mutagenesis using a relatively high concentration of EMS. Following this treatment, it would be expected for a plant to carry multiple different mutations, and it is certainly possible that more than one of them might impact the same phenotype. Indeed, several visible phenotypes that complicated disease analysis

(i.e. spontaneous chlorosis and dwarfing) were observed in the *jah1* mutant population following crosses to wild-type Col-0, and these were selected against when choosing the backcrossed line for characterization. Because the second phenotype resulting in reduced disease susceptibility in the *jah2* population was observed in a sibling line not exhibiting jasmonate hypersensitivity, it is likely that the mutation responsible for this effect is unlinked to the *jah2* mutation and can be separated from it in further backcrossing.

Once the *jah2* mutant has been backcrossed to remove this complicating second mutation, the first step will be reassessing its responses to jasmonate treatment and DC3000 infection. Both of these responses differed between the original mutant and the line that had undergone a single backcross, so it is important to examine these phenotypes again following additional backcrossing. If the reduced susceptibility phenotype is observed in the backcrossed *jah2* mutants, this will support the exciting hypothesis that this mutant represents a gene involved in *JIN1*-independent signaling. I would suggest gene expression studies as one way to examine this hypothesis. This hypothesis predicts that *jah2* represents a gene involved in negative regulation of *JIN1*-independent signaling that may involve suppression of *JIN1* or its downstream targets. If this is the case, we would expect the *jah2* mutation to result in increased signaling through this *JIN1*-independent pathway, resulting in increased inhibition of *JIN1* or its downstream targets. Thus, a *jah2* mutant would be predicted to exhibit gene expression patterns similar to those seen in a *jin1* mutant. Because this *JIN1*-independent pathway is proposed to be regulated by the ethylene-responsive transcription factor *ERF1* and likely serves to

integrate jasmonate and ethylene signaling, it would also be worthwhile to test the *jah2* mutants for enhanced sensitivity to ethylene treatment.

Of course, the most significant open question regarding *jah2* is the identity of the affected gene. I have crossed the original *coh-23* mutant to the ecotype Landsberg *erecta*, and an F2 population is available for mapping. Once a rough map position is established, allelism tests should be conducted with any mutants in the region that have a role in jasmonate or ethylene signaling before proceeding to more detailed mapping.

Because the response of *jah2* mutants to infection is currently unclear, it is difficult to speculate about known genes that might be affected by this mutation. However, there are some possibilities that should be investigated, particularly if rough mapping places the mutation near their location. These include MPK6, a kinase involved in map kinase cascades. Mutations in the *MPK6* gene result in increased sensitivity to jasmonate treatment (Takahasi *et al.* 2007), making this a possible candidate gene for *JAH2*. Experiments are underway in our laboratory to confirm the increased jasmonate sensitivity of *mpk6* mutants in our assays and to examine their response to our infection protocols. If these experiments yield results similar to those observed in *jah2* mutants, it will become important to cross these two mutants to examine allelism. An alternative candidate gene for *JAH2* is a member of the *JAZ* family. These genes are negative regulators of jasmonate signaling, as the gene affected in *jah2* mutants is proposed to be. While few phenotypes have been reported for mutations in individual *JAZ* genes (Chini *et al.* 2006; Thines *et al.* 2006), recent work in our lab suggests that these mutants may have subtle phenotypes (Agnes Demianski, unpublished data). Thus, it is possible that *jah2*

might represent a mutation in a member of the *JAZ* family involved in jasmonate signaling impacted by COR. A combination of mapping and phenotypic characterization should reveal whether the mutation in *jah2* affects *MPK6*, a *JAZ* family member, or some novel gene yet to be implicated in jasmonate signaling.

Further characterization of *jah1* mutants

The data regarding *jah1* mutants presented in Chapter 4 raise hypotheses that suggest several further experiments to carry out using this mutant. One significant question is whether *JAH1* acts downstream or independently of *JIN1*. This could most easily be examined by studying a *jin1 jah1* double mutant. If *jah1* blocks the pathway downstream of *JIN1*, this double mutant would be predicted to resemble *jah1* mutants. On the other hand, if the gene represented by *jah1* acts independently of *JIN1*, this double mutant would exhibit phenotypes that are additive or somewhere between the extremes exhibited by the two parents. It would be particularly interesting to examine *PR-1* and other markers of SA signaling in these mutants to determine whether *JIN1* exerts its inhibition of SA-mediated defenses via *JAH1*. It is also possible that the *jin1 jah1* double mutants might resemble the *jin1* parent, suggesting that *jah1* is impacting the jasmonate signaling pathway upstream of *JIN1*. While this is unlikely given the expression data showing that *jah1* mutation has no impact on *JIN1* expression (Ch. 4, Fig 3), it is not entirely implausible. The RT-PCR assay used in that experiment is not quantitative and may lack sensitivity, as evidenced by the failure to observe induction of *JIN1* during

infection. Also, *JAH1* could act upstream of *JIN1* through post-transcriptional regulation, similar to that observed in the JIN1/JAZ3 interaction.

The response of *jah1* mutants to DC3000 infection also suggests questions first raised by *jin1* mutants regarding the extent to which disease symptom development is correlated to bacterial growth. In Chapter 4, I propose two different hypotheses about the cause of the increased disease symptoms that develop in *jah1* mutant plants: direct effects on symptom production resulting from hyper-activation of jasmonate-responsive genes and indirect effects on symptom production resulting from increased bacterial growth at earlier stages of infection. I also suggest that these hypotheses are not mutually exclusive and that the increased symptoms may result from a combination of these effects. In that chapter, I suggest exogenous application of SA during infection to limit bacterial growth and allow observation of increased jasmonate signaling in plants with normal bacterial levels.

As part of the hypothesis that symptoms are an indirect result of increased bacterial growth, I suggest that the increased accumulation of bacteria in *jah1* plants earlier in infection may trigger a change in the bacteria that results in premature activation of bacterial activities resulting in symptom production. Unfortunately, this is not a hypothesis that can be directly tested at this point. Ideally, we would want to examine expression of bacterial virulence genes during the infection process with an eye towards those that are expressed earlier in bacteria from *jah1* plants than those from wild-type. As things currently stand, it is not feasible to examine expression of multiple genes in bacteria during the infection process, and we do not know which bacterial genes are

specifically required for production of disease symptoms. As the technology in the field improves, perhaps experiments of this type will become possible and allow us to better understand the processes in the bacteria that contribute to symptom development in the host plant.

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Appendix 1

Expression of selected pathogen-induced genes in *jin1* mutant plants

To further investigate how mutation of *JIN1* alters expression of genes induced during DC3000 infection, we examined expression of additional genes in wild-type and *jin1* mutant plants following infection. *JIN1* itself has been shown to be induced during infection (Thilmony *et al.*, 2006), but this study relied on an infiltration method of infection which produces tissue damage and may stimulate jasmonate signaling independently of the effect of the bacteria. To confirm the induction pattern of *JIN1* transcript during *P. syringae* infection, I examined its levels in wild-type plants that had been infected using the L-77 dip procedure, via reverse transcription PCR (RT-PCR) using methods similar to those presented in Chapter 4. Consistent with earlier experiments, *JIN1* transcript levels are elevated 1 and 2 days following infection (Fig 1).

Work in our lab has demonstrated that expression of the auxin-conjugase family member *IAR3* is elevated following infection and that this induction is dependent on the production of coronatine (COR) (D. Brooks and B. Kunkel, unpublished data). Because some of the phenotypic effects of COR, such root inhibition, are at least partially independent of JIN1 activity, we were interested in determining whether the COR-dependent induction of *IAR3* required *JIN1*. RT-PCR was used to examine this question, and *IAR3* expression during infection was shown to be partially dependent on *JIN1* as *jin1* mutant plants exhibited delayed and decreased elevation of *IAR3* transcript levels (Fig 1).

As referred to in Chapter 2, another study has shown that SA levels are elevated in *jin1* mutant plants compared to wild-type during infection by *P. syringae* (Nickstadt *et al.*, 2005). The increased SA levels in wild-type plants are dependent upon the activity of

the *ICS1* gene (Wildermuth *et al.*, 2002). We hypothesized that the increase in SA levels in *jin1* mutants might be correlated with a further increase in *ICS1* gene expression. To examine this, we examined *ICS1* transcript levels during infection (Fig 1). We did not observe a difference in *ICS1* transcript levels between wild-type and *jin1* plants during infection (Fig 1), suggesting that either the increased SA levels in the mutant plants are not produced by ICS1 or that SA levels may not be regulated solely at the level of *ICS1* transcription, but may involve post-transcriptional regulation.

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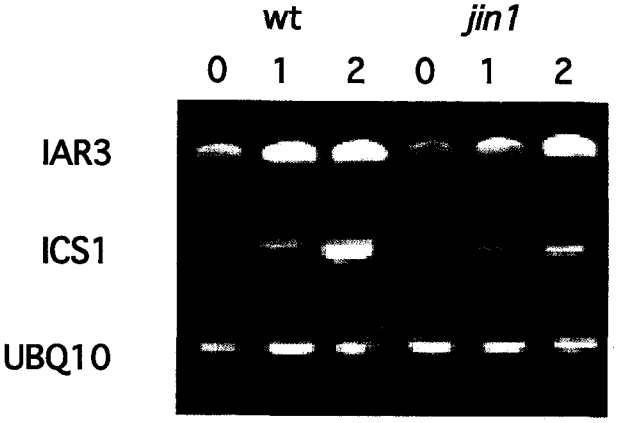
Figure 1

Expression of JA-dependent (*JIN1* and *IAR3*) and SA synthesis (*ICS1*) genes in wild-type and *jin1* mutant plants after dip infiltration with DC3000.

Gene expression is examined using RT-PCR. *UBQ10* is included as a control for equal loading. Similar results were seen in a second independent experiment.


wt
jin1
0 1 2 0 1 2
IAR3
ICS1
UBQ10

Appendix 2

Informative recombinants used for mapping the *jah1* mutation

Plants with recombination in the CIW to T10J-T7 interval

Plant #	CIW2	7242	**3298**	7310	**9608**	T13H18-T7	T10J7-T7	JA response
39	C	H					H	het?
88	C						H	
108	C						H	
125	C						H	
128	C						H	
159	C						H	
161	C						H	
163	C						H	
164	C						H	
170	C						H	
172	C						H	
198	C						H	
96	C						L	
104	C						L	
105	C						L	
A43	C		H		H		H	
A53	C		H		H		H	
A74	C		H		H		H	
A102	C		H		H		L	
A109	C		H		H		L	
A108	C		H		H		L	
A116	C		H		H		H	
A138	C		H		H		H	
A133	C		H		H		H	
A121	C		H		H		H	
46	H						C	het
71	H						C	
120	H						C	
121	H						C	
144	H						C	
145	H						C	
148	H						C	
156	H						C	
184	H						C	
185	H						C	
195	H						C	
A34	H						C	
22	H						C/H	
61	H						C?	
63	H						C?	
136	H						C?	
38	H						H?	het?
11	H						L	jah?
74	H						L	
A35	H		C		C		C	
A149	H		C		C		C	
A38	H		C		C			
A139	H		L		L		L	
177	L						C	
A52	L		H		H		C	
A24		C	H		H		C	

Plant #	CIW2	7242	**3298**	7310	**9608**	T13H18-T7	T10J7-T7	JA response
A17		C	H		H		H	
A19		C	H		H		H	
50	C	C	H		H		H	het
52	C	C	H		H		H	het
57	C	C	H		H		H	het?
48	C				H		H	jah
126	C				H		L?	
135	C				H		H	
160	C				H		H	
162	C				H			
165	C				H		H	
194	H				C		C	
A28	H				C		C	
95	L				C		C/H	
73	H					C	C	
83	C	C					H	
21	H		C?	C				jah
206	H			C				
94	C	C					H	
A148	H		H		C		C	
A167			C		H		H	
A128	C		C		H		H	
51	C	C	C		H		H	jah
A164	C		C		H		H	
A166	C		C		H			
A134	H		H		L		L	
A169			H		L		L	
A27	C				C		H	
70	H	H	H		H?		C	het?
76	C	C			C		H	
103	C				C		H	
106	C				C		H	
90	C				C		H	
47	H				H		C	jah?
A25	H		H		H		C	
A37	H		H		H		C	
A158	H				H		C	
A152	H				H		C	
37	H				H		C	het
A49	H		H		H		C	
A54	H		H		H		C	
A62	H		H		H		C	
A69	H		H		H		C	
A72	H		H		H		L	
A76	H		H		H		C	
A78	H		H		H		C	
A90	H		H		H		C	
A95	H		H		H		C	
A93	H		H		H		C	
A97	H		H		H		C	
A100	H		H		H		L	
A176			H		H		C/H	

Plant #	CIW2	7242	**3298**	7310	**9608**	T13H18-T7	T10J7-T7	JA response
A3	H				H		C	
A83	H				H		C	
A103	H				H		C	
A150	H				H		C	
A123	H				H		L	
A131	H				H		L	
A142	H		H		H		C	
A55	H		H		H		C	
A145	H		H		H		L	
A119	H		H		H		L	
A146	H		H		H		C	
A91	H		H		H		C	
A160	C				C		L	
12	C					C	H?	
A136	C				C		L	

Plants with unknown recombination state across the CIW2 to T10J7-T7 interval

Plant #	CIW2	7242	**3298**	7310	**9608**	T13H18-T7	T10J7-T7	JA response
41	C							
44	H							
45					L		L	het?
84							H	
85							H	
87							H	
93							H	
98							C	
101							C	
102							L	
107	L							
109							C	
119	L							
127	L							
137	C							
149	L							
150	H							
152	H							
158	H							
166	C							
173	L							
174	L							
175	C							
178	C							
179	L							
180	L							
183	H							
186	H							
197	L							
199	C							
200	C							
202	C							
207	L							

Plant #	CIW2	7242	**3298**	7310	**9608**	T13H18-T7	T10J7-T7	JA response
208	C							
209							C	
211	L							
212	C							
213	L							
214	H							
216	H							
217	H							
219	L							
221	C							
223	H							
224	C							
230	L							
231	H							
232	H							
233	H							
234	H							
235	H							
236	H							
237	L							
238	L							
240	C							
241	C							
243	H							
246	H							
248	C							
249	H							
250	H							
252	H							
254	C							
255	C							
256	C							
257	C							
258	L							
259	C							
260	C							
262	C							
264	C							
265	C							
267	L							
268	C							
269	C							
271	H							
272	H							
273	L							
274	C							
279	L							
280	L							
281	C							
282	C							
283	C							
284	C							

Plant #	CIW2	7242	**3298**	7310	**9608**	T13H18-T7	T10J7-T7	JA response
285	C							
286	C							
287	C							
288	C							
289	C							
291	C							
292	C							
293	L							
294	C							
295	C							
296	C							
297	C							
298	C							
299	L							
300	C							
301	C							
302	L							
303	L							
304	C							
305	L							
306	C							
307	C							
308	C							
309	H							
310	C							
311	L							
312	H							
313	L							
314	L							
315	H							
316	L							
317	C							
318	L							
319	C							
320	H							
321	H							
322	C							
323	H							
324	L							
325	L							
326	H							
327	H							
329	C							
330	L							
331	C							
332	C							
333	C/H							
334	C							
335	C							
336	C							
337	L							
338	L							

Plant #	CIW2	7242	**3298**	7310	**9608**	T13H18-T7	T10J7-T7	JA response
339	C							
340	C							
341	H							
342	H							
343	C							
344	C							
345	L							
346	L							
347	H							
348	H							
349	L							
350	C							
351	H							
352	C							
353	L							
354	L							
355	L							
356	H							
357	L							
390	H							
392	H							
393	L							
394	L							
A170						H		L
A172						L		H
A71	L							
A177						H		
A178						H		
A196				H?				
A197				L				
A198				L				
A204				C				
A205				L				
A206				C				

C=Col at this locus
H=Heterozygous at this locus
L=Ler at this locus
7242=J2-3107242
3298=J2-3833298
7310=J2-4377310
9608=J2-4449608
Markers flanking the interval containing COH-36 (3298 and 9608) are bolded.
Individuals with recombination in the 600 kb interval containing COH-36 are bolded.