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WASHINGTON UNIVERSITY IN ST. LOUIS

Division of Biology and Biomedical Sciences

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Investigating auxin biosynthesis and its contribution to virulence by *Pseudomonas syringae* pv.

tomato DC3000

by

Sheri McClerklin

A dissertation presented to the
Graduate School of Arts & Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

August 2016

St. Louis, Missouri

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education

ABSTRACT OF THE DISSERTATION

Investigating the role of auxin production by *Pseudomonas syringae* during pathogenesis

by

Sheri McClerklin

Doctor of Philosophy in Plant and Microbial Biosciences

Washington University in St. Louis, 2016

Professor Barbara N. Kunkel, Chair

Auxin is a major hormone that regulates many processes in plant development and has been shown to be important in several plant-pathogen interactions. Many microbes, including multiple pathovars of *Pseudomonas syringae* produce indole-3-acetic acid (IAA), a naturally occurring auxin. However IAA production and its biosynthesis pathway remains undetermined for many bacteria including *P. syringae* strain DC3000, the subject of this thesis. To investigate IAA biosynthesis by DC3000, I examined IAA production in cultures fed with different pathway intermediates. I show that DC3000 produces IAA in culture and cultures supplemented with Indole-3-acetaldehyde (IAAld) produced high levels of IAA, indicating that IAAld is an important intermediate for DC3000 IAA biosynthesis. Subsequently I investigated the IAA pathway(s) that utilize IAAld as an intermediate focusing on enzymes that convert IAAld to IAA. Through bioinformatics, I identified 6 putative DC3000 aldehyde dehydrogenase enzymes and showed that *E. coli* expressing genes encoding three of these, *PSPTO_0092*, *PSPTO_2673*, and *PSPTO_3644*, displayed IAAld dehydrogenase activity. Mutations in each of these aldehyde dehydrogenase genes, which we designated as *aldA*, *aldB* and *aldC* respectively, were made and tested for IAA production. The *aldA* and *aldB* mutants displayed reductions in IAA when grown in cultures supplemented with IAAld, however the *aldC* mutant did not. These results indicate that DC3000 utilizes IAAld as an important intermediate for IAA production and *AldA* and

AldB function in DC3000 IAA biosynthesis as indole-3-acetaldehyde dehydrogenases that convert IAAld to IAA.

Additionally I tested whether IAA derived from *P. syringae* DC3000, a bacterial speck causing bacteria, contributes to its pathogenesis. To investigate the role of DC3000 derived IAA during virulence, I utilized the DC3000 IAA biosynthesis mutants *aldA* and *aldB*. I showed that both *ald* mutants display reduced growth in Arabidopsis plants and that the severity of the reduced virulence is correlated with the degree of reduced IAA production. Additionally I showed that the *ald* mutants do not display reduced growth on a SA deficient mutant, *sid2-2* suggesting that DC3000 derived auxin functions to suppress SA-mediated host defenses. Elevated endogenous IAA in transgenic plants overexpression the Arabidopsis *YUCCA1* auxin biosynthesis gene did not rescue the *ald* mutant phenotype, indicating that plant derived auxin and pathogen derived auxin function to regulate DC3000 virulence in independent manners.

CHAPTER 1
INTRODUCTION

Bacterial plant pathogens utilize multiple strategies that allow them to colonize intercellular spaces, multiply and acquire nutrients within their host and ultimately lead to disease on susceptible plant species. Plants, however, have evolved the ability to mount defense strategies to prevent pathogenesis. In this introduction, I provide background into the virulence mechanisms pathogens utilize to successfully infect their hosts and the strategies plants have evolved as defense mechanisms. I focus mainly on the bacterial pathogen *Pseudomonas syringae* in my thesis research, and the strategies it uses during infection, including secretion of type III effectors and other virulence factors and modulation of host hormone physiology. I am especially interested in how *P. syringae* modulates host auxin physiology. Auxin is a major plant hormone and has recently been shown to contribute to disease development. I give insights into the roles of auxin during plant development and in plant microbe interactions including auxin production by *P. syringae* and the importance of auxin during pathogenesis. Furthermore, I present a summary of the main results and conclusions described in this thesis.

***Pseudomonas syringae*, a model plant pathogen**

P. syringae is Gram-negative plant pathogen that mainly affects the aerial portions of plants, including leaves and fruits and causes economically important diseases on a range of plant species including *Arabidopsis thaliana*, *Solanum lycopersicum* (tomato) and other *Brassica* species (Cuppels, 1986; Zhao et al., 2000). The pathogenesis process includes epiphytic colonization of the leaf surface, entry into the leaf through wound sites or stomata, bacterial growth in the apoplast and disease symptom production. The success of *P. syringae* as a pathogen depends on its ability to suppress host defense, acquire nutrients and alter host physiology in order to multiply to high levels within the plant (Xin and He., 2013). If this is

accomplished, disease symptoms consisting of chlorosis (yellowing of the leaf) and grey water soaked disease lesions develop (Preston, 2000). *P. syringae* infection is contained locally around the initial site of infection. It is considered a hemibiotrophic pathogen because at early stages of infection it obtains its nutrients from living cells (Glazebrook., 2005; Melotto et al., 2008), but eventually the infected areas will develop necrosis and death during later stages of infection (Preston, 2000; Xin and He, 2013). This is in contrast to true biotrophs that acquire nutrients from living cells without causing host cell death and to necrotrophic pathogens that acquire nutrients by killing host cells and feeding on their contents (Agrios, 1997; Melotto et al., 2008).

In the Kunkel lab we utilize *P. syringae* as a model organism for investigating plant-pathogen interactions due to its ability to be modified genetically. More specifically, we utilize *P. syringae* pv tomato strain DC3000, which has been studied over several decades and has contributed to our understanding of bacterial pathogenesis including the type III secretion system (T3SS), a needle-like apparatus used to secrete virulence proteins or “effectors” across the bacterial envelope, secreted effectors, toxin production and production of phytohormones (Melotto et al., 2008; Xin and He, 2013; Zhou and Chai, 2008). The genetic tractability of *P. syringae* makes it an important model system to investigate the mechanisms governing pathogenesis and disease. Furthermore, because *P. syringae* infects a range of economically important crops (Mansfield et al., 2012), it is important to understand the infection process and how we can target different aspects of virulence in order to prevent yield loss.

Plant pathogen interactions

Throughout their life cycle, plants are exposed to many biotic stresses including infection by pathogenic organisms. Plant pathogen interactions include multi-layered steps of virulence

strategies utilized by the pathogen and corresponding plant defense mechanisms that have evolved to thwart the invading pathogen (Chisholm et al., 2006; Jones and Dangl, 2006). During the infection process, the success of many pathogens like *P. syringae* depends on their ability to dampen host defense, acquire nutrients and water and multiply to high levels (Kunkel and Chen, 2006; Melotto et al., 2013). Plants as a first level of defense can perceive microbes at the cell surface by recognizing pathogen-associated molecular patterns (PAMPs) (Zipfel, 2009). PAMP-triggered immunity (PTI) is activated when plant cell surface receptors recognize conserved microbial features, such as lipopolysaccharides and flagellin (Dangl and Jones, 2001; Jones and Dangl, 2006), and induce MAP kinase-signaling that leads to activation of host defenses, ultimately contributing to inhibition of bacterial growth (Chisholm et al., 2006; Xin and He, 2013). However, pathogens have evolved mechanisms to actively suppress PTI, which include secretion of type III effector proteins and small molecules that inhibit PTI and alter host physiology (Block and Alfano, 2011; Guo et al., 2009). Many type III effectors directly target components of the PTI pathway to dampen host defense and other processes. As a second level of defense, plants evolved a “more specialized mechanism to detect microbes” (Chisholm et al., 2006). Effector triggered immunity (ETI) involves the recognition of Type III secreted effectors by plant resistance (R) proteins most of which are nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins (Mchale et al., 2006; Xin and He, 2013). Recognition of effector activity in the host cell by these R proteins triggers defenses including localized cell death at the site of infection (the hypersensitive response or HR) and production of antimicrobial activities that act collectively to limit pathogen growth (Dodds and Rathjen, 2010); not surprisingly pathogens have evolved another layer of virulence strategies to allow them to successfully avoid ETI and

many effectors may have overlapping roles in targeting components of ETI or PTI (Dangl and Jones, 2006).

Research investigating virulence and defense mechanisms has given us insights into the complexities of the ever-evolving strategies of virulence vs. defense. Perception of flagellin, the protein subunit of flagella, by the plant receptor-like kinase, FLS2, has given us a detailed look into how plants respond to PAMPs. Additionally, analysis of pathogen effectors and their cognate R proteins has elucidated the specification and diversification of functions and targets of the effector proteins (Chisholm et al., 2006). For example, the *P. syringae* effector AvrRpt2 is a protease that targets the host protein RIN4 (Axtell et al., 2003). Although the virulence targets of AvrRpt2 are not known, proteolysis of the RIN4 protein by AvrRpt2 is monitored by the plant resistance protein RPS2 and activates immunity. Furthermore, the effector AvrPtoB is an E3 ligase (ubiquitin conjugating enzyme) that targets many PTI receptors including FLS2. AvrPto-triggered protein turnover of these receptors impairs host basal defenses (Chisholm et al., 2006; Jones and Dangl, 2006). Many effectors have redundant targets within the plant and multiple plant R proteins are able to monitor the same key target, therefore recognizing a range of modifications of the target by secreted effectors. In addition to AvrRpt2 targeting RIN4, AvrB and AvrRpm1 can also target RIN4 and the corresponding plant R proteins, RPS2 and RPM1 recognize different modifications of RIN4, including proteolysis and phosphorylation, respectively (Liu et al., 2009). Therefore RIN4 is an important protein in plant defense and is heavily monitored given its importance in the activation of resistance signaling.

Examples like that of RIN4 and its demonstrated importance for both PTI and ETI as a central target for multiple effectors shows us the overlap that has evolved between resistance pathways. This has led to the molecular arms race model between plants and pathogens that both

are constantly evolving mechanisms of recognition and evasion, not only in response to each other but to gain the upper hand in the evolutionary war (Jones and Dangl, 2006). This evolutionary war has generated “highly polymorphic repertoires of R proteins and effectors” (Cunnac et al., 2009) and could be the cause of pathogen-host specificity and the ability of pathogens to be limited to infection of certain plant species.

***P. syringae* virulence factors**

Type III-secreted effectors

Secretion of effectors and other virulence factors is a major strategy underlying the success of bacterial pathogens including *P. syringae*. (Melotto and Kunkel, 2013) *P. syringae* utilizes the T3SS to secrete up to 30 effectors into the plant cell (Chang et al., 2005; Chisholm et al., 2006). Initial identification of the role of effectors during pathogenesis proved challenging due to redundancy in function. However combinatorial effector deletions have elucidated the importance of effector delivery and function to successful pathogenesis (Cunnac et al., 2009). Many effectors target a wide range of processes in the plant including MAPK signaling proteins in the PTI pathway, vesicle trafficking, hormone signaling and targeting of the PTI/ETI regulator RIN4. The repertoire of effectors secreted represents a wide range of biochemical activities including proteases, phosphatases and E3 ligases and function collectively to suppress host defenses and alter plant physiology to promote pathogen growth (Xin and He, 2013). For example AvrPto and AvrPtoB directly target PAMP receptors FLS2 and EFR to block PTI (Feng and Zhou, 2012), and HopM1 targets AtMIN7 and disrupts vesicle trafficking (Nomura et al., 2006). Additionally HopI1 localizes to the chloroplast and disrupts SA synthesis to promote virulence (Jelenska et al., 2007) and AvrPphB manipulates the jasmonic acid (JA) signaling

pathway. Although effectors function to promote pathogenesis most functions of effectors are recognized by various resistant plant lines, resulting in activation of immunity (Xin and He, 2013). However examining the role of effectors during pathogenesis in plants lacking their cognate R protein has expanded our understanding in the field and has given insights into the complexities of effector and the fact that they can have multiple functions within the plant.

***P. syringae* effector, AvrRpt2**

The *P. syringae* effector, AvrRpt2 is a cysteine protease (Axtell et al., 2003) that functions to cleave RIN4 (Coaker et al., 2005). In plants with a functional copy of RPS2, the corresponding R protein, this cleavage is recognized and triggers the hypersensitive response (HR). In mutant plants lacking a functional copy of RPS2, *rps2*, AvrRpt2 has been shown to promote virulence of *P. syringae* strain DC3000 (Chen et al., 2000). *rps2* mutant plants infected with DC3000 expressing AvrRpt2 support increased bacterial growth and symptoms compared to plants infected with DC3000 lacking AvrRpt2. AvrRpt2 has also been shown to modulate auxin sensitivity within the plant. AvrRpt2 promotes auxin induced-gene expression and *A. thaliana* plants expressing AvrRpt2 display increased sensitivity to exogenous auxin (Chen et al., 2007). Furthermore AvrRpt2 stimulates the degradation of Aux/IAA proteins (proteins that negatively regulate auxin signaling). The ability of AvrRpt2 to promote DC3000 pathogenesis may be due to its stimulation of Aux/IAA degradation (Cui et al., 2013). Interestingly the virulence activity of AvrRpt2 remains even in the absence of RIN4. DC3000 strains expressing AvrRpt2 display increased virulence compared to DC3000 on *rin4 rps2* double mutant plants (Lim and Kunkel, 2004). These results indicate that AvrRpt2 targets multiple proteins and

pathways to promote pathogenesis. One of my early research projects was to investigate the importance of AvrRpt2 protease activity in its virulence activity (see Appendix 2).

Coronatine

The T3SS and its secreted effectors are not the only factors secreted by pathogens that help promote pathogenesis. The phytotoxin coronatine (Cor) is a small molecule that functions as a molecular mimic of jasmonic acid-isoleucine (the active form of JA) and binds directly to the JA receptor complex (Katsir et al., 2008). *P. syringae* carrying mutations in coronatine biosynthesis genes display decreased virulence (Brooks et al., 2004; Uppalapati et al., 2007). Decreased virulence in Cor-deficient mutants is due to the modulation of the JA signaling pathway. JA and SA signaling are mutually antagonistic in Arabidopsis and in DC3000 infections, stimulation of JA signaling by secretion of Cor results in down regulation of SA signaling. (Brooks et al., 2005). Cor is also important for disease symptom production and stomatal opening. Cor-deficient mutants fail to develop normal disease symptoms and Cor is responsible for the re-opening of stomata in response to PAMP triggered stomatal closure (Melotto et al., 2006). Thus Cor contributes to virulence at many stages during *P. syringae* pathogenesis and provides an example of how bacterial hormone mimics and modulation of host hormone physiology is a key mechanism for successful pathogenesis.

***Pseudomonas iaaL* gene**

IAA production by plants and microbes is well established (Spaepen and Vanderleyden, 2011). Additionally, plants can conjugate IAA to amino acids and sugars for storage and degradation (Korasick et al., 2013) and IAA conjugates have been reported to play a role in *P.*

syringae pathogenesis. For example, IAA-Asp was shown to promote DC3000 pathogenesis and *Arabidopsis gh3.2* mutants, encoding an enzyme responsible for synthesizing IAA-Asp, was less susceptible to DC3000 infection (Gonzalez-Lamothe et al., 2012). *P. syringae* genome encodes, IAA conjugation enzymes, such as *iaaL* and recently has been shown to be important for virulence in *P. savastanoi* (Glass and Kosuge, 1986; Spaepen et al., 2011). The *iaaL* gene, which encodes an IAA-Lys synthase, is capable of conjugating IAA to the amino acid Lysine. Mutations of the *iaaL* gene in *Pseudomonas savastanoi* pv. *nerii* resulted in accumulation of IAA in culture (Glass and Kosuge, 1988). The *iaaL* gene was shown to be dependent on HrpL, an alternative sigma factor that is required for transcription of T3SS, effector genes and other pathogenesis related factors (Castillo-Lizardo et al., 2015). Furthermore mutations in *iaaL* and the upstream gene (in the same operon), *mateE* resulted in decreased virulence of DC3000 on tomato plants. These results indicate that the pathogen is influencing IAA homeostasis by conjugating it. However the role of IAA-Lys during pathogenesis remains to be determined. Furthermore the ability of other pathogens to conjugate hormones like IAA and the mechanism by which these conjugates function during pathogenesis requires further examination.

Plant hormones regulate defense responses

Salicylic acid and jasmonic acid

Plant hormones are not only important for regulating plant development and growth, they are also major regulators of plant defense against pathogens (Lopez et al., 2008). Two important defense hormones are jasmonic acid (JA), which is primarily involved in defense against necrotrophic pathogens, and salicylic acid (SA), which is required for both PTI and ETI and defense against biotrophic and hemibiotrophic pathogens such as *P. syringae* (Vlot et al., 2009;

Xin and He, 2013). Following pathogen attack, synthesis of the SA biosynthesis enzyme, Isochorismate synthetase 1 (*ICS1*), is induced leading to increased SA synthesis (Wildermuth et al., 2001). Exogenous application of SA also increased plant resistance to a wide range of pathogens (Durrant and Dong, 2004). Plants defective in SA biosynthesis, such as the *sid2-2* mutant, display increased susceptibility to *P. syringae* and *Peronospora parasitica* (Mutka et al., 2013; Wildermuth et al., 2001). Furthermore expression of *Pathogenesis Related 1 (PR1)* gene, a commonly used marker for SA mediated defenses is up-regulated during pathogen infection. Although JA and SA primarily regulate different defense pathways there is crosstalk between the two in which the JA and SA signaling pathways have antagonistic roles (Thaler et al., 2012). For example stimulation of JA signaling by Cor can lead to suppression of SA mediated defenses (Brooks et al., 2005). Additionally many JA signaling plant mutants including *jin1* and *coi1* are less susceptible to *P. syringae* infection because they are insensitive to Cor (Kloek et al., 2001; Laurie-Berry et al., 2006); however bacterial growth is restored to wild-type levels in *jin1 sid2-2* double mutant plants. This suggests that modulation of JA signaling by Cor is required to suppress SA mediated defenses to promote *P. syringae* virulence. It is possible that the mutual antagonistic nature of the JA and SA signaling pathways could allow for specificity in the regulation of only those processes needed to fight off a particular pathogens without wasting those that are not required.

Hormone crosstalk

JA and SA, although major hormones involved in plant defense, are not the only plant hormones that modulate the outcome of plant-pathogen interactions. There is evidence of almost every plant hormone playing a role in defense including ethylene, abscisic acid, cytokin and

auxin. (Jameson, 2000; Robert-Seilaniantz et al., 2011) Furthermore there seems to be a prevailing theme in which most of these hormones function antagonistically to SA. For example ethylene is a plant hormone involved in fruit ripening and senescence, and has been shown to function to positively regulate defense against necrotrophic pathogens. Additionally, it negatively regulates SA-mediated biotrophic defenses. EIN3 and EIL1 regulators of ethylene signaling repress SA synthesis through direct binding to the promoter of *ICS1*. Consequently *ein3 eil1* double mutant plants display increased resistance to *P. syringae* (Chen et al., 2009). Furthermore abscisic acid (ABA) is an important hormone known for its role in seed development, stomatal opening and drought stress responses. It has also been implicated in antagonizing SA in which the ABA biosynthesis mutant *aao3* exhibits increased SA levels and reduced susceptibility to DC3000 (de Torres Zabala et al., 2009).

The observation that many hormones regulate both plant development and defense pathways is well established. However the molecular mechanisms that govern these processes including crosstalk between hormone signaling pathways requires further investigation. However it is clear that many of these pathways are key targets for successful pathogenesis of pathogens like *P. syringae*. As seen previously with the ability of Cor to modulate the JA pathway, IAA production and modulation of auxin physiology is likely to be an additional strategy for successful pathogenesis.

Microbial IAA biosynthesis

Auxin is an important hormone plant hormone that contributes to the regulation of a plethora of plant growth and developmental processes (Zhao, 2010). The most common naturally occurring auxin is indole-3-acetic acid (IAA). However other natural and synthetic

auxins including phenyl acetic acid (PAA), naphthalene acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2-4-D) have been identified and have similar physiological effects as IAA in plants (Korasick et al., 2013). Additionally IAA can be conjugated to amino acids and sugars, which leads to its storage or degradation. The synthesis and regulation of auxin is not only important for plant development but is also important in regulation of plant interactions with microbes.

Examples of microbial synthesis of IAA have been known for quite some time and as additional bacterial species are examined, it is becoming evident that many bacteria can produce IAA. Interestingly it is suggested that up to 80% of bacteria isolated from the rhizosphere synthesize IAA (Spaepen and Vanderleyden, 2011). It is often found that microbes can synthesize IAA from one or more pathways starting with tryptophan (Trp), however at least in culture, IAA synthesis is usually low unless there is excess Trp available. Five different pathways that use Trp as a starting substrate for IAA synthesis have been described in microbes (Spaepen et al, 2007; Zhao, 2010), each designated by the major intermediate in the pathway. These include the indole-3-acetamide (IAM) pathway, the indole-3- pyruvate (IPA) pathway, the tryptamine (TAM) pathway, the tryptophan side-chain oxidase (TSO) pathway and the indole-3-acetonitrile (IAN) pathway (Figure 1.1) (Patten et al., 2013).

Two of the primary IAA pathways, the IAM and IPA pathways, have been identified and well characterized in bacteria and have helped elucidate our understanding of bacterial IAA production and the role of bacterial-derived IAA in plant-pathogen interactions. The tumor and gall forming bacteria *P. syringae* pv *syringae* and *Pantoea agglomerans* pv *gypsophila*, utilize the IAM pathway to produce IAA and this pathway has been shown through mutational analysis to be responsible for gall formation (Barash and Manulis-Sasson, 2007; White and Ziegler,

1991). The IAA biosynthesis genes *iaaM* and *iaaH* encode tryptophan 2-monooxygenase and indoleacetamide hydrolase enzymes, respectively, and are responsible for converting Trp to IAA via an IAM intermediate. The IPA pathway is another major pathway that has been well characterized in bacteria in which IAA is produced via an IPyA intermediate. In bacteria the *ipdc* gene encoding the indole-pyruvate decarboxylase enzyme that catalyzes that second step of the pathway from IPyA to IAAld has been identified and characterized in a range of bacteria from *A. brasilense*, *E. cloacae* and *P. agglomerans* and is important for epiphytic colonization of certain bacteria (Koga et al., 1992; Manulis et al., 1998; Spaepen et al., 2007; Spaepen et al, 2011).

Although the IAM and IPyA pathways have been the most well characterized pathways of microbial IAA biosynthesis to date, the roles of the IAN, TAM and TSO pathways are less clear. In the IAN pathway, a putative nitrilase enzyme, which catalyzes the conversion of IAN to IAA, was mutated in *P. syringae* B728a and showed reductions in IAA synthesis (Howden et al., 2009). However this pathway is poorly understood in bacteria. Likewise enzymes of the TAM and TSO pathways remain unidentified, although enzymatic activity has been shown in culture lysates. Although multiple IAA biosynthesis pathways have been observed in a broad range of bacteria, the specific pathways that lead to IAA production in some bacteria like DC3000 are undetermined. A primary focus of my thesis was to identify and characterize the biosynthetic pathways used by DC3000 to produce IAA (Chapter 2).

Auxin and *P. syringae* pathogenesis

IAA is well established as an important hormone in plant pathogen interactions. In the gall-forming bacteria *Rhizobium radiobacter* (formerly *Agrobacterium tumefaciens*), disease production is, in large part, due to the production of IAA and CK by the plant, which is mediated

by the transfer of IAA biosynthesis genes from the bacterium into the host (Yamada, 1993). *Pantoea. agglomerans*, another gall-forming bacteria, produces IAA and this IAA contributes to gall formation. Both *R. radiobacter* and *Pantoea* utilize the IAM pathway for auxin production. Although it is clear that IAA production by these two pathogens is important for their virulence, the contribution of IAA to pathogenesis of other pathogens including *P. syringae* requires further elucidation.

Although the role of IAA production and its contribution to pathogenesis in the gall-forming bacteria has been researched extensively, the role of IAA in bacterial speck disease-causing pathogens such as *P. syringae* remains unclear, although there is evidence from other lines of research that auxin promotes *P. syringae* pathogenesis. For example, it has been observed that exogenous application of IAA and other forms of auxin promote disease susceptibility to DC3000 and *P. syringae* pv. *maculicola* (Chen et al., 2007; Wang et al., 2007, Novarro et al., 2006). Transgenic *A. thaliana* lines over-expressing *YUCCA1*, a plant auxin biosynthesis gene, which accumulate increased endogenous IAA levels, exhibit enhanced susceptibility to DC3000 (Mutka et al., 2013). Additionally, IAA levels increase in *A. thaliana* and tomato plants infected with DC3000. This increase was also observed in plants infected with *Xanthomonas* (Chen et al., 2007; Ding et al., 2008), although these results are mainly correlative, this suggests that modulation of auxin homeostasis could be a shared virulence strategy among pathogens.

Modulation of host auxin physiology has also been shown to be important for *P. syringae* pathogenesis. Impairment of auxin signaling in the plant results in reduced disease susceptibility. For example *axr2* mutant plants displayed decreased susceptibility when infected with *P. syringae* (Wang et al., 2007). Furthermore there are examples in which pathogen derived

proteins can modulate host auxin physiology. For example, the *P. syringae* Type III-secreted effector protein, AvrRpt2, promotes virulence of DC3000 and modulates *A. thaliana* auxin physiology and signaling (Chen et al, 2007; Cui et al., 2013). It is suggested that the promotion of DC3000 virulence by AvrRpt2 could in part be due to its modulation of host auxin physiology. Additionally, as described above, a gene encoding a putative IAA-Lys enzyme, *iaaL* has been shown to be important for DC3000 pathogenesis (Castillo-Lizardo et al., 2105). Although there are many examples of auxin being important for the promotion of pathogenesis, its role in many plant-pathogen interactions, like that of DC3000 and its hosts, is not well understood. Furthermore, because the process of pathogenesis, involves a multi-step process it remains to be elucidated whether the role for IAA during pathogenesis might be regulation of these processes.

Summary of chapters

In this thesis, I present my research on IAA biosynthesis in DC3000 and the contribution of this pathogen-derived auxin to pathogenesis. Chapter 2 presents my results on the DC3000 IAA biosynthesis pathway. I demonstrated that DC3000 produces IAA in culture, and indentified the auxin intermediate indole-3-acetaldehyde (IAAld) as an important intermediate for auxin production in culture. Using bioinformatics, I identified three IAA dehydrogenase (Ald) enzymes that catalyze the conversion of IAAld to IAA. Furthermore, DC3000 carrying mutations in the genes encoding these enzymes led to reduced IAA production.

Chapter 3 presents results on the contribution of DC3000-derived auxin to its pathogenesis. I inoculated *A. thaliana* plants with DC3000 IAA biosynthesis mutants, *aldA* and *aldB*, and monitored growth and disease symptom production. I observed that the *ald* mutants displayed decreased growth in planta and reduced disease symptom production. I also observed

that reduced growth of the *ald* mutants was restored on a SA-deficient plant mutant, *sid2-2* and wild-type plants infected with the *ald* mutants displayed increased SA-dependent defense gene expression. This indicates that the role of DC3000 IAA production is to suppress SA mediated defenses. Lastly, I utilized a transgenic *A. thaliana* line with increased endogenous auxin levels shown previously to promote DC3000 pathogenesis, to examine whether elevated endogenous auxin could restore full virulence to the *ald* mutants. However, bacterial growth of *ald* mutants was not restored on the transgenic *A. thaliana* plant lines. This suggests that plant and microbial IAA can function to promote DC3000 via independent pathways.

Chapter 4 presents a summary of major conclusions of chapters 2 and 3. In particular I discuss how my research fits with our current understanding of microbial IAA biosynthesis and how it broadens our view on the pathways bacteria use to produce IAA. Furthermore I discuss the implications that auxin can play multiple independent roles in promoting pathogenesis, depending on the source.

Appendix 1 presents preliminary data on the role of the Alds in the synthesis of additional auxins, and summarizes work done in collaboration with Soon-Goo Lee in the laboratory of Joseph Jez. Appendix 2 describes my unpublished results on the requirement of the cysteine protease activity of AvrRpt2 in its ability to promote DC3000 virulence. These data indicate that the cysteine protease activity is required for AvrRpt2's ability to modulate host auxin physiology but may not be required to promote pathogenesis. I discuss how this data compares to what has been reported previously. These experiments provide a starting point for future experiments to be carried out in the future.

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FIGURES

Figure 1.1. A diagram of the infection cycle of *Pseudomonas syringae*. Pathogenesis by *P. syringae* includes colonization of the leaf surface, entry into the leaf through stomata (natural openings on the leaf) or wound sites, growth in the apoplast (intercellular spaces within between leaf cells) where bacteria are using T3SS to secrete virulence factors into the cell that promote pathogenesis and nutrient and water acquisition. Successful pathogenesis of susceptible hosts culminates disease symptoms.

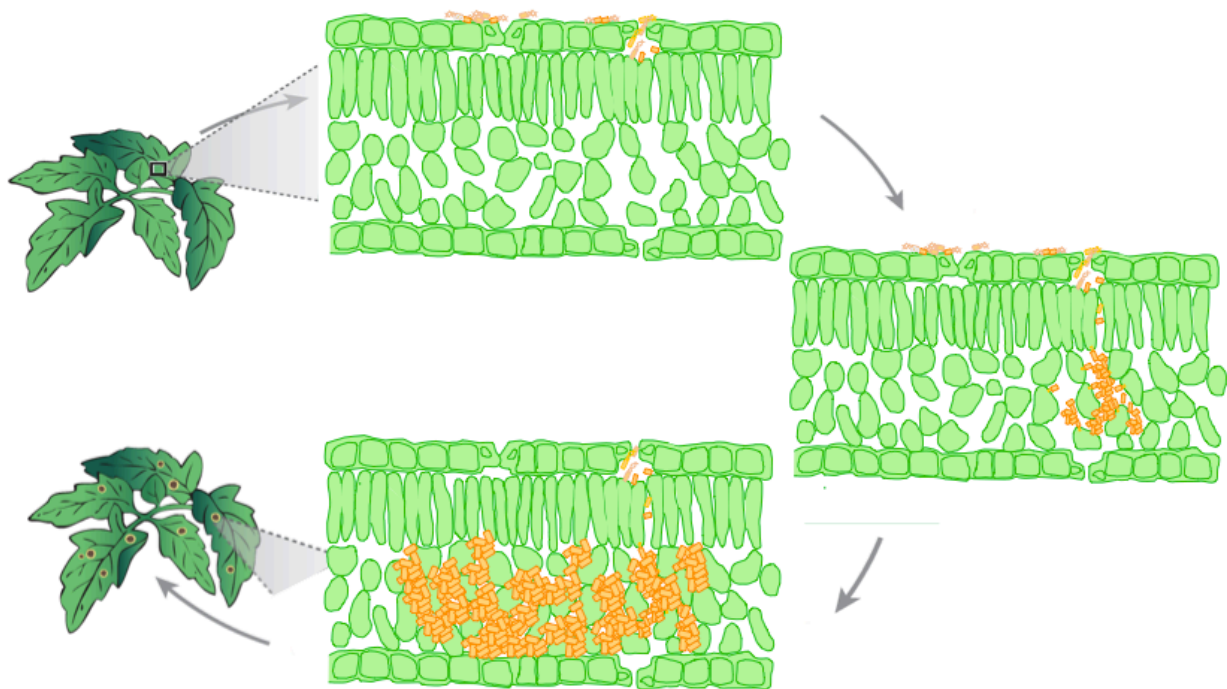
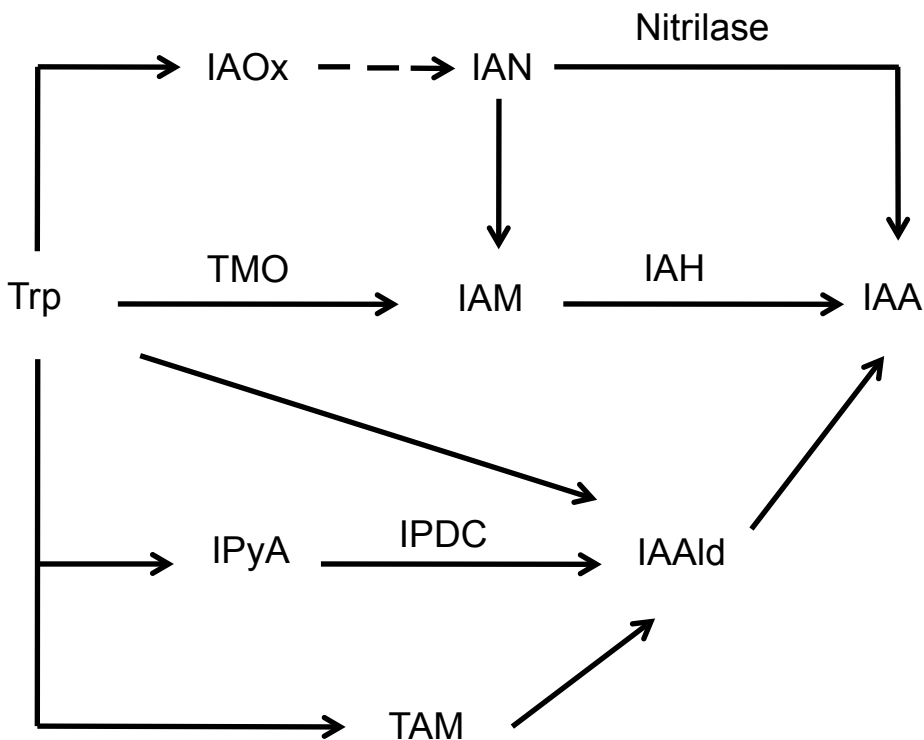


Figure 1.2. Overview of indole-3 acetic acid (IAA) biosynthesis pathway(s) in bacteria.

Enzymes with demonstrated biochemical activities are indicated. Enzyme abbreviations are: tryptophan 2-monooxygenase (TMO), indole acetamide hydrolase (IAH), and indole pyruvate decarboxylase (IPDC). Compound abbreviations are: tryptophan (Trp), indole-3-acetaldoxime (IAOx), indole-3-acetonitrile (IAN), indole-3-acetamide (IAM), indole-3-pyruvate (IPyA), indole-3-acetaldehyde (IAAld), and tryptamine (TAM). Adapted from Spaepen et al 2011.



Chapter 2

Identification of Aldehyde dehydrogenase (Ald) genes involved in DC3000 IAA biosynthesis

This chapter is a collaborative work that was submitted for publication as:

Indole-3-acetaldehyde dehydrogenase, AldA, functions in IAA biosynthesis in *Pseudomonas syringae* pv tomato DC3000 and contributes to virulence on *Arabidopsis*

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Author contributions:

S.M. designed and performed the majority of the experiments and wrote and edited the majority of the manuscript. S.G.L. designed and performed the experiments for Figure 2.4 and Table 2.2, wrote the manuscript and methods section describing the data related to those experiments. R.N. assisted with the experiments for Figure 3.4 and Table 2.2. J.M.J. designed experiments for Figure 2.4 and Table 2.2 and edited the manuscript. B.N.K. designed experiments, wrote the Discussion section, and edited the manuscript.

ABSTRACT

Although it is known that many *P. syringae* pathovars can produce IAA in culture (Glickmann et al, 1998), it remains to be determined whether *P. syringae* strain DC3000 produces IAA and what pathway(s) it utilizes for IAA synthesis. In this chapter, I show that DC3000 produces IAA in culture and synthesis was increased when supplemented with exogenous Tryptophan (Trp), as shown previously for other *P. syringae* strains and auxin-producing bacteria. In feeding studies to identify the pathway(s) DC3000 uses to synthesize IAA, I demonstrated that IAAld is an important intermediate for DC3000 IAA biosynthesis. Subsequently I used a bioinformatic approach to identify enzymes that convert IAAld to IAA and identified 6 putative DC3000 aldehyde dehydrogenase enzymes. *E. coli* expressing 3 of these proteins, PSPTO_0092, PSPTO_2673, PSPTO_3644, displayed increased IAA levels over empty vector controls in cultures supplemented with IAAld. Furthermore I generated plasmid disruption mutants in each aldehyde dehydrogenase gene, which we designated as *aldA*, *aldB* and *aldC* respectively, and tested whether these strains had decreased IAA production. The *aldA* and *aldB* mutants displayed significant reductions in IAA when supplemented with IAAld, however the *aldC* mutant did not. These results indicate that DC3000 utilizes IAAld as an important intermediate for IAA production and AldA and AldB function in DC3000 IAA biosynthesis as indole-3-acetaldehyde dehydrogenases that convert IAAld to IAA.

INTRODUCTION

Bacterial IAA synthesis involves at least five different pathways utilizing Tryptophan (Trp) as a precursor (Figure 2.1). There are two primary pathways that have been well characterized not only in their role in IAA biosynthesis but in plant-microbe interactions. The indole-3-acetamide (IAM) pathway converts Trp to IAA in two steps. The IAA biosynthesis genes *iaaM* and *iaaH*, which encode tryptophan 2-monooxygenase and indoleacetamide hydrolase enzymes, respectively, catalyze the conversion of Trp to IAA via an IAM intermediate (Spaepen and Vanderleyden, 2011). The IAM pathway is characteristic of the gall forming bacteria, including *Pantoea agglomerans* and *Pseudomonas savastanoi* (Barash and Manulis-Sasson, 2007; Aragon et al., 2014). The indolepyruvate (IPyA) pathway is the second main pathway and involves the conversion of Trp to IAA via an IPyA intermediate. The conversion of IPyA to indole-3-acetaldehyde (IAAld) is the rate-limiting step in this reaction and is carried out by indolepyruvate decarboxylase enzyme encoded by the *ipdc* gene (Patten et al., 2013). This gene is found in a range of bacteria including *Azospirillum brasilense* and *E. cloacae* (Spaepen et al., 2007; Koga et al., 1992). The IPyA pathway is important for epiphytic colonization of plant-associated bacteria (Manulis et al., 1998; Spaepen et al., 2011). An additional IAA biosynthesis pathway is the IAN pathway, which involves the conversion of IAN to IAA and is mediated by nitrilases. This pathway is far less understood than the IAM and IPyA pathways, however nitrilases that function in IAA biosynthesis have been identified in *Alcaligenes faecalis* and *P. syringae* pv *syringae* (Kobayashi et al., 1993; Howden et al., 2009).

IAA production and genes involved in IAA biosynthesis pathways have been identified in many *P. syringae* pathovars (Glickmann et al., 1998). In the Kunkel lab, we utilize *Pseudomonas syringae* pv *tomato* DC3000 to study the mechanisms that govern pathogenesis.

We were interested in investigating whether DC3000 produces auxin, as has been reported for other *P. syringae* strains, and if auxin contributes to pathogenesis. IAA production by DC3000 was previously investigated by Andrew Mutka and it was observed that DC3000 produces IAA in culture. Subsequently he investigated which pathway DC3000 used to produce IAA. The DC3000 genome is annotated as encoding a tryptophan 2-monooxygenase (TMO) enzyme (*PSPTO0518*; *iaaM*; Buell et al., 2003). To test if the predicted IaaM/TMO protein contributes to IAA synthesis, DC3000 deletion mutants in the *PSPTO0518* gene were assayed for IAA production; however, no changes in IAA levels were observed. This suggests that the IAM pathway does not contribute to IAA production in DC3000. Additionally, DC3000 does not carry an obvious *ipdc* gene, suggesting that DC3000 does not use the IPyA pathway to produce IAA. Thus, the IAM and IPyA pathways do not appear to be involved in IAA biosynthesis in DC3000.

In this study, I further explored IAA production by DC3000. To investigate IAA biosynthesis pathway(s) involved, I carried out culture feeding studies in which DC3000 was supplemented with IAA intermediates. I observed that cultures supplemented with IAAlld led to increased IAA production, indicating its importance in DC3000 IAA biosynthesis. I identified 3 aldehyde dehydrogenase enzymes that exhibited IAA synthesis activity in *E. coli* cells and demonstrated that mutation of the genes encoding two of these enzymes led to decreased IAA production in culture. In collaboration with Dr. Lee in the laboratory of J. Jez, the biochemical activity of these 2 enzymes was characterized and we observed that they are able to convert IAAlld to IAA. Thus, I identified two new indole-3-acetaldehyde dehydrogenase enzymes that function in DC3000 IAA biosynthesis.

RESULTS

***Pseudomonas syringae* pv. tomato strain DC3000 synthesizes IAA in culture via an indole-3-acetaldehyde intermediate**

Many *P. syringae* strains produce IAA in culture and IAA concentrations increased when bacterial cultures were supplemented with Trp (Glickmann et al, 1998); however, it has not been determined whether *P. syringae* pv. tomato strain DC3000 can synthesize IAA. To examine this I grew wild-type DC3000 in Hoitkin-Sinden minimal media containing citrate (HSC) with shaking for 48 hours at 28°C. I chose this media as it is reported to more accurately reflect growth conditions in the apoplast of leaves (Rico and Preston, 2008). IAA concentrations in culture supernatants harvested at 24 and 48 hours were determined by LC-MS/MS. As observed for many other *P. syringae* strains, the level of IAA produced by DC3000 when provided with Trp was significantly higher than in unsupplemented media (Table 2.1).

The observation that DC3000 produces IAA in culture raised the question as to which pathway(s) DC3000 uses to produce IAA (Figure 2.1). To identify the IAA biosynthetic pathway(s) used by DC3000, I performed IAA precursor feeding experiments (Table 2.1). Cultures grown in HSC media were supplemented with 0.25 mM Trp, IAM, IAN, IPyA, TAM, or IAAlD and analyzed for IAA production by LC-MS/MS. DC3000 cultures supplemented with IAM, IAN, and TAM produced small but detectable amounts of IAA compared to cultures grown in HSC alone; however, these levels were relatively low compared to cultures fed with Trp. In contrast, high levels of IAA were produced when DC3000 was grown in media supplemented with IAAlD. This indicates that IAAlD is an important intermediate for DC3000 IAA synthesis in culture.

The feeding experiments with IPyA were inconclusive, as IPyA is unstable in solution (Bentley et al., 1956; Mashiguchi et al., 2011) and high amounts of IAA accumulated spontaneously in HSC media containing IPyA, but lacking DC3000 (Table 2.1). Given the absence of an obvious *ipdc* gene in the DC3000 genome, it is unlikely that DC3000 uses IPyA to synthesize IAAld. Thus, we hypothesize that DC3000 synthesizes IAA via a pathway involving conversion of Trp to IAAld through TSO activity (Oberhansli et al., 1991; Spaepen et al., 2007) (Figure 2.1). We cannot rule out the ability of DC3000 to produce small amounts of IAA through alternative pathways using IAM, IAN and/or TAM; however, based on the results of our feeding studies these pathways do not contribute significantly to IAA synthesis in culture.

Identification of putative *Pst*DC3000 aldehyde dehydrogenase genes

My studies indicate that DC3000 synthesizes IAA via one or more pathways that involve IAAld as an intermediate (Table 2.1). Thus, I predicted that disrupting the final step, which converts IAAld to IAA, would lead to a reduction in DC3000 IAA biosynthesis. To investigate this, we sought to identify the gene(s) encoding the enzyme(s) responsible for the conversion of IAAld to IAA. Previously, an *Azospirillum brasilense* mutant (*aldA*) with decreased IAA production was identified and the mutation mapped to a gene encoding a protein with ~80% amino acid identity to an annotated aldehyde dehydrogenase from *Xanthobacter autotrophicus* GJ10 (Xie et al., 2005). Aldehyde dehydrogenases (ALDs) generally catalyze the conversion of aldehydes to carboxylic acids (Ho and Weiner, 2005; Weiner and Wang, 1994). We predicted that a similar enzyme might catalyze the conversion of IAAld to IAA, and thus utilized the amino acid sequences of the ALDs from *A. brasilense* and *X. autotrophicus* to perform a BLAST search to identify putative aldehyde dehydrogenases in DC3000.

The DC3000 protein, PSPTO_0728, was identified as the protein with the highest amino acid identity (~70%) to the query and is also annotated as a putative ALD. We subsequently used the PSPTO_0728 amino acid sequence to search the DC3000 genome and the top 5 putative ALDs, PSPTO_0092, PSPTO_2673, PSPTO_3064, PSPTO_3323 and PSPTO_3644, with the highest similarity to PSPTO_0728 (~30-40% amino acid identity) were chosen. None of these proteins have previously been demonstrated to have dehydrogenase activity, nor were described as being involved in either auxin biosynthesis or DC3000 virulence.

I examined whether these proteins could convert IAAld to IAA by expressing each gene individually in *E. coli*, growing the strains in LB media supplemented with 0.25 mM IAAld for 24 hrs at 37°C, and then assaying the resulting strains for IAA production by LC-MS/MS. We observed a small amount of IAA produced by *E. coli* carrying the empty expression vector (pET-21a) (Figure 2.2), consistent with previous reports (Glickmann et al, 1998; Brandl and Lindow, 1996). Upon induction of expression of the ALDs from DC3000, we observed increased IAA levels over the control for three of the six proteins. Cells expressing PSPTO_0092 showed the greatest accumulation of IAA with an ~100-fold increase in IAA over the empty vector control (Figure 2.2b). The strains expressing either PSPTO_2673 or PSPTO_3644 showed 10- and 5-fold increases in IAA levels, respectively (Figure 2.2a). Differences in IAA levels between these strains were not due to large variations in protein expression, as comparable protein levels were observed on SDS-PAGE gels for all strains tested (Figure 2.3). Thus, PSPTO_0092, PSPTO_2673, and PSPTO_3644 can convert IAAld to IAA and likely function in DC3000 auxin biosynthesis. We refer to PSPTO_0092, PSPTO_2673, and PSPTO_3644 as AldA, AldB and AldC, respectively, throughout this study.

Biochemical analysis of putative IAld dehydrogenases

In collaboration with the laboratory of J. Jez, Dr. Lee, examined the biochemical activity of the three putative ALDs from DC3000. These proteins were expressed in *E. coli* as a N-terminal hexahistidine-tagged protein and purified by nickel-affinity and size-exclusion chromatographies. In vitro assays of purified AldA, AldB, and AldC using IAld with either NAD^+ or NADP^+ as substrates confirm the major activity of AldA as that of an IAld dehydrogenase, as each protein converted NAD(P)^+ to NAD(P)H only in the presence of the IAld (Figure 2.4). Each Ald used NAD^+ with a 10- to 40-fold preference versus NADP^+ , but AldA had a specific activity ($3.52 \mu\text{mol min}^{-1} \text{mg protein}^{-1}$) using IAld as a substrate that was 100- and 800-fold higher than AldB and AldC, respectively. AldA-C displayed no changes in specific activities in the presence of calcium, magnesium, manganese, cobalt, nickel, and copper, which suggests that these proteins function as non-metallo NAD^+ -dependent ALDs. In addition, none of the three Alds showed any detectable activity with NADH (at 200 mM) and IAA (at 1 mM), indicating a clear preference for the formation of IAA compared to the reverse reaction. Steady-state kinetic analysis showed that AldA had a catalytic efficiency (k_{cat}/K_m) with IAld as a substrate that was 130- and 710-fold higher than AldB and AldC, respectively (Table 2.2). AldA also showed more than a 300-fold higher k_{cat}/K_m with NAD^+ compared to NADP^+ . A similar cofactor preference was observed for AldB and AldC. The low activities of AldB and AldC did not allow for accurate determination of steady-state kinetic parameters for NADP^+ . These biochemical comparisons suggest that AldA functions as an IAld dehydrogenase and that AldB and AldC likely prefer other aldehyde substrates in vivo.

IAA production is disrupted in DC3000 *ald* mutants

To study the role of these ALDs in DC3000 IAA biosynthesis, I generated plasmid disruption mutants in *aldA* (PSPTO_0092), *aldB* (PSPTO_2673) and *aldC* (PSPTO_3644) (Figure 2.5). We monitored the ability of each mutant strain to produce IAA in culture when provided IAAlD in the growth media. Only two mutants displayed reduced levels of IAA when compared to DC3000 (Figure 2.6a). The *aldA* mutant displayed a ~75% reduction in IAA levels compared with DC3000, whereas the *aldB* mutants exhibited a ~15% reduction in IAA levels. The *aldC* mutant did not display a statistically significant reduction in IAA levels, which is consistent with the observation that the AldC enzyme does not exhibit aldehyde dehydrogenase activity in the presence of IAAlD in vitro (Figure 2.4). Furthermore, we generated an *aldA aldB* double mutant and tested whether they have additive effects on IAA biosynthesis. We observed that the *aldA aldB* double mutant displayed an even greater reduction in IAA when grown in culture than either single mutant alone (Figure 2.6c). These results indicate that AldA and AldB proteins contribute to IAA synthesis in DC3000, that AldC does not, and that AldA and AldB function additively in DC3000 IAA biosynthesis. A plasmid carrying the coding region of AldA, *pAldA*, was expressed in the *aldA* mutant, and tested for its ability to complement the *aldA* mutant phenotypes. IAA production in culture and bacterial growth *in planta* following infection was restored by introduction of *pAldA* in the *aldA* single mutant (Figure 2.6d). In fact, IAA production of the *aldA* complemented strain was higher than wild-type DC3000. This may be due to nature of the pME6031 vector, which is a multi-copy replicating plasmid. Complementation of the *aldB* mutant is in progress, and a clone carrying the wild type *AldB* genomic region, *pAldB*, has been made.

We also investigated the role of the *ald* genes on DC3000 biology by examining growth of DC3000 *ald* mutants in culture. The *ald* single mutants showed no difference in growth in both NYG and HSC cultures (Figure 2.5 e-f). The *aldA aldB* double mutant displayed reduced growth compared to wt and both single mutants in HSC. In cultures supplemented with IAAlD, DC3000 exhibited reduced growth rates in HSC media supplemented with IAAlD compared to DC3000 grown in HSC alone (Figure 2.6b). This could be due to a toxic effect of IAAlD at the concentration used (0.25 mM). All three *ald* mutant strains also displayed a similar reduction in growth rates in HSC media supplemented with IAAlD. In this case, the *aldA* and *aldB* knockout strains were slightly more sensitive than DC3000. The increased sensitivity of the *ald* mutants may be due to the fact that they are less efficient at converting IAAlD to IAA.

DISCUSSION

In summary, I showed that IAAlD is an important intermediate for IAA production in culture. We identified two novel indole-3-acetaldehyde enzymes that function to convert IAAlD to IAA, which we designated as AldA and AldB. I generated mutations in the genes encoding these enzymes in DC3000 and showed that there was a decrease in IAA production in culture in these mutants. Our findings indicate that unlike the gall forming strains of *P. syringae* that utilize the IAM pathway, DC3000 synthesizes IAA via IAAlD intermediate. Furthermore, I have identified the first indole-3-acetaldehyde dehydrogenases that function in IAA biosynthesis.

Aldehyde dehydrogenases and IAA biosynthesis

Many plant pathogenic bacteria including, *P. syringae* have been shown to produce IAA, often using one of the two well-characterized IAM or IPyA pathways (Spaepen et al., 2011). The

observation that DC3000 utilizes an IAAld intermediate and aldehyde dehydrogenase enzymes function to synthesize IAA is a novel discovery. Their role in IAA biosynthesis in plants was hypothesized, however no biochemical evidence had been reported confirming their role. There are reports of aldehyde dehydrogenase involvement in bacterial IAA biosynthesis (Xi et al, 2005), however, this is the first reported evidence of a *bona fide* indole-3-acetaldehyde dehydrogenase in bacteria or plants. This is contrary to the hypothesis that DC3000 uses the IAM pathway. The predicted DC3000 IaaM/TMO protein shares only ~30% amino acid identity to enzymes with demonstrated IAA biosynthetic activity (Patten et al, 2013) and is more closely related to a second group of IaaM homologs that may function in pathways other than IAA synthesis. Based on my feeding experiments, DC3000 is likely utilizing the tryptophan side chain oxidase (TSO) pathway, in which Trp is directly converted to IAAld (Figure 2.1). It is of importance to note that although cultures supplemented with TAM did not lead to high levels of IAA in culture, if conversion of TAM to IAAld is a rate-limiting step, then feeding with IAAld will not lead to increased IAA production in culture. Therefore, we cannot fully rule out a role for the TAM pathway. Therefore, additional enzymes in the TAM and TSO pathways must be identified and characterized, to determine which IAAld containing pathway(s) DC3000 is using, starting from Trp. Furthermore, it remains to be determined whether IAAld in microbial biosynthesis is limited to certain bacteria like DC3000 or whether additional bacteria have the same ability. However, what is clear is that there are alternate pathways to IAA production, in addition to those that have been previously described, that are important.

The observation that the *aldA aldB* double mutant retains some ability to make IAA, indicates that there are additional proteins that function in the conversion of IAAld to IAA, for example unidentified aldehyde dehydrogenase proteins. Bioinformatic searches using AldA and

AldB amino acid sequences, indicate that there are additional proteins in the DC3000 genome that are annotated as aldehyde dehydrogenases. Furthermore, the ability of DC3000 to make IAA in culture when supplemented with intermediates other than IAAlde, suggests that DC3000 may utilize other pathways to produce IAA. DC3000 cultures fed with Trp and examined for the accumulation of individual auxin intermediates, could shed some light on alternate IAA synthesis pathways.

Aldehyde dehydrogenases/oxidases and plant IAA biosynthesis

It has long been proposed that IAAlde is involved in plant IAA biosynthesis (Korasick et al., 2013). As for microbes, IAAlde was originally thought to be involved in the IPA branch; where IPA is converted to IAA via an IAAlde intermediate. This reaction was thought to be catalyzed by the flavin monooxygenase like enzymes encoded by the *A. thaliana* YUCCA genes (Zhao et al., 2001). Aldehyde oxidase (AO) enzymes, which also convert aldehydes to carboxylic acids, but that differ in their use of oxygen as an electron acceptor instead of NAD/NADP⁺, were thought to catalyze the last step in the reaction, from IAAlde to IAA. More specifically, AAO1, an oxidase enzyme of the AO family was shown to convert IAAlde to IAA in vitro (Seo et al., 2000). However our understanding of plant auxin biosynthesis has since evolved. It is now understood that the YUCCA enzymes catalyze the direct conversion of IPA to IAA (Mashiguchi et al., 2011; Stepanova et al., 2011). Furthermore, the *A. thaliana* AO family mutant *aba3*, which encodes a molybdenum cofactor sulfuryase that is required for AO family activity (Bittner et al., 2001; Xiong et al., 2001), does not show altered IAAlde levels compared to wild-type plants. This indicates that the AO family is likely not involved in the conversion of IAAlde to IAA (Mashiguchi et al., 2011). The role of IAAlde in plant IAA biosynthesis requires further investigation, including the identification of aldehyde dehydrogenase/oxidase enzymes

that function in auxin biosynthesis. It is suggested that IAAld is produced from TAM in peas, indicating that IAAld is involved in the TAM branch of IAA biosynthesis (Quittenden et al., 2009). This is in contrast to the results of my DC3000 feeding studies, which indicate that TAM does not lead to high levels of IAA. These results suggest that DC3000 utilizes the TSO pathway in which Trp is directly converted to IAAld via a presently unidentified tryptophan side chain oxidase enzyme.

Microbial IAA biosynthesis and pathogenesis

Because our understanding of microbial IAA biosynthesis outside of the IAM and IPyA pathways require further investigation, it is challenging to understand why DC3000 utilizes IAAld as a major IAA intermediate. However, the use of alternative pathways for IAA biosynthesis by different pathogens could be indicative of the virulence strategies and the nature of the diseases they cause. For example, the IAM pathway is characteristic of the gall-forming bacteria and the IPyA pathway is commonly associated with successful epiphytic colonization of pathogens (Spaepen et al., 2011). However, DC3000 does not produce galls (Preston, 2000) and is a relatively poor epiphyte (Boureau et al., 2002); therefore it might not be surprising that DC3000 uses a different pathway for IAA production. It is also possible that DC3000 is producing a different form of auxin *in planta*, such as Phenylacetic acid (PAA). It would be of interest to determine whether additional pathogens that cause bacterial speck like symptoms, including certain species of *Xanthomonas* utilize similar IAA pathways as DC3000. Additionally, phylogenetic analysis of the Ald proteins could provide insight into which bacteria are associated with the occurrence of these proteins and their role in pathogenesis.

The observation that DC3000 produces IAA, leads us to wonder whether DC3000 derived auxin contributes to its pathogenesis, as has been observed with several pathogens. DC3000 IAA biosynthesis mutants provide us with a valuable tool to investigate pathogen derived auxin during infection (Chapter 3). Additionally we can examine the role of auxin on other aspects of DC3000 biology.

MATERIALS AND METHODS

Bacterial strains and plasmids

The bacterial strains and plasmids used in the present study are shown in Table 2.2. *P. syringae* strain DC3000 wild-type and mutant strains were grown on Nutrient Yeast Glycerol Medium (NYG) (ref) or Hoitkin Sinden (HS) Medium with citrate added (HSC) at 28°C. HSC was prepared as described in (Sreedharan et al., 2006). *Escherichia coli* strains were maintained on Luria Broth (LB) medium at 37°C. Antibiotics used for selection of *P. syringae* strains include: rifampicin (Rif, 100 µg/ml), kanamycin (Km, 25 µg/ml), and tetracycline (Tet, 16 µg/ml). A modified version of the pJP5603 suicide vector (Penfold and Pemberton, 1992), pJP5603-Tet, in which the Kanamycin resistance cassette was replaced with the tetracycline resistance gene, was constructed for generation of double insertion/disruption mutants. The pJP5603-Tet vector was made by digesting pJP5603 with XbaI and BglII to release the ~1.3kb Kan^R cassette, and an ~2.9kb XbaI and BglII fragment containing the Tet^R gene from pME6031 was inserted in its place.

Quantification of Indole-3-acetic acid (IAA) production in culture

P. syringae strains were grown in NYG medium with Rif in overnight cultures. Cells were collected by centrifugation from each overnight culture, washed twice with 10mM MgCl₂, re-suspended at a density of $\sim 1 \times 10^7$ cells/ml in HS minimal media containing 10mM citrate and incubated with shaking for 48hrs at 28°C. The culture medium was supplemented with 0.25mM tryptophan, indole-3-acetamide, indole-3-acetonitrile, tryptamine, or indole-3-acetaldehyde as indicated. One ml samples were taken at 24 and 48hrs after incubation, centrifuged to pellet the cells and the resulting supernatants frozen in liquid nitrogen and stored at -80°C. Growth of cultures was monitored by reading the OD₆₀₀ at regular intervals with a spectrophotometer. The samples were analyzed for IAA production by LC-MS/MS.

Bioinformatics, nucleotide sequences, and accession numbers

Amino acid similarity (BLASTP) searches were performed using the National Center for Biotechnology Information (NCBI) server to search non-redundant databases for *P. syringae* DC3000 specific sequences. *P. syringae* strain DC3000 sequence information was obtained from Kyoto Encyclopedia of Genes and Genomes (KEGG; www.genome.jp/kegg) and the Pseudomonas-Plant Interaction website (PPI; www.pseudomonas-syringae.org). The accession numbers for the following proteins were as follows: *Azospirillum brasilense* Yu62 AldA, AY850388, *Pseudomonas syringae* DC3000 AAO54270.1

Construction and expression of *E. coli* expressing *P. syringae* putative aldehyde dehydrogenase genes

To make the pET21a-PSPTO_0092 expression plasmid, the full-length coding sequence (CDS) from *PSPTO_0092* was amplified from *P. syringae* strain DC3000 genomic DNA with primers 0092NdeIF and 0092XhoIR (Table 2.3). The resulting ~ 1.5 kb PCR fragment was cloned into the pBlunt II-TOPO vector (Invitrogen), transformed into *E. coli* DH5 α and plated on LB media containing kanamycin. The resulting pTOPO-0092 plasmid was sequenced to confirm that no PCR-derived mutations were introduced into the clone, and then was digested with NdeI and XhoI and the approximately ~ 1.5 kb insert (corresponding to the PSPTO 0092 CDS) was ligated into the pET21a vector cut with the same enzymes to generate pET21a-0092. The pET21a-0092 plasmid was transformed into *E. coli* DH5 α DE3. The same strategy was used to generate pET21a-0728, pET21a-2673, pET21a-3064, pET21a-3323 and pET21a-3364.

For *E. coli* expression assays to monitor IAA production, the *E. coli* strains carrying the pET21a-DC3000 putative Aldehyde dehydrogenase (Ald) constructs were grown in triplicate cultures overnight in LB media containing Amp with shaking at 37°C. Overnight cultures were diluted 1/100 and incubated with shaking until an OD₆₀₀ 0.4-0.6 was reached. Cultures were induced with 1mM IPTG, supplemented with 0.25mM IAAl and incubated with shaking for an additional 24hrs. A 1ml sample was taken 1.5 hrs after IPTG induction to verify induction of the putative Ald proteins. This was done by centrifuging the samples, boiling the resulting cell pellets in SDS-PAGE buffer and loading equal amounts of cell lysate on an acrylamide gel for visualization of protein. Additional 1ml samples were taken at 24 hrs after IPTG induction, centrifuged to pellet cells and the resulting supernatants were frozen in liquid nitrogen and stored at -80°C. The samples were analyzed for IAA production by LC-MS/MS.

Protein expression and purification

The pET28a-AldA, pET28a-AldB, and pET28a-AldC constructs were generated using NdeI and NotI enzyme sites and transformed into *E. coli* BL21 (DE3) cells (Agilent Technologies). Cells were grown at 37 °C in Terrific broth containing 50 mg mL⁻¹ Kan until A_{600nm} = 0.8 and induced with 1 mM IPTG at 18 °C. Cells were harvested by centrifugation (4,500 x g; 15 min) and re-suspended in lysis buffer (50 mM Tris, pH 8.0, 500 mM NaCl, 25 mM imidazole, 10% glycerol, and 1% Tween-20). After sonication and centrifugation (11,000 x g; 30 min), the supernatant was loaded onto a Ni²⁺-NTA column (Qiagen) previously equilibrated with lysis buffer. Wash buffer (lysis buffer without Tween-20) was used to remove unbound proteins, and then bound ALD protein was eluted using wash buffer containing 250 mM imidazole. The His-tagged ALD protein was loaded onto a Superdex-200 26/60 size-exclusion column (GE healthcare) equilibrated in 25 mM Hepes (pH 7.5) and 100 mM NaCl. Fractions with ALD activity were pooled, concentrated to 10 mg mL⁻¹, and stored at -80 °C. Protein concentrations were determined using molar extinction coefficients at A_{280nm} for each ALD, as calculated using ProtParam.

Enzyme assays

Enzymatic activity of each ALD was measured by monitoring NADH formation ($\epsilon_{340} = 6220 \text{ M}^{-1} \text{ cm}^{-1}$) at A_{340nm} on an Infinite M200 Pro plate reader (Tecan). Standard assay conditions for ALD were 100 mM Tris•HCl (pH 8.0), 100 mM KCl in 200 μL at 25 °C. For specific activity determinations, the following substrate concentrations were used: 1 mM IAld and either 1 mM NAD⁺ or 1 mM NADP⁺. For determination of steady-state kinetic parameters, reactions were performed in standard assay conditions with either fixed NAD⁺ (1.0 mM) and varied IAld (0.05-2.5 mM) or with fixed IAld (1.0 mM) and varied NAD⁺ (0.05-2.5 mM).

All data were fit to the Michaelis-Menten equation, $v = (k_{cat}[S])/(K_m + [S])$, using SigmaPlot.

Construction of *P. syringae ald* gene plasmid disruption mutants

The scheme used to generate the *ald* plasmid disruption mutants is illustrated in Figure 2.4a-b) To generate the *aldA*::pJP5603 insertion disruption strain, an ~0.5 kb *SacI*-*XbaI* genomic fragment internal to *aldA* (*PSPTO_0092*) was amplified from *P. syringae* DC3000 genomic DNA with the primers 0092*SacI*F and 0092*XbaI*R. The resulting PCR fragment was cloned into the pBlunt II-TOPO vector (Invitrogen), transformed into *E. coli* DH5 α and plated on LB media containing Km. Several pTOPO-0092int clones were sequenced to verify that there were no PCR-derived mutations. The genomic fragment was then cloned into the pJP5603 Km^r suicide vector (Penfold and Pemberton, 1992) by digesting the pTOPO-0092int clone with *SacI* and *XbaI* and ligating the resulting genomic fragment into pJP5603 digested with *SacI* and *XbaI* to generate pJP5603-0092int. The pJP5603-0092int plasmid was transformed into *E. coli* DH5a λ pir and introduced into *P. syringae* DC3000 via bacterial conjugation using the helper strain MM294A(pRK2013) (Finan et al., 1986). DC3000 trans-conjugates were selected for Rif^r and Km^r resistance on NYG media containing Rif and Km at 28°C. The same strategy was used to generate *aldB*::pJP5603 and *aldC*::pJP5603 single mutants, as well as *aldA*::pJP5603 *aldB*::pJP5603-Tet, double mutant strains. To generate double mutants, a Tet^R version of the pJP5603-*aldB* insertion disruption suicide plasmid was used.

Plasmid disruption of *aldA* by pJP5603 was confirmed by PCR using primers M13F, 0092seqF, and 0092seqR. Disruption of the wild-type genomic copy was verified by amplification of an ~1.1 kb fragment with M13F and 0092seqF primers in the *aldA*:pJP56023 strain and the absence of a band of this size in wild-type DC3000 and *aldB*::pJP5603 and *aldC*::pJP5603 strains (Figure 2.4 c-d). Additionally, to confirm disruption of only the *aldA*

gene, the presence of the wild-type gene was confirmed (using 0092seqF/R primers) by the amplification of an ~1.5 kb band in wild-type, *aldB*::pJP5603 and *aldC*::pJP5603 strains and the absence of a band of this size in *aldA*::pJP5603 strains. The same strategy was used to confirm all of the additional single and double *ald* mutants

To generate the *aldA* complementing clone, *pAldA*, the *aldA* coding sequence and 5' regulatory region were amplified from genomic DNA using primers 0092XhoIF and 0092EcoRIR. The resulting ~2 kb PCR product was cloned into the pBlunt II-TOPO vector (Invitrogen) to generate pTOPO-0092comp. This plasmid was then digested with XhoI and EcoRI and the 2 kb insert ligated into the broad host range plasmid pME6031 vector with XhoI and EcoRI compatible ends to generate pME6031-0092 (*pAldA*). The *pAldA* plasmid was introduced into the *aldA*::pJP5603 mutant strain via bacterial conjugation using the helper strain MM294A(pRK2013). DC3000 trans-conjugates were selected for Rif^r, Km^r and Tet^r resistance on NYG media containing Rif, Kan and Tet at 28°C. A similar strategy was used to generate *pAldB*, a complementing clone for *aldB*.

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FIGURES

Figure 2.1. Overview of tryptophan-dependent indole-3 acetic acid (IAA) biosynthesis pathway(s) in bacteria. Enzymes with demonstrated biochemical activities are indicated.

Enzyme abbreviations are: tryptophan 2-monooxygenase (TMO), indole acetamide hydrolase (IAH), and indole pyruvate decarboxylase (IPDC). Two aldehyde dehydrogenases (ALD), AldA and AldB, which catalyzes the conversion of IAAld to IAA are described in this chapter.

Compound abbreviations are: tryptophan (Trp), indole-3-acetaldoxime (IAOx), indole-3-acetonitrile (IAN), indole-3-acetamide (IAM), indole-3-pyruvate (IpyA), indole-3-acetaldehyde (IAAld), and tryptamine (TAM). Adapted from Spaepen et al 2011.

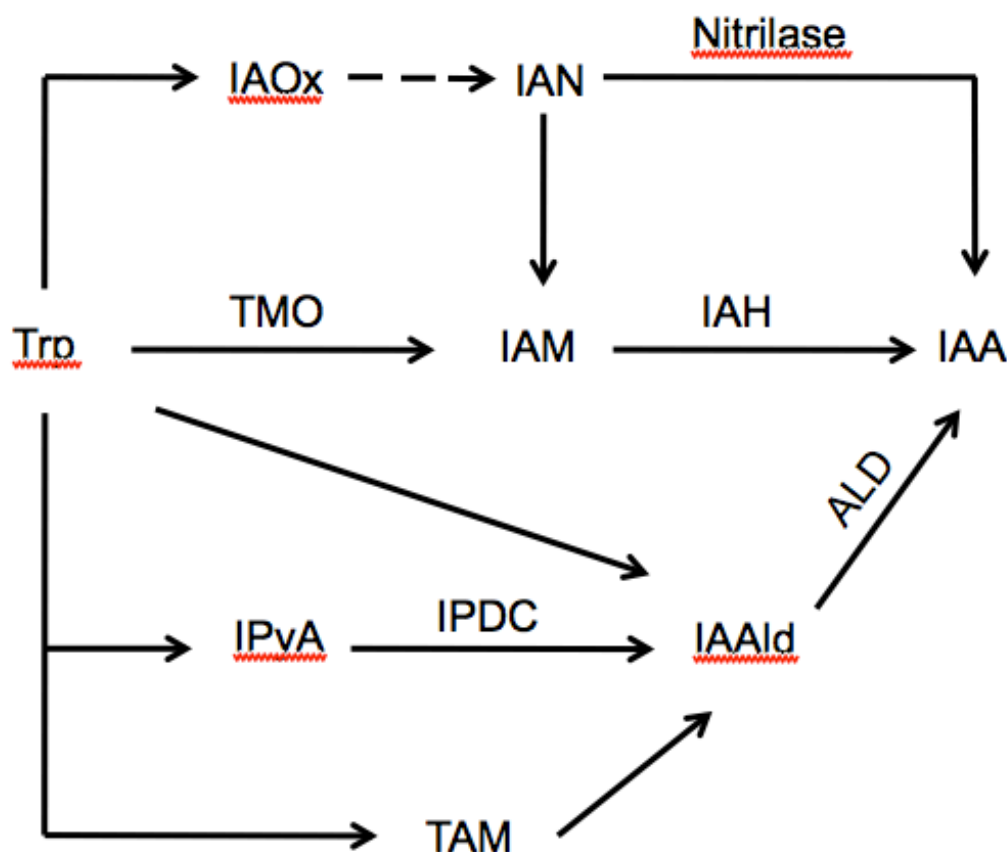


Table 2.1: Indole-3-acetic acid (IAA) levels in culture						
Strain	Media^a	Supplement	IAA ng/ml 24hr^b	SEM	IAA ng/ml 48hr^b	SEM
DC3000	HSC	N/A	28.9	4.59	30.6	3.46
DC3000	HSC	Trp	2520	245.02	2763.3	258.99
DC3000	HSC	IAAld	3703.3	169	11666.7	656.6
DC3000	HSC	IAM	144.33	17.84	100.83	11.19
DC3000	HSC	IAN	190.33 ^c	7.45	301.33 ^c	9.53
DC3000	HSC	IpyA	8820 ^d	330.8	14133.3 ^d	284.8
DC3000	HSC	TAM	107.2	20.4	147.3	10.4

^aDC3000 cultures grown in Hoitken-Sinden media with 10mM citrate (HSC) and 0.25mM of the indicated supplement.

^bHSC media supplemented with Trp, IAAld, IAM, or TAM accumulated no detectable levels of IAA in the absence of bacteria after 24 or 48 hrs of incubation.

^cHSC media containing IAN but lacking DC3000 accumulated 111ng/ml and 124 ng/ml of IAA at 24 and 48 hrs of incubation, respectively

^dHSC media supplemented with IpyA but lacking DC3000 accumulated 17,000 ng/ml and 16,300 ng/ml of IAA at 24 hrs and 48 hrs of incubation, respectively.

Figure 2.2. Heterologous expression of putative DC3000 aldehyde dehydrogenases in *E. coli*. DC3000 genes encoding putative aldehyde dehydrogenase proteins were cloned into pET-21a and introduced into *E. coli* BL21 (DE3) cells. A) Quantification of IAA produced by strains expressing PSPTO_0728, PSPTO_2673 and PSPTO_3644 and pET-21a as a negative control. B) Quantification of IAA produced by strains expressing PSPTO_0092, PSPTO_3064 and PSPTO_3323. The inset focuses in on strains accumulating lower levels of IAA. PSPTO_2673 was included as a control for comparison with results shown in A. IAA levels were measured in *E. coli* supernatants 24 hrs after addition of 1mM IPTG (final concentration) and 0.25 mM indole-3-acetaldehyde (IAAld). Values are an average of three biological replicates +/- SEM. Similar results were obtained from two additional independent experiments.

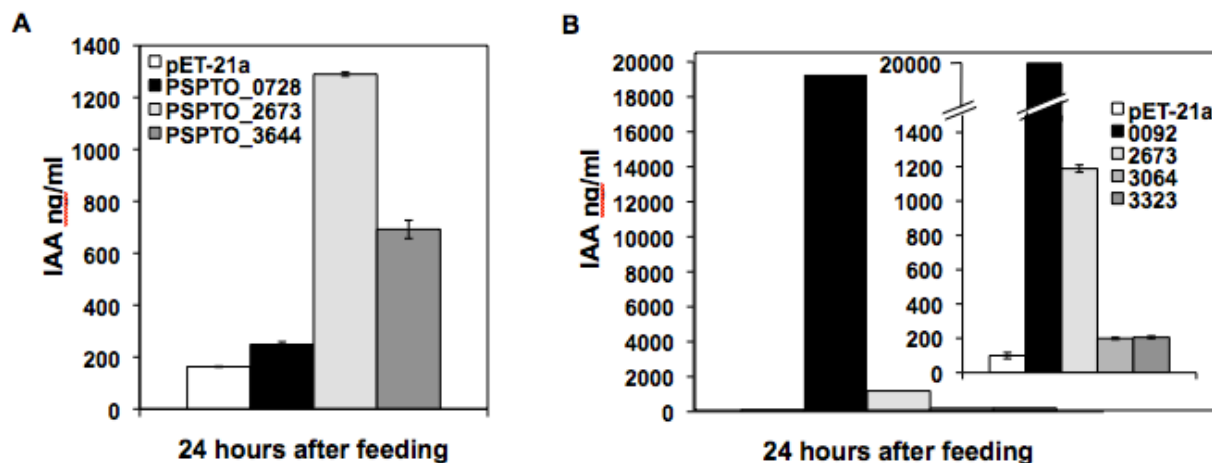


Figure 2.3. IPTG induction of Ald protein expression in *E. coli* cultures assayed in Figure 2.

Putative DC3000 Ald proteins were expressed from the pET-21a vector in *E. coli*. Protein expression was induced with IPTG (1 mM final concentration). Protein lysates (20ul) were separated by electrophoresis on a 12% polyacrylamide gel and visualized by staining with Coomassie blue. Ald proteins of expected size (~53kDa) are indicated by bands between 49 and 64 kDa.

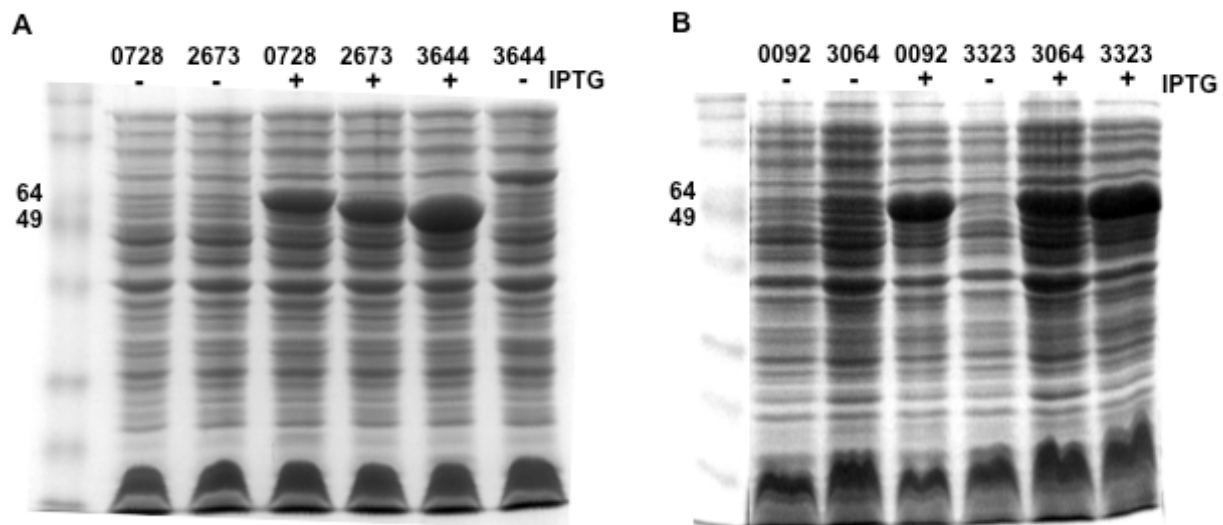


Figure 2.4. Enzymatic activity of AldA, AldB, and AldC. Specific activities were determined using standard assay conditions using IAAlD and either NAD⁺ or NADP⁺ as substrates, as described in the experimental methods. Relative activity is shown using AldA with IAAlD and NAD⁺ as 100% (3.52 mmol min⁻¹ mg protein⁻¹). The inset zooms in on the lower specific activities for AldB and AldC. Values are expressed as a mean \pm S.E. ($n = 3$).

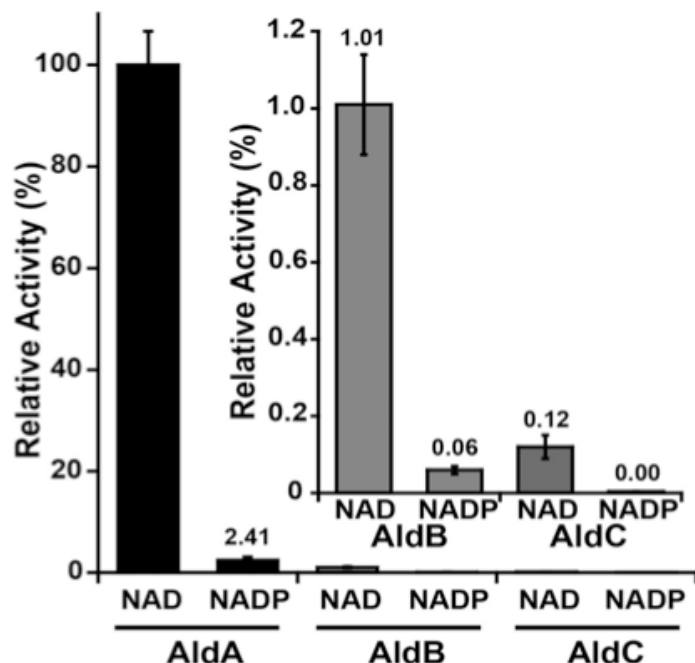


Table 2.2. Steady-state kinetic analysis of AldA, AldB, and AldC

All values are expressed as a mean \pm S.E. ($n = 3$).

Protein	Substrate	k_{cat} (min ⁻¹)	K_{m} (μM)	$k_{\text{cat}}/K_{\text{m}}$ (M ⁻¹ s ⁻¹)
AldA	IAAlD	234 \pm 21	119 \pm 37	32,770
	NAD ⁺	194 \pm 9	42 \pm 8	77,350
	NADP ⁺	30 \pm 1	1,955 \pm 182	252
AldB	IAAlD	8.7 \pm 0.2	595 \pm 39	244
	NAD ⁺	2.1 \pm 0.1	33.4 \pm 9.2	1,044
AldC	IAAlD	3.6 \pm 0.2	1,282 \pm 161	46
	NAD ⁺	0.40 \pm 0.01	445 \pm 99	14

Figure 2.5. Generation and confirmation of DC3000 *ald* plasmid insertion mutants. A) The *PSPTO_0092* region of DC3000 genome showing neighboring genes (blue arrows) and plasmid pJP5603-0092int containing a ~530 bp internal fragment of *PSPTO_0092* (gray box) used to generate *aldA*. B) Schematic diagram illustrating the result of a single homologous recombination event between pJP5603-0092int and the chromosomal copy of *PSPTO_0092*, leading to disruption of the gene. C) Amplification of the plasmid-disrupted *PSPTO_0092* gene using primer pairs M13F and 0092seqF. D) Amplification of wild-type *PSPTO_0092* using primer pairs 0092 seq F/R. Primer pairs used in PCR reactions shown in panels C and D are illustrated by arrowheads. Growth of *ald* single and double mutants in culture. E) Growth of *ald* mutants in NYG media. Cell growth was monitored at indicated intervals for 24 hrs. F) Growth of *ald* mutants in HSC media. Cell growth was monitored by measuring OD600 at indicated intervals for 48 hrs. Values are an average of three biological replicates +/- SEM. Similar results were obtained in two of three experiments.

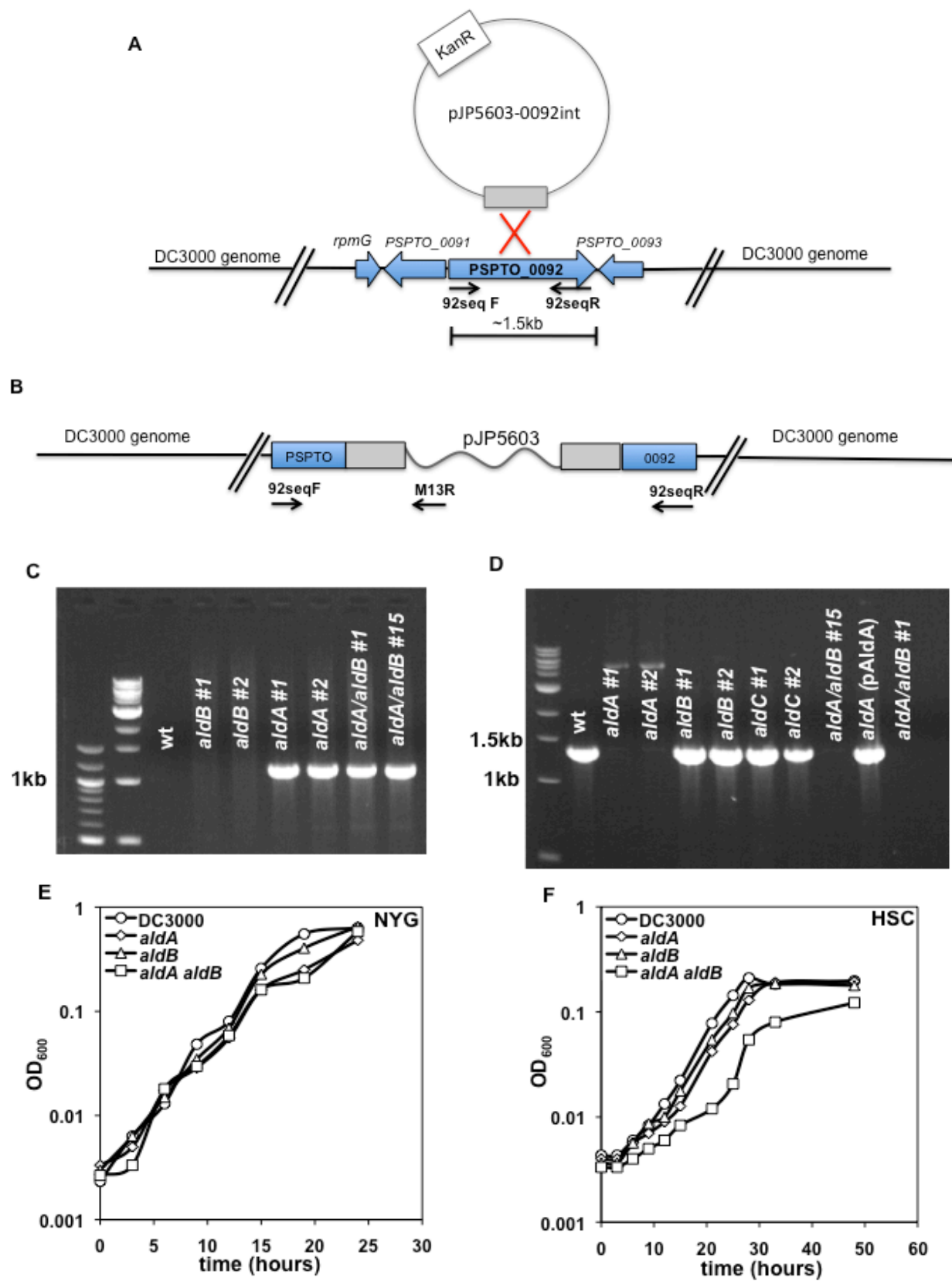


Figure 2.6. Quantification of IAA production in DC3000 *ald* mutants. A) Measurement of IAA accumulation in supernatants of DC3000 *ald* single mutants grown for 48 hrs in HSC media supplemented with 0.25 mM IAAld. B) Growth of *ald* mutants in HSC media supplemented with 0.25 mM IAAld. Cultures were used to quantify IAA shown in panel A. C) Measurement of IAA accumulation in supernatants of two independent *aldA aldB* double mutants grown for 48 hrs in HSC media supplemented with 0.25 mM IAAld. D) Measurement of IAA accumulation in supernatants of *aldA* complementing strain grown for 48hrs in HSC media supplemented with 0.25mM IAAld. For panels A-D, values are an average of three biological replicates +/- SEM (error bars too small to see in panels B, C). Letters indicate significant difference between samples within a given time point ($p<0.05$).

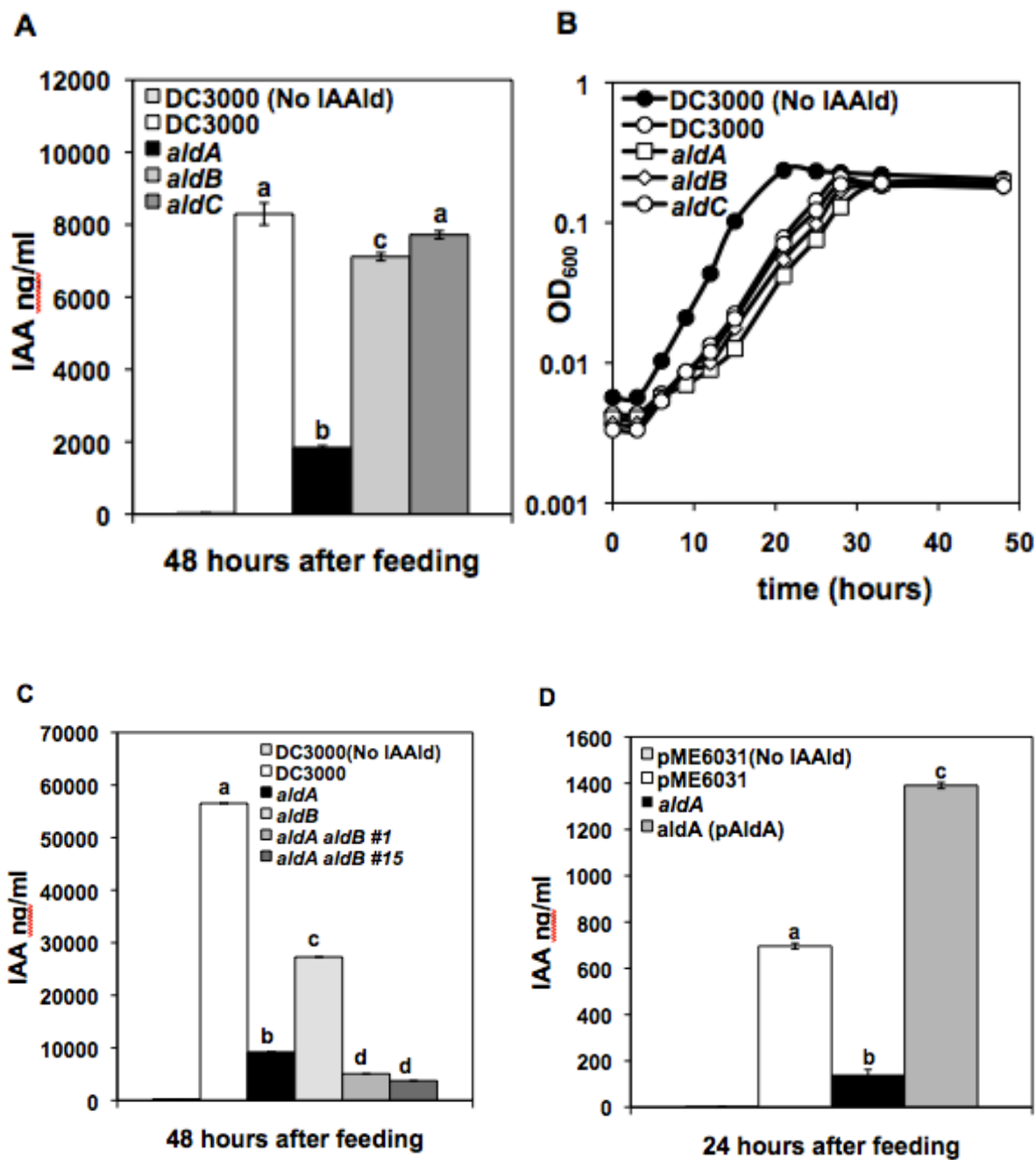


Table 2.3: Bacterial strains and vectors used in this study

Strain or Plasmid	Characteristics	Reference or Source
<i>Pseudomonas syringae</i> strains		
<i>P. syringae</i> pv. <i>tomato</i> DC3000	Derivative of NCPPB1106; Rif ^r	Cuppels, 1986
<i>aldA</i> ::pJP5603	<i>PSPTO_0092</i> disrupted with pJP5603; Rif ^r , Km ^r	This study
<i>aldB</i> ::pJP5603	<i>PSPTO_2673</i> disrupted with pJP5603; Rif ^r , Km ^r	This study
<i>aldC</i> ::pJP5603	<i>PSPTO_3644</i> disrupted with pJP5603; Rif ^r , Km ^r	This study
<i>aldB</i> ::pJP5603-Tet	<i>PSPTO_2673</i> disrupted with pJP5603-Tet; Rif ^r , Tet ^r	This study
<i>aldA</i> ::pJP5603 <i>aldB</i> ::pJP5603-Tet	double mutant; Rif ^r , Km ^r , Tet ^r	This study
<i>aldA</i> ::pJP5603 p <i>AldA</i>	<i>aldA</i> mutant with complementing clone; Rif ^r , Km ^r , Tet ^r	This study
<i>aldB</i> ::pJP5603 p <i>AldB</i>	<i>aldB</i> mutant with complementing clone; Rif ^r , Km ^r , Tet ^r	This study
<i>Escherichia coli</i> strains		
DH5 α	recA, lacZAM15	Invitrogen
BL21(DE3)	F ⁻ <i>ompT hsdS_B(r_B⁻ m_B⁻) gal dcm</i> (DE3)	Novagen
DH5 α l pir	recA, lacZAM15, l pir	Miller and Mekalanos, 1988
MM294A (pRK2013)	Triparental mating helper strain; Cm ^r	Finan, 1986
Plasmids		
pCR BLUNT-II-TOPO	Cloning Vector; Km ^r	Invitrogen
pET-21a	Expression vector; Amp ^r	
pTOPO-0092	<i>PSPTO_0092</i> CDS in pTOPO; Km ^r	This study
pET-21a-0092	<i>PSPTO_0092</i> CDS in pET21a; Amp ^r	This study
pTOPO-0728	<i>PSPTO_0728</i> CDS in pTOPO; Km ^r	This study
pET-21a-0728	<i>PSPTO_0728</i> CDS in pET21a; Amp ^r	This study
pTOPO-2673	<i>PSPTO_2673</i> CDS in pTOPO; Km ^r	This study
pET-21a-2673	<i>PSPTO_2673</i> CDS in pET21a; Amp ^r	This study
pTOPO-3064	<i>PSPTO_3064</i> CDS in pTOPO; Km ^r	This study
pET-21a-3064	<i>PSPTO_3064</i> CDS in pET21a; Amp ^r	This study
pTOPO-3323	<i>PSPTO_3323</i> CDS in pTOPO; Km ^r	This study
pET-21a-3323	<i>PSPTO_3323</i> CDS in pET21a; Amp ^r	This study
pTOPO-3364	<i>PSPTO_3364</i> CDS in pTOPO; Km ^r	This study
pET-21a-3364	<i>PSPTO_3364</i> CDS in pET21a; Amp ^r	This study
pJP5603	Suicide vector; Km ^r	Penfold, 1992
pJP5603-Tet	pJP5603 with Tet ^r in place of Km ^r ; Tet ^r	This study
pTOPO-0092int	Construct used to make <i>aldA</i> ::pJP5603 strain; Km ^r	This study
pJP5603-0092int	Construct used to make <i>aldA</i> ::pJP5603 strain; Km ^r	This study
pTOPO-2673int	Construct used to make <i>aldB</i> ::pJP5603 strain; Km ^r	This study
pJP5603-2673int	Construct used to make <i>aldB</i> ::pJP5603 strain; Km ^r	This study
pJP5603-Tet-2673int	Construct used to make <i>aldB</i> ::pJP5603-Tet strain; Km ^r	This study
pTOPO-3644int	Construct used to make <i>aldC</i> ::pJP5603 strain; Km ^r	This study
pJP5603-3644int	Construct used to make <i>aldC</i> ::pJP5603 strain; Km ^r	This study
pTOPO-0092comp	Construct used to make p <i>AldA</i> complement clone; Km ^r	This study
pTOPO-2673comp	Construct used to make p <i>AldB</i> complement clone; Km ^r	This study
pME6031	Replicating vector, stable in <i>P. syringae</i> ; Tet ^r	Heeb, 2000
p <i>AldA</i>	<i>PSPTO_0092</i> CDS and promoter region in pME6031; Tet ^r	This study
p <i>AldB</i>	<i>PSPTO_2673</i> CDS and promoter region in pME6031; Tet ^r	This study

Table 2.4: List of primers used in this study

For cloning		
Name	Sequence	comments
0092NdeIF	5' ATATCATATGACCACCCTGACCC 3'	NdeI site underlined
0092XhoIR	5' TTA <u>ACTCGAG</u> TTACA <u>ACTTGAT</u> CCAGGTGG 3'	XhoI site underlined
0728NdeIF	5' ATATCATATGCGTTACGCTCATCCCG 3'	NdeI site underlined
0728XhoIR	5' TTA <u>ACTCGAG</u> CTAGAAAAAACCAGCGGG 3'	XhoI site underlined
2673NdeIF	5' ATATCATATGAGCCTTACCCGCTTTCA3'	NdeI site underlined
2673XhoIR	5' TTA <u>ACTCGAG</u> TTAACGCATGACAAATGGATCAGG 3'	XhoI site underlined
3064NdeIF	5' ATATCATATGCATGATCTTCTTACCGCAG 3'	NdeI site underlined
3064XhoIR	5' TTA <u>ACTCGAG</u> TCATTTTACGGTCTCGTCAACC 3'	XhoI site underlined
3323NdeIF	5' ATATCATATGCCTAACATTCTCGGCC 3'	NdeI site underlined
3323XhoIR	5' TTA <u>ACTCGAG</u> TCACGCGACAGGCTGATC 3'	XhoI site underlined
3644NdeIF	5' ATATCATATGACCACACAGCCCTTGAAC 3'	NdeI site underlined
3644XhoIR	5' TTA <u>ACTCGAG</u> TTACATCAAGTCAGCCGGAGC 3'	XhoI site underlined
0092SacIF	5' ATATGAGCTCAAGGCCACCATGATCCGTTTC 3'	SacI site underlined
0092XbaIR	5' TTAATCTAGAGCTTCATGTTTCGATTCGCCC 3'	XbaI site underlined
2673SacIF	5' ATATGAGCTCTCTGATCGCCGAGAACAAAGG 3'	SacI site underlined
2673XbaIR	5' TTAATCTAGACAGTTCCAGCGACAGTTTGG 3'	XbaI site underlined
3644SacIF	5' ATATGAGCTCCCTGGGGTGTTCATCTGGTC 3'	SacI site underlined
3644XbaIR	5' TTAATCTAGACAAAAATCTCCTCACGGGCGATG3'	XbaI site underlined
0092XhoIF	5' CTCGAGGCTGGATCCTTGCGCTGAATAC 3'	XhoI site underlined
0092EcoRI R	5' GAATTCGGACCTGCGCAATTCTTTGCG 3'	EcoRI site underlined
For genotyping		
Name	Sequence	Organism and gene
M13F	5' GTAAAACGACGGCCAG 3'	
M13R	5' CAGGAAACAGCTATGACC 3'	
0092seqF	5' CGTACTGGTTGACCCACAC 3'	<i>P. syringae</i> strain DC3000 PSPTO_0092
0092seqR	5' GAACAACGCGCCCAAATC 3'	<i>P. syringae</i> strain DC3000 PSPTO_0092
2673seqF	5' ATCCCTGAACGAATGTCCCG 3'	<i>P. syringae</i> strain DC3000 PSPTO_2673
2673seqR	5' TGAGCCTTACCCGCTTTTCAG 3'	<i>P. syringae</i> strain DC3000 PSPTO_2673
3644seqF	5' CAAACCCACAAATCGCCTTG 3'	<i>P. syringae</i> strain DC3000 PSPTO_3644
3644seqR	5' TGGACCTGGCCGCTCTATC 3'	<i>P. syringae</i> strain DC3000 PSPTO_3644

Chapter 3

Examining *ald* mutant virulence phenotype

This chapter is a collaborative work that was submitted for publication as:

Indole-3-acetaldehyde dehydrogenase, AldA, functions in IAA biosynthesis in *Pseudomonas syringae* pv tomato DC3000 and contributes to virulence on *Arabidopsis*

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Author contributions:

S.M. designed and performed the majority of the experiments, and wrote and edited the majority of the manuscript. J.M.J. edited the manuscript. B.N.K. designed and performed pathogen infection experiments, wrote the Discussion section and edited the manuscript.

ABSTRACT

Auxin is an important hormone that regulates many processes in plant development and has been shown to be important for plant-pathogen interactions. Modulation of host auxin physiology seems to be an important virulence strategy for *Pseudomonas syringae*, including both gall-forming and leaf spotting strains. However it is unclear whether auxin production by *P. syringae* DC3000, a bacterial speck-causing bacteria, contributes to pathogenesis. To investigate the role of DC3000 derived indole-3-acetic acid (IAA), a predominant form of auxin during virulence, we utilized the DC3000 IAA biosynthesis mutants, *aldA* and *aldB*. We show that both *ald* mutants display reduced growth and symptom production on *Arabidopsis thaliana* and the severity of the reduction in virulence is correlated with the degree of reduced IAA production by the mutants. Additionally, we show that the growth of *ald* mutants is restored on a SA-deficient *A. thaliana* mutant, *sid2-2*, suggesting that DC3000-derived auxin functions to suppress SA mediated defenses. Elevated levels of endogenous IAA in transgenic plants that over-express the *YUCCA1* auxin biosynthesis gene, did not restore growth of the *ald* mutant to high levels in planta, indicating that plant-derived IAA and pathogen derived auxin function to regulate DC3000 virulence in independent mechanisms.

INTRODUCTION

Pathogens have evolved a variety of strategies to ensure a successful interaction with their host. This includes protein secretion pathways like the type III secretion system to deliver virulence proteins (also known as “effectors”) into host cells and production of plant hormones or hormone mimics, both of which are important for suppressing host defenses and/or modulating host physiology to promote colonization and disease (Chisholm et al., 2006; Jones and Dangl, 2006; Xin and He, 2013). For example, several *P. syringae* strains, the causal agents of a variety of leaf spotting diseases (Cuppels, 1986; Preston, 2000) produce the phytotoxin coronatine, a molecular mimic of the plant hormone jasmonic acid-isoleucine (Staswick, 2008). The production and secretion of coronatine modulates host jasmonic acid signaling and is important for *P. syringae* pathogenesis (Brooks et al, 2004; Brooks et al, 2005; Uppalapati et al, 2007). Many plant-associated microbes also have the ability to synthesize indole-3-acetic acid (IAA), a common form of the phytohormone auxin (Spaepen et al 2011), and in some cases production of IAA has been implicated in virulence of pathogenic strains (Duca et al, 2014).

Auxin is an integral plant hormone involved in the regulation of a broad range of growth and developmental processes, including cell division and expansion and root growth and development (Woodward and Bartel, 2005). IAA is also important in several plant-pathogen interactions. For example IAA is responsible for gall formation caused by *Rhizobium radiobacter* (formerly *Agrobacterium tumefaciens*)(Jameson 2000) and *P. savastanoi* (Aragon et al., 2014). More recently auxin has been shown to promote virulence of *P. syringae*. Exogenous application of auxin enhances disease susceptibility on *A. thaliana* (Chen et al, 2007; Wang et al, 2007), and transgenic *A. thaliana* lines over-expressing *YUCCA1*, an auxin biosynthesis gene, accumulate elevated levels of IAA and exhibit enhanced susceptibility to *P. syringae* (Mutka et

al, 2013). Additionally, impairment of auxin signaling in the plant has been reported to reduce susceptibility to *P. syringae* pv. tomato and maculicola strains (Novarro et al, 2005; Wang et al, 2007). Although the role of IAA in promoting gall formation is well understood, its role in promoting bacterial speck disease remains to be elucidated.

In Chapter 2, I demonstrate that *P. syringae* pv. tomato strain DC3000 produces IAA and identify two indole-3-acetaldehyde dehydrogenases, AldA and AldB, that function in DC3000 IAA biosynthesis. However it is unknown whether DC300-derived IAA contributes to its virulence. In this Chapter, I utilize the DC3000 *ald* mutants described in Chapter 2 to investigate the role of pathogen-derived IAA during pathogenesis. I show that disruptions of *aldA* and *aldB* lead to reduced virulence in *A. thaliana*. Furthermore, I explore the mechanism by which pathogen derived auxin contributes to DC3000 virulence and show that auxin produced by DC3000 suppresses salicylic acid (SA)-mediated defenses in *A. thaliana*.

RESULTS

DC3000 IAA biosynthesis mutants exhibit reduced virulence on *Arabidopsis thaliana*

Previous studies indicate that auxin can promote susceptibility to *P. syringae* strains DC3000 and *P. syringae* pv *maculicola* 4326 (Chen et al, 2007; Wang et al 2007; Novarro et al, 2006; Mutka et al, 2013); however, it is unknown whether auxin produced by these strains contributes to their virulence. To examine this, I assayed the *aldA* and *aldB* mutants for altered virulence on *A. thaliana* plants. DC3000 grew to high levels when infiltrated into *A. thaliana* plants (Figure 3.1a), while the *aldA* and *aldB* mutants exhibited a ~10-fold reduction in growth (Figure 3.1a). Surface inoculation experiments were also performed to monitor development of disease symptoms. Plants dip-inoculated with DC3000 exhibited characteristic disease symptoms consisting of many individual water-soaked lesions surrounded by yellowing of the

leaf (chlorosis) (Figure 3.1 b-c). Plants infected with the *aldA* mutant displayed reduced disease symptom severity compared to DC3000, manifested primarily as a decrease in the percentage of leaves developing high levels of chlorosis. Plants infected with the *aldB* mutant also displayed a reduction in symptom severity, although to a lesser degree than plants infected with the *aldA* mutant (Figure 3.1b-c). These results indicate that DC3000-derived IAA contributes to its virulence.

We tested whether the *ald* genes have additive affects on virulence by testing the *aldA aldB* double mutant in DC3000. The *aldA aldB* double mutant exhibited a further reduction in bacterial growth on *A. thaliana* plants compared to the *aldA* or *aldB* single mutants (Figure 3.1a). This correlates with the fact that IAA production was significantly lower in *aldA aldB* double mutants than in either single mutant (see Figure 2.6c). The additive nature of these mutant phenotypes suggests that AldA and AldB contribute to DC3000 IAA biosynthesis and virulence in a partially redundant manner.

Pathogen-derived IAA suppresses SA-mediated defenses

IAA may contribute to pathogenesis by suppressing host defenses mediated by the defense hormone SA (Wang et al 2007; Kazan and Manners, 2009). We hypothesized that if pathogen-derived IAA promotes pathogen growth in planta by suppressing SA-mediated defenses, then the DC3000 *ald* mutants would exhibit reduced growth in planta due to an impairment in the ability to suppress SA-mediated defenses. We further predicted that the growth of *ald* mutants would be restored to wild-type levels on *A. thaliana* mutants that fail to accumulate SA, and therefore have disrupted SA mediated defenses. To test this, we inoculated the *sid2-2* mutant that is impaired in SA synthesis due to disruption of the *ICS1* SA biosynthesis gene (Wildermuth et al., 2001) with DC3000 and the *ald* mutants and monitored bacterial

growth. Wild-type DC3000 grew to higher levels in *sid2-2* mutants plants than in wild-type Col-0 (Figure 3.2a), consistent with previous reports that the *sid2-2* mutant exhibits increased disease susceptibility (Mutka et al., 2013; Wildermuth et al., 2001). Consistent with our earlier results, the *aldA* and *aldB* mutants exhibited significantly reduced growth on wild-type plants compared to DC3000; however, each mutant grew to levels comparable to wild-type DC3000 on *sid2-2* plants (Figure 3.2a). Thus, reduced growth of the *ald* mutants is restored to normal levels in plants impaired for SA-mediated defenses.

To further investigate the relationship between DC3000-derived IAA and SA-mediated defenses I monitored the expression of *PR1*, a commonly used marker for SA-mediated defenses in *A. thaliana* (Mutka et al, 2013). I examined *PR1* transcript levels in plants infected with wild-type DC3000 and the *aldA* and *aldB* mutants 24 hours after inoculation. *PR1* expression was induced by 24 hrs in plants infected wild-type DC3000 compared to mock treatment (Figure 3.2b). Expression of *PR1* was significantly higher in plants infected with the *aldA* mutant. There was also a small but significant increase in *PR1* expression in plants infected with *aldB* mutant; however, this was not as large as observed for the *aldA* mutant. These results suggest that DC3000-derived IAA is required for normal virulence as it suppresses SA-mediated defenses (Figure 3.4).

Increased endogenous IAA in YUCCA over-expressing lines do not restore growth of *ald* mutants

Hormone biosynthesis mutants are distinguished by their ability to be rescued by application of exogenous hormone. This indicates that the mutant phenotypes are a result of impairment in hormone biosynthesis, rather than in the ability to sense or respond to the

hormone. In order to test whether the DC3000 *ald* mutant virulence phenotype is due to impaired auxin biosynthesis, we utilized the *YUCCA* over-expressing (35S:YUC1) lines (Zhao et al, 2001; Mashiguchi et al, 2011; Mutka et al., 2013) with increased endogenous IAA to test whether increased IAA levels in plant tissue can rescue the decreased virulence phenotype. We inoculated the 35S:YUC1 lines with DC3000 and the *ald* mutants and monitored bacterial growth. Wild-type DC3000 grew to higher levels in 35S:YUC1 plants than in wild-type Col-0 (Figure 3.3), as had been previously reported by Mutka et al (2013). Consistent with my earlier results, the *aldA* and *aldB* mutants exhibited significantly reduced growth on Col-0 plants compared to DC3000. Surprisingly, each mutant displayed the same reduced growth on the 35S:YUC1 plants as observed in wild-type Col-0, and failed to grow to levels comparable to DC3000. These results indicate that increased endogenous IAA in 35S:YUC1 plants does not restore normal growth to the *ald* mutants and suggests that DC3000-derived auxin functions via a different mechanism to promote pathogenesis (Figure 3.4).

DISCUSSION

In summary, I showed that plants inoculated with DC3000 IAA biosynthesis mutants, *aldA* and *aldB*, displayed decreased bacterial growth and disease symptoms (Figure 3.1). The decreased virulence of the *ald* mutants observed on wild-type plants was restored on SA deficient, *sid2-2* plants (Figure 3.2a). *PR1* expression was increased in plants infected with wild-type DC3000; this expression was further increased in plants infected with the *ald* mutants (Figure 3.2b). My findings indicate that auxin production by DC3000 contributes to its virulence and functions to suppress SA mediated defenses. Furthermore, elevated auxin levels in transgenic 35S:YUC1 plants that accumulate elevated levels of IAA did not restore growth of *ald* mutants during infection (Figure 3.3). This suggests that there are multiple roles for IAA during

P. syringae infection, and that the source of IAA, whether produced by plant or by pathogen, may be an important factor.

Pathogen-derived auxin contributes to pathogenesis.

Several studies have found that pathogen-derived auxin contributes to virulence. My results indicate that IAA produced by DC3000 contributes to its pathogenesis, as observed in other pathogens, including several *Pseudomonas* strains. It is of interest to note that although plants infected with the *aldA* and *aldB* single mutants show similar reductions in bacterial growth during infection, they display different reductions in disease symptom development. One hypothesis for the discrepancy in disease symptom severity is that the AldA enzyme uses additional substrates besides IAAld and these molecules are required for normal disease symptoms. Work done by Dr. Lee in the lab of J. Jez indicates that AldA can utilize phenylacetaldehyde (PAAld) as a substrate with a higher affinity than AldB (see Appendix 1). The product of this reaction, phenylacetic acid (PAA), is also a naturally occurring auxin found in plants (Sugawara et al., 2015; Wightman and Lighty, 1982; Schneider et al., 1985). If PAA, like other auxins, contributes to pathogenesis, then this could explain the difference in disease symptom severity between the *aldA* and *aldB* mutants. However, the role of PAA during infection requires further investigation. Likewise, further characterization of Ald enzymes is required to understand their function during pathogenesis or other aspects of DC3000 biology.

Pathogen-derived auxin suppresses SA mediated defenses

The molecular mechanisms underlying the role(s) of auxin during infection are not well understood. A popular hypothesis is that auxin suppresses SA mediated defenses (Kazan and Manners, 2009). Previous work showed that application of exogenous auxin can suppress SA-

responsive gene expression (Park et al, 2007; Wang et al 2007). However little is known about the function of pathogen-derived auxin during infection. The observation that DC3000 IAA biosynthesis mutants have restored growth on SA-deficient mutants and increased *PR1* gene expression supports the hypothesis that auxin functions to suppress SA-mediated defenses. Although the antagonistic role of pathogen-derived IAA and SA is unknown, it has been observed that pathogens utilize other hormones to suppress SA. For example, Coronatine, the jasmonic acid-Ile (JA-Ile) mimic, is secreted by *P. syringae* and modulates JA signaling, thus leading to suppression of SA mediated defenses (Brooks et al., 2004; Brooks et al., 2005). Additionally, it has been observed in plants that altered auxin signaling can impact SA-mediated defenses (Robert-Seilaniantz et al, 2011). The mechanisms underlying IAA and SA antagonism during infection requires further study, thus the DC3000 *ald* mutants provide us with novel tools to investigate the role of pathogen-derived IAA in IAA/SA crosstalk during infection.

Plant-derived auxin enhances susceptibility in an SA-independent manner

Elevated auxin levels in plants has been shown to promote disease susceptibility. Previously the molecular mechanism of the role of increased auxin in the promotion of pathogenesis was investigated. Mutka et al. (2013) showed that transgenic plants over expressing the *YUCCA1* IAA biosynthesis gene (*35S:YUC1*) accumulate elevated IAA, and these plants exhibit enhanced susceptibility to DC3000. However, they concluded from their studies that the primary function of auxin during infection is NOT to suppress SA-mediated defenses. The *35S:YUC1* plants did not exhibit significant suppression of SA levels or SA-responsive gene expression. This suggests that elevated IAA in plants functions to promote pathogenesis independent of suppression of SA. This finding is in contrast to what I observed to be the role of pathogen-derived auxin during infection. Although initially this discrepancy seemed unexpected,

our differing results can be explained by a model in which plant and pathogen-derived auxin play different roles. If pathogen derived IAA functions to suppress SA-mediated defenses, but increased IAA in plants functions via an SA-independent manner, then one would not expect that the virulence defects of DC3000 *ald* mutants would be restored by elevated IAA in the plant. In fact, I observed that *35S:YUC1* plants did not restore growth defects of *ald* mutants during infection.

The distinction between our findings and that of Mutka et al (2013), indicate that there are multiple roles for auxin during infection (Kazan and Manners, 2009). Furthermore, the roles of auxin during infection could be dependent on the source of auxin production. Mutka et al proposed that elevated auxin could function to regulate a number of processes during infection including cell wall loosening (Cosgrove, 2005), allowing pathogens easier access to plant cells for delivery of Type-III secreted effectors. Additionally, they proposed that auxin may directly affect pathogen biology. This is an interesting hypothesis and IAA has been shown to be a microbial signaling compound (Spaepen et al., 2011; Spaepen et al, 2007). IAA can regulate expression of genes involved in virulence and promote microbial survival under stress conditions (Bianco et al., 2006; Spaepen et al., 2009; Van Puyvelde et al 2011). Preliminary work done in the Kunkel lab also suggests that IAA regulates gene expression in DC3000 (G. Harrison). Further studies examining the effects of auxin on pathogen biology are required. However, *35S:YUC1* plants and DC3000 *ald* provide useful tools to investigate auxin during pathogenesis and source dependent contributions.

MATERIALS AND METHODS

Bacterial strains and plasmids

The bacterial strains used in the present study, DC3000, *aldA*, *aldB* and the *aldA aldB* double mutant are described in **Chapter 2**. *P. syringae* strain DC3000 wild-type and mutant strains were grown on Nutrient Yeast Glycerol Medium (NYG) (ref) at 28°C. Antibiotics used for selection of *P. syringae* strains include: rifampicin (Rif, 100 µg/ml), kanamycin (Km, 25 µg/ml), and tetracycline (Tet, 16 µg/ml).

Plant material and growth conditions

All *Arabidopsis thaliana* transgenic lines and mutants used in this study were in the Col-0 background. The 35S:*YUC1* overexpression line (Zhao et al., 2001) was obtained from Yunde Zhao. The *sid2-2* mutant (Wildermuth et al., 2001) was obtained from Mary Wildermuth. Plants were grown on soil in a growth chamber with a short-day photoperiod (8-h light/16-h dark) at 21°C and 75% relative humidity, with a light intensity of approximately 130 µEinstein sec⁻¹ m⁻².

P. syringae inoculation and quantification of bacterial growth

A. thaliana plants were infected at approximately 4 weeks of age. For surface inoculations, plants were dipped into a solution containing *P. syringae* at approximately 3x10⁸ cells ml⁻¹ (OD₆₀₀ = 0.3), 10 mM MgCl₂ and 0.02% Silwet L-77. To quantify bacterial growth in the plant, whole leaves were sampled at various time points after inoculation, weighed to determine leaf mass, ground in 10 mM MgCl₂ and then plated in serial dilutions on NYG media with rifampicin. Between four and six leaves were sampled per treatment, depending on the experiment. On the day of inoculation, leaves were sampled at 2 h after inoculation, surface

sterilized with 15% H₂O₂ and then washed three times with sterile water before grinding to remove bacteria from the surface of the leaf. For syringe infiltrations, a solution containing 10⁴–10⁵ cells ml⁻¹ (OD₆₀₀ = 10⁻⁵–10⁻⁴) in 10 mM MgCl₂ was injected into leaves using a 1-ml needleless syringe. Bacterial growth was monitored as described for dip inoculations, with the exception that leaves sampled on the day of inoculation were not subject to surface sterilization.

Quantification of disease symptoms following dip inoculation was carried out 4 days post inoculation. Leaves were categorized based on presence and amount of chlorosis or yellowing of the leaf. For ~ 10 plants per each treatment, each leaf was individually assessed for percent of the leaf exhibiting chlorosis.

Quantitative RT-PCR

For the analysis of defense related gene expression in infected plants, I infiltrated entire rosette leaves of 4 week old plants with DC3000 (OD₆₀₀ = 10⁻⁴) or a mock solution of 10mM MgCl₂. Approximately 100mg of tissue was isolated at 24 h post infiltration, and frozen immediately in liquid nitrogen. Three biological replicates per treatment were harvested and analyzed. Total RNA was isolated with the RNeasy Plant Mini Kit (Qiagen), and genomic DNA was removed with DNase I (Life Technologies). cDNA was performed using the RNA, Superscript III Reverse Transcriptase (Life Technologies) and oligo (dT)₂₀ primers. Negative control reactions lacking reverse transcriptase were run in parallel to verify that there was no contamination from the genomic DNA. Quantitative RT-PCR reactions were then set up with the cDNA and SYBR Green Jump Start Taq ReadyMix (Sigma-Aldrich), using a final reaction volume of 20 µL. See table 3.1 for qPCR primers used. Three technical replicates were run per sample. Reactions were run on the Applied Biosystems 7500 Real-Time PCR system. Expression was normalized to the reference gene *PP2A3* (At1g13320) (Czechowski et al., 2005).

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FIGURES

Figure 3.1. Growth and symptom production of *ald* mutants on *A. thaliana*. A) Growth of DC3000 and *aldA*, *aldB* and *aldA aldB* double mutants following syringe infiltration of Col-0 ($OD_{600} = 1 \times 10^{-4}$). Similar results were seen in two additional experiments. Letters indicate significant difference between samples within a given time point ($p < 0.05$). B) Disease symptom severity 4 days after dip inoculation with *ald* mutants. Disease symptom severity was quantified as the average percentage of affected leaves per plant exhibiting the indicated amount of chlorosis. Approximately 10 plants were assayed for each treatment. The results were plotted as the average percentage of leaves from each genotype exhibiting the indicated degree of chlorosis. C) Photographs taken 4 days after dip inoculation. Plants shown were used to quantify disease symptom severity in panel B. Similar results were obtained in two additional experiments.

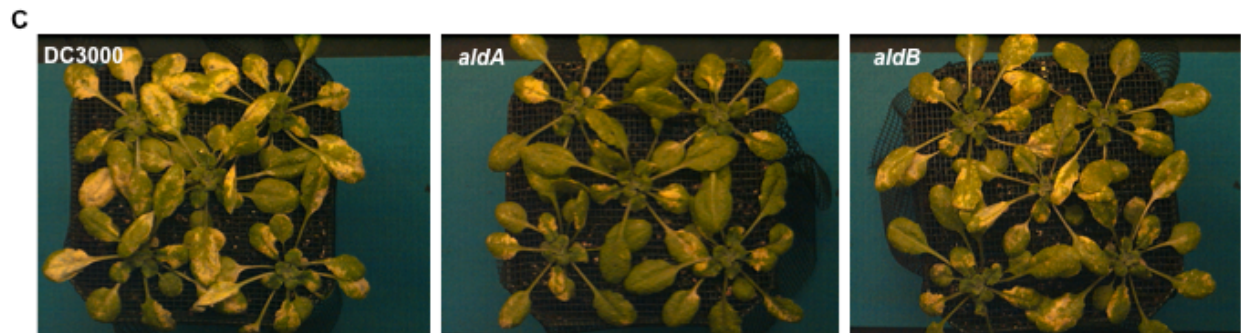
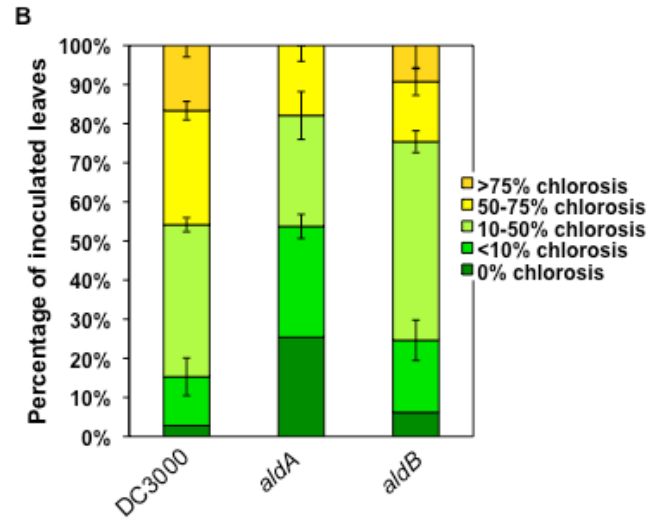
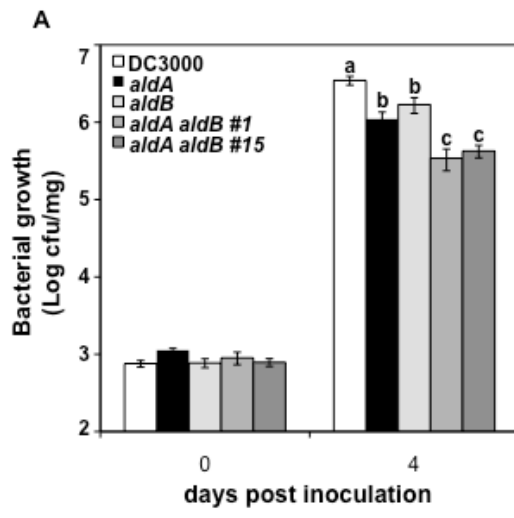


Figure 3.2. Growth of *ald* mutants on SA deficient *sid2-2* plants and *PR1* expression in plants inoculated by *ald* mutants. A) Growth of *ald* mutants on wild type *A. thaliana* (Col-0) and *sid2-2* mutant plants following syringe infiltration ($OD_{600} = 1 \times 10^{-4}$). Similar growth differences were observed in two additional experiments. B) *PR1* expression in Col-0 plants at 24 hrs following syringe infiltration ($OD_{600} = 1 \times 10^{-5}$). Significant elevation of *PR1* expression in *aldA*-infected plants was observed in three experiments, and in two experiments for *aldB*-infected plants. Letters indicate significant difference between samples within a given time point ($p < 0.05$).

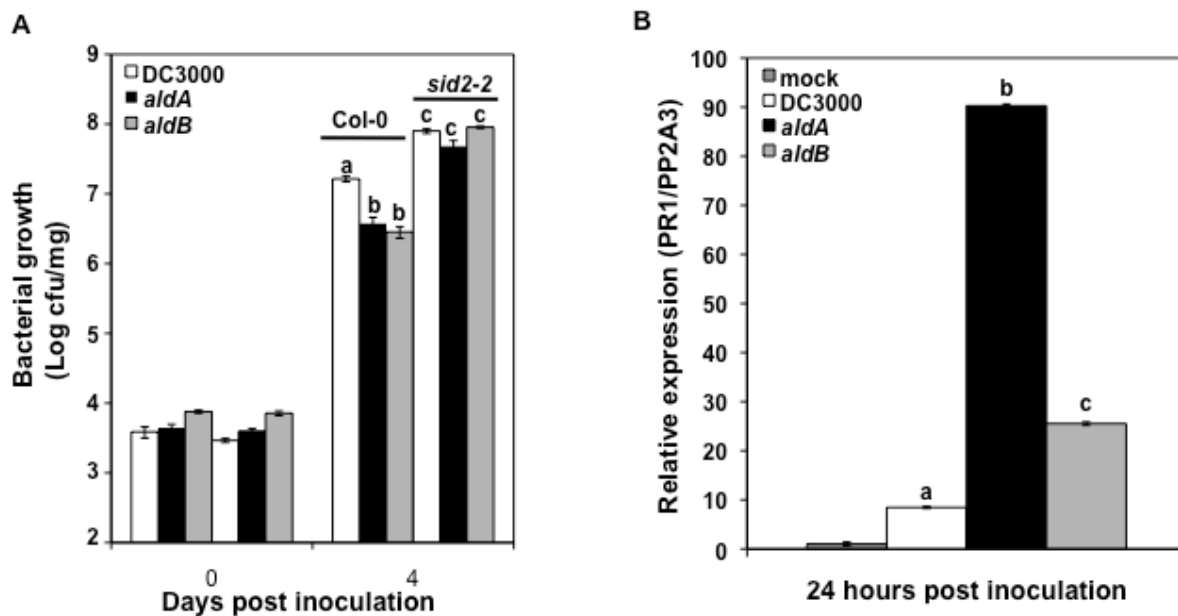


Figure 3.3. Growth of *ald* mutants on Col-0 and *YUCCA1*-over expressing plants. Growth of *ald* mutants on wild type *A. thaliana* (Col-0) and *YUCCA1* transgenic plants was monitored 4 days following syringe infiltration ($OD_{600} = 1 \times 10^{-4}$). Letters indicate significant difference between samples within a given time point ($p < 0.05$).

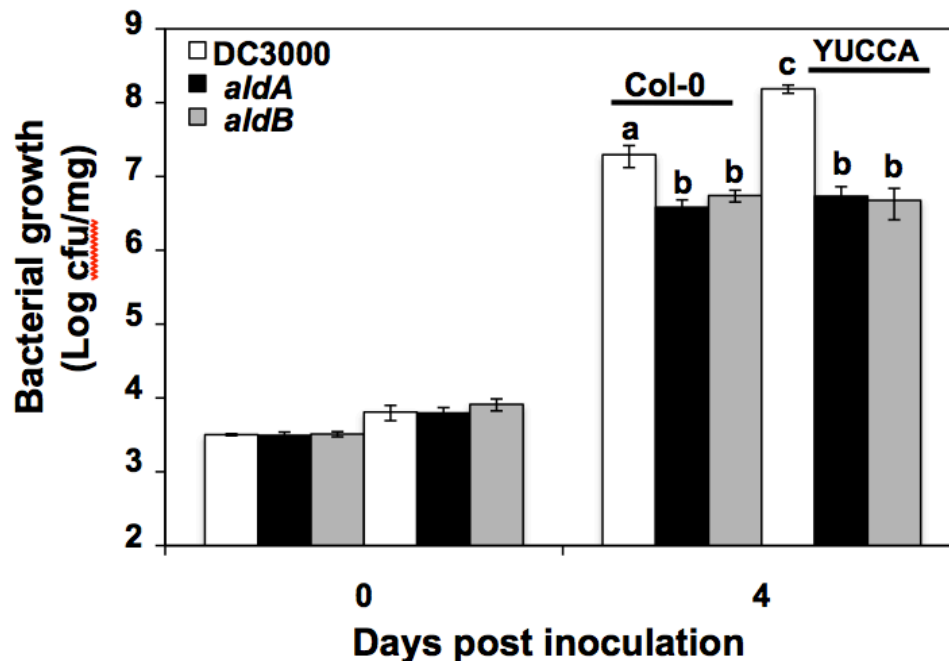


Figure 3.4. Working model for the role of auxin during DC3000 pathogenesis. Upon pathogen attack, expression of the salicylic acid (SA) biosynthesis gene *ICS1* is up-regulated, SA levels increase, and plant defenses (e.g. *PR1* expression) are induced to restrict pathogen growth and disease in the host. DC3000 produces auxin (IAA) through ALD enzymes, which promotes pathogen growth and disease symptom development by suppressing SA mediated defenses. In plants over-expressing the *YUCCA* (*YUC1*) auxin biosynthesis gene, IAA levels are elevated, which promotes pathogen growth and disease development via a mechanisms independent of suppression of SA mediated defenses. Abbreviations: IAA, indole-3-acetic acid; *ICS1*, *ISOCHORISMATE SYNTHASE 1*; SA, salicylic acid; *PR1*, *PATHOGENESIS-RELATED GENE 1*; ALD, aldehyde dehydrogenase; Trp, tryptophan; IPA, indole-pyruvic acid.

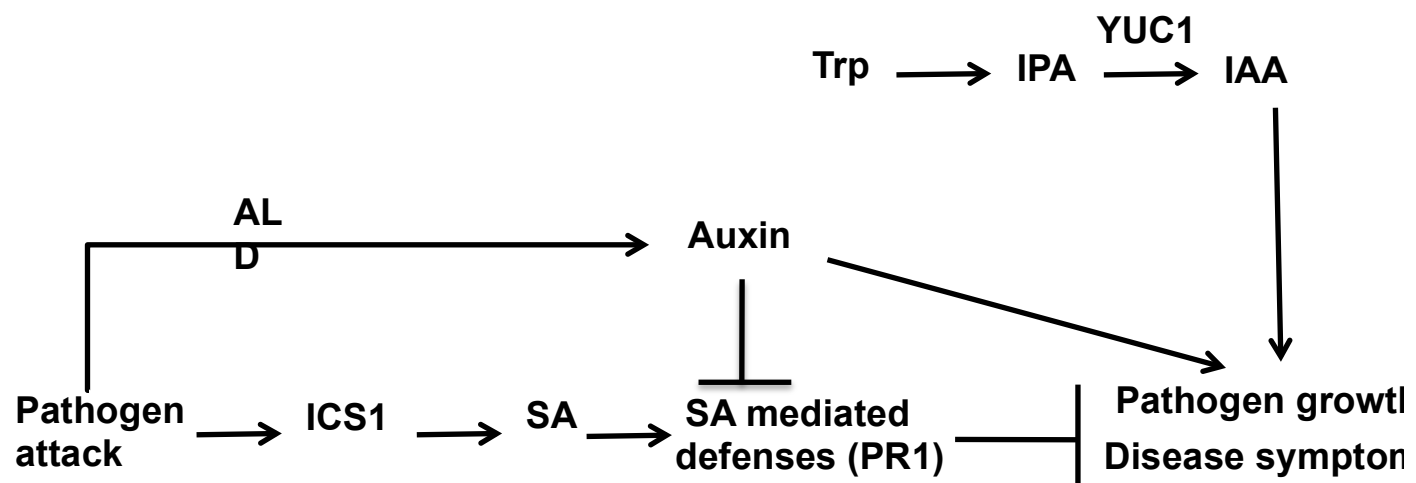


Table 3.1. Quantitative real-time PCR (qRT-PCR) primers

Name	Sequence	Organism and gene
PP2A3F	5' AACGTGGCCAAAATGATGC 3'	<i>A. thaliana</i> AT1G13320
PP2A3R	5' AACCGCTTGGTCGACTATCG 3'	<i>A. thaliana</i> AT1G13320
PR1F	5' GGAGCTACGCAGAACAATAA 3'	<i>A. thaliana</i> AT2G14610
PR1R	5' CCCACGAGGATCATAGTTGCAACTGA 3'	<i>A. thaliana</i> AT2G14610

Chapter 4

Conclusions and Future Directions

In this thesis, I have presented my research on auxin biosynthesis in *Pseudomonas syringae* strain DC3000 and the role of pathogen-derived auxin during infection. In particular, I have identified an important intermediate for IAA production in culture and identified two novel enzymes that function in DC3000 IAA biosynthesis. Additionally, I determined that pathogen-derived auxin contributes to virulence and its role seems to be the suppression of SA-mediated defenses. Here I summarize the major conclusions from this work and present additional future directions that were not addressed in Chapter 2 or Chapter 3.

IAA biosynthesis in DC3000

I investigated IAA biosynthesis in DC3000 and the biochemical nature of the enzymes involved. DC3000 makes IAA in culture and indole-3-acetaldehyde (IAAld) is an important intermediate for IAA production (Table 2.1). There are three IAA biosynthesis pathways that are reported to utilize IAAld as an intermediate (Figure 2.1), however the enzyme(s) responsible for converting IAAld to IAA have not been characterized. I identified 6 putative aldehyde dehydrogenase (ALD) enzymes in DC3000, and showed that *E. coli* expressing 3 of these proteins showed increased IAA levels when fed with IAAld (Figure 2.2). In collaboration with Soon-Goo Lee of the Jez lab, we demonstrated that the putative ALD enzymes encoded by the *PSPTO_0092* and *PSPTO_2673* synthesize IAA using IAAld and NAD^+ in *in vitro* enzyme assays (Figure 2.4). These are the first demonstrated IAAld dehydrogenase enzymes, and we named them AldA and AldB, respectively. Additionally, I generated mutations in the DC3000 genes encoding these enzymes (Figure 2.5) and assayed the mutants for altered IAA production. The *aldA*, *aldB* and the *aldA aldB* double mutants displayed reduced IAA in culture when

supplemented with IAAlD (Figure 2.6). Thus, I conclude that the IAAlD dehydrogenases, AldA and AldB, function in DC3000 IAA biosynthesis to convert IAAlD to IAA.

Future directions for investigating the role of Alds in IAA biosynthesis

The ability of the *aldA aldB* double mutant to produce low levels of IAA when fed with IAAlD (Figure 2.6) indicates there are ALD enzymes, other than AldA and AldB, that function in IAA biosynthesis. Bioinformatics, using AldA and AldB sequence can help identify additional enzymes in DC3000. Preliminarily, I found at least two additional enzymes that are annotated as aldehyde dehydrogenases, encoded by the genes *PSPTO_1881* and *PSPTO_3323*, which were not included in my studies summarized in Chapter 2. To investigate whether these proteins can function in IAA biosynthesis, *E. coli* expression and feeding tests will have to be performed. This will help indicate if these enzymes can convert IAAlD to IAA. If IAA production is increased in cells expressing these proteins, then I recommend making mutations in DC3000 in the corresponding genes. Ultimately ALL indole-3-acetaldehyde dehydrogenase enzymes identified will have to be disrupted in DC3000, to understand their roles during IAA biosynthesis, and ultimately the role of pathogen-derived IAA during DC3000 pathogenesis. Currently the existing *ald* single and double mutants are disrupted by a single homologous recombination, incorporating the pJP5603 suicide vector into the chromosome (non-replicating in DC3000) (Penfold and Pemberton, 1992). There is a low rate of loop out of this vector and all strains must be used relatively fresh and kept under their respective antibiotic selections to ensure that the plasmid disruption is present in these strains. In the future, I would recommend generating whole gene knockouts or antibiotic cassette replacement strains when making higher order mutant combinations. This will help alleviate any worries of a mixed population of bacteria due to plasmid loop out and reversion of the mutations, during experiments.

Future directions for investigating the role of Alds on DC3000 biology.

Reduced bacterial growth of the *aldA aldB* double mutants in culture suggests that disruption of this activity negatively impacts DC3000 biology. However, it is unclear whether the reduced growth is the result of reduced IAA production, increased accumulation of an intermediate such as IAALd, or the role of Alds in some other important aspect of DC3000 biology independent of its role in IAA biosynthesis. To test whether IAA is important for growth of DC3000, IAA feeding experiments can be performed to determine if exogenous IAA can rescue the growth defects of the *ald* mutants in culture. If exogenous IAA can rescue the growth defects, then this would indicate that IAA is required for normal growth. Subsequent experiments could include whether auxin regulates the expression of genes involved in metabolism or cell growth. However, if exogenous IAA does not restore growth defects, this suggests that AldA and AldB regulate normal biology of the bacteria either dependently or independently of its dehydrogenase activity. In collaboration with the Jez lab, we have begun experiments to determine the specificity of the Ald proteins for IAALd. In preliminary experiments, Dr. Lee has determined that AldA can utilize phenylacetaldehyde (PAAld) as a substrate to produce phenylacetic acid (PAA), another known auxin, in *in vitro* assays (see Appendix 1). Further studies to test the additional substrates used by the Ald proteins are required and may provide insight into the role of Alds in other processes involved in DC3000 biology.

Alternatively, the Ald proteins might function in DC3000 biology independent of its aldehyde dehydrogenase activity. Crystal structures of AldA (McClerklin et al, in prep),

identified key residues that are important for dehydrogenase activity, including Cys302. To identify dehydrogenase-independent activities of Ald proteins, amino acid substitutions (to Alanine) can be made in these key residues. It would be interesting to introduce plasmids carrying proteins variants into DC3000 *ald* mutant strains and assay for complementation of the growth phenotype. If *aldA*: pAldA-C302A strains exhibit reduced IAA synthesis, but show normal growth, then this would indicate that the Ald proteins have multiple roles in DC3000 biology and have dehydrogenase dependent and independent activities. In contrast, if DC3000 strains carrying Ald protein variants disrupted in dehydrogenase activity display the same reduced growth phenotypes observed for *ald* mutant strains, this would indicate that the aldehyde dehydrogenase activity is required for its role in DC3000 biology.

Future directions for investigating additional IAA biosynthesis pathways in DC3000

The ability of DC3000 to produce small amounts of IAA using other auxin intermediates (IAM, IAN, TAM) suggests that DC3000 might use other IAA biosynthesis pathways in addition to the IAALd pathway studied in this thesis. To identify additional IAA biosynthesis enzymes, a high throughput Tn5 mutagenesis could be performed. This would include use of transposons (mobile elements) that are randomly incorporated into the genome leading to gene disruptions. Tn5 mutants would then be tested for altered IAA production upon feeding with Trp. I have developed a high-throughput colorimetric screen based on Salkowski's reagent, which leads to a visual color change when reacting with IAA (Glickmann and Dessaux, 1995), which could be use for this purpose. Once mutants have been identified, genomic sequencing, using primers specific to the Tn5 insertion, could be used to determine the position of the insertion. This approach may reveal novel genes that not only function in IAA biosynthesis but also possibly in auxin transport, or regulation of auxin related genes. For example Xie et al (2005) identified

AldA in *Azospirillum* in a transposon mutagenesis using the Salkowski reagent. One challenge in this approach is that there may be multiple enzymes that carry out the same function, and thus a single mutation might not reduce IAA production enough to allow identification of such a mutant in a colorimetric screen. However I think it is likely that we can identify some auxin synthesis related mutants, based on the reduced IAA levels I observed in the *aldA* and *aldB* single mutants. If this approach fails, we can use culture-feeding studies and examine production of auxin intermediates in culture using GC/MS-MS. This might help reveal additional pathways in DC3000. Additionally, a bioinformatics approach focusing on identification of genes encoding enzymes that catalyze these other steps might be worthwhile.

Pathogen derived auxin contributes to pathogenesis

I took advantage of the IAA synthesis mutants generated in the first part of my thesis project to investigate whether pathogen-derived auxin contributes to *P. syringae* virulence. Plants infected with DC3000 *aldA* and *aldB* mutants displayed reduced bacterial growth and disease symptom development on *A. thaliana* (Figure 3.1). Reduced bacterial growth of *ald* mutants on Col-0 plants was restored on SA-deficient *sid2-2* plants (Figure 3.2a). Furthermore, transcript levels of *PR1*, a marker for SA-mediated defenses, were increased in plants infected with the *ald* mutants compared to plants infected with wild-type DC3000 (Figure 3.2b). Thus, one role of pathogen-derived auxin is to promote pathogenesis by suppressing SA-mediated defenses. Surprisingly, the growth defect of the *ald* mutants on Col-0 was not restored in *35S::YUC1* plants that accumulate elevated levels of IAA (Figure 3.3). This suggests that there are multiple roles for IAA during infection and that the source of auxin production may be an important factor (Figure 4.1).

Future experiments for investigating the role of pathogen-derived auxin on virulence and disease

Little is known about the development of disease symptoms in plants infected with *P. syringae* strain DC3000. It is widely understood that molecules produced by the pathogen, including Coronatine and several Type III effector proteins, promote disease symptom development (Brooks et al., 2005; Chen et al., 2007; Badel et al., 2003). Coronatine can stimulate expression of auxin-related genes and impact IAA levels (Uppalapati et al., 2005). Additionally auxin has been shown to increase disease symptom development (Navarro et al., 2006; Chen et al., 2007). Given these results it is possible that pathogen-derived auxin directly promotes disease symptom development. This could contribute to the impaired disease we observe in the *aldA* mutant, as the reduction in disease symptom severity seems more pronounced than one might expect given that the small (~ 5-fold) reduction in growth (Figs 3.1). The *STAYGREEN* (*SGR*) gene of *A. thaliana* has been shown to be involved in symptom production and is required for normal development of chlorosis during infection by DC3000 (Mecey et al., 2011). *SGR* expression could be monitored in plants that are infected with wild-type DC3000 and *ald* mutants. If expression of *SGR* is increased in wild-type DC3000 compared to *aldA*, this would indicate that pathogen derived auxin is functioning to directly promote symptom production.

It is widely shown that natural and synthetic auxins promote disease susceptibility to *P. syringae* (Chen et al., 2007; Wang et al., 2007; Navarro et al., 2006). Dr. Lee's results demonstrating the ability of AldA to utilize PAAld as a substrate, leads us to wonder whether

PAA can promote DC3000 virulence. PAA is a naturally occurring auxin found in plants (Korasick et al., 2013). PAA binds to the auxin co-receptor in *in vitro* studies (Sugawara et al., 2015) and has been shown to have biological activity in several auxin assays (Haagen Smit and Went 1935; Muir et al. 1967). Preliminary evidence from the Kunkel lab indicates that exogenous application of PAA increases susceptibility to DC3000, as *A. thaliana* plants co-inoculated with DC3000 and PAA displayed increased disease symptom production compared to control plants (B. Kunkel, see Appendix 1). Further experiments are required to determine if PAA also impacts bacterial growth in planta. If the Ald enzymes catalyze the production of PAA during infection, PAA could help promote virulence of DC3000. In order to test this we could monitor PAA levels in planta after infection with DC3000 or the *ald* mutants. Altered PAA levels in plants infected with the *ald* mutants would suggest that they contribute to PAA synthesis during infection. An additional way to investigate this is to test whether co-inoculation with PAA can restore virulence to the *ald* mutants. If we observe that co-inoculation of *A. thaliana* plants with *ald* mutants and PAA restores the virulence phenotypes, then this would suggest that the function of the ALD enzymes is to synthesize PAA and PAA is required for normal pathogenesis.

The nature of PAA synthesis is still in question. In *A. thaliana* it was recently demonstrated that a *YUCCA* family member, *YUC6*, could convert phenylpyruvate (PPA) to PAA when expressed in *E.coli* (Dai et al., 2013). This suggests that in plants, PAA is produced from PPA. In order to understand PAA biosynthesis in *P. syringae* it is necessary to determine if PAAld is an auxin intermediate found in plants and bacteria. Additionally, in culture feeding studies in which *ald* mutants are supplemented with PAAld and monitored for PAA production compared to wild-type would help us determine if the Ald enzymes identified in this study

function in PAA biosynthesis. Furthermore, examination of PAA levels during infection could help us determine its role during pathogenesis.

Future experiments for investigating alternate roles of pathogen-derived auxin during infection

Although my results suggest that pathogen-derived auxin functions to suppress SA-mediated defenses, this does not rule out additional roles for pathogen-derived auxin during infection. IAA may have multiple roles during infection that impact virulence, including regulating pathogen gene expression. It has been observed that *P. syringae* virulence gene expression is induced in planta (for example, see Yu et al., 2013); additionally work in the Kunkel lab indicates that IAA regulates bacterial gene expression in culture (G. Harrison, personal communication). In preliminary experiments monitoring pathogen gene expression during infection, I (along with former member A. Mutka) have observed increased expression of *hrpA* and *hrpL*, which encode a component of the T3SS and a transcriptional activator of virulence genes, respectively (Boureau et al., 2002; Tang et al., 2006). Several different types of experiments to monitor pathogen gene expression in response to altered levels of auxin could be performed. For example, to test if DC3000 responds to pathogen-derived auxin, this could be done by infecting wild-type plants with DC3000 and the *ald* mutants and isolating bacteria RNA at various times after infection. Comparison of expression of virulence-related genes *hrpA*, *hrpL* and *avrPto* (encoding an T3SS secreted effector protein) could indicate whether pathogen-derived auxin functions to regulate virulence gene expression during infection. This, however, is only a limited number of genes to test, and probably does not cover a majority of genes that are expressed during infection. In order to understand the larger effects of auxin on gene expression, RNA-seq experiments could be performed on wild-type DC3000 and *ald* mutants

grown in culture and *in planta*. This may reveal novel genes regulated by pathogen-derived auxin, including novel virulence-related genes, and genes required for nutrient acquisition or stress tolerance. In other experiments, pathogen gene expression could be monitored during infection of plants that accumulated elevated levels of auxin, to assess the impacts of host-derived auxin on pathogen gene expression.

Conclusion

Auxin and other hormones play an important role in plant development and plant-pathogen interactions. Although investigation of the role of hormones has proven to be challenging, due to redundancy in protein functions, and a requirement for hormones in normal development, continued research into their roles in pathogen virulence is important. Auxin is an essential hormone in plant development, however its importance in pathogen virulence is becoming increasingly evident. Therefore, further investigation of the role of auxin during plant-pathogen interactions is essential in understanding mechanisms that govern pathogenesis.

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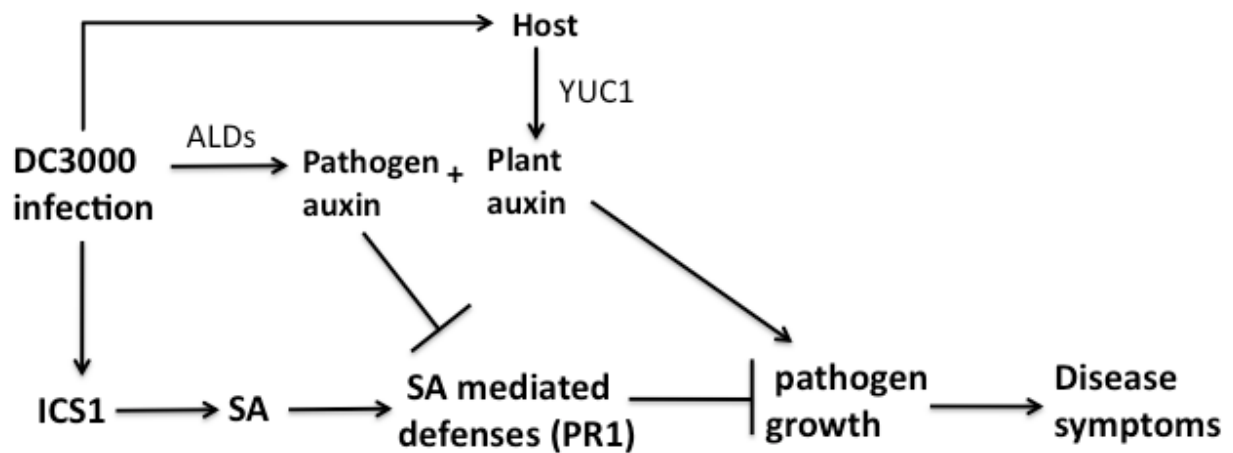
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Figure 4.1. Working model for independent roles of auxin during DC3000 pathogenesis.

During infection, DC3000 produces auxin through one or more aldehyde dehydrogenase (ALD) enzymes, which promotes pathogen growth and disease development by suppressing SA mediated defenses. In plants over-expressing the *YUCCA* (*YUC1*) auxin biosynthesis gene, IAA levels are elevated which promotes pathogen growth via a mechanism independent of suppression of SA mediated defenses. Auxin likely plays multiple roles, depending on its source during pathogenesis. It is also possible that different forms of auxin are produced by pathogen and host.



APPENDIX 1

Examining the effects of IAAld and PAAld on DC3000 pathogenesis

Examining the effect of IAAlD on DC3000 pathogenesis

In this thesis I have shown that the DC3000 ALD enzymes synthesize IAA using the precursor IAAlD and are required for normal pathogenesis of DC3000. Disruptions in the *ald* genes lead to decreased IAA in culture and reduced virulence on *A. thaliana*. At present we have not tested directly whether disrupted auxin biosynthesis by DC3000 leads to decreased IAA levels in plants infected with the *ald* mutants. However, we are able to take advantage of co-inoculation experiments, by applying exogenous auxin or IAAlD at the time of inoculation, to examine whether the reduced virulence of the *ald* mutants is due to reduced IAA biosynthesis. Previously, we and others have shown that exogenous application of auxin (NAA or 2,4-D) can promote disease symptom development (Chen et al., 2007; Wang et al., 2007; Navarro et al., 2006). Our hypothesis is that if exogenous application of IAAlD can also promote pathogenesis of wild-type, then if the *ald* mutants can no longer synthesize IAA via IAAlD then one would not see promotion of DC3000 virulence in co-inoculations with *ald* mutants and IAAlD. The following experiments were performed to address these questions.

For co-inoculation experiments, *A. thaliana* plants at approximately 4 weeks of age were dipped into a solution containing *P. syringae* at approximately 3×10^8 cells ml⁻¹ (OD₆₀₀ = 0.3), 10 mM MgCl₂ and 0.02% Silwet L-77. *P. syringae* solutions were supplemented with either 50 µM IAAlD or DMSO. As a positive control, plants were co-inoculated with DC3000 and 50 µM NAA, which has been previously reported to increase DC3000 pathogenesis (Chen et al, 2007). Quantification of disease symptoms following dip inoculation was carried out 4 days post inoculation. Leaves were categorized based on presence and amount of chlorosis or yellowing of

the leaf (Figure 1).

A. thaliana plants inoculated with wt DC3000 and no additional auxin displayed normal disease symptom production. Co-inoculation with DC3000 and NAA lead to increased disease symptom production, as indicated by the decrease in leaves that displayed little to no chlorosis (<10%) and an increase in leaves with higher amounts of chlorosis. Plants co-infected with DC3000 and IAAld displayed and even further increase in disease symptom production compared to both DMSO and NAA treated plants. This was indicated by a large reduction in the percentage of leaves lacking observable symptom production. This result indicates that IAAld promotes disease susceptibility to DC3000, and is consistent with our feeding and biochemical studies indicating that IAAld is an important intermediate for IAA synthesis by DC3000.

For future experiments, I would recommend repeating the co-inoculation experiments to confirm these results. Lower concentrations of IAAld might be needed to make sure that experiments are performed in a biologically relevant manner.

The next step would be to perform co-inoculation experiments with *ald* mutants and IAAld and IAA. To test if the reduced virulence is due to the inability of the *ald* mutants to convert IAAld to IAA. If co-inoculation experiments IAA restores virulence, but co-inoculation with IAAld does not lead to increased disease symptoms, then this would suggest that the reduced virulence of the *ald* mutants is due to their inability to convert IAAld to IAA.

Examining the contribution of PAA to DC3000 pathogenesis

The observation that the *aldA* and *aldB* single mutants display similar reductions in bacterial growth in planta but exhibit differences in disease symptom development, lead us to wonder whether the two different ALD enzymes function in the synthesis of alternative

compounds. More specifically, we wondered whether the ALD enzymes might function in the synthesis of alternative auxins, both of which may contribute to DC3000 pathogenesis in different ways. To address this, we investigated whether the ALD enzymes could synthesize phenylacetic acid (PAA), a naturally occurring auxin found in plants (Sugawara et al., 2015; Wightman and Lighty, 1982; Schneider et al., 1985). Additionally we also investigated whether PAA is important for DC3000 pathogenesis. The following experiments were performed to address these questions.

In collaboration with the Jez lab, we have begun experiments to determine the specificity of the ALD proteins for IAAld. Dr. Soon Goo Lee utilized *in vitro* assays in which the ALD enzymes were purified from *E. coli* and assayed for aldehyde dehydrogenase activity in the presence of PAA. IAAld was used as a control in these experiments (Figure 2). In preliminary experiments, Dr. Lee determined that AldA can utilize phenylacetaldehyde (PAAld) as a substrate to produce PAA, and in fact utilizes PAAld at a higher efficiency than IAAld. Additionally AldB and AldC can utilize PAAld as a substrate although at a lower rate. This data suggests that AldA prefers PAAld over IAAld and thus that AldA could synthesize PAA in planta in addition to, or perhaps or instead of, IAA.

As we have shown that AldA can utilize PAAld as a substrate to make PAA, we investigated whether PAA might be involved in DC3000 pathogenesis. To test this Barbara Kunkel and Sarah Decou, a Bio-500 student, performed co-inoculation experiments on *A. thaliana* plants with PAA. *A. thaliana* plants at approximately 4 weeks of age were dipped into a solution containing *P. syringae* at approximately 3×10^8 cells ml^{-1} ($\text{OD}_{600} = 0.3$), 10 mM MgCl_2 and 0.02% Silwet L-77. *P. syringae* cultures were supplemented with either 10uM NAA, 10uM PAA or DMSO. Quantification of disease symptoms following dip inoculation was carried out 4

days post inoculation. Leaves were categorized based on presence and amount of chlorosis or yellowing of the leaf (Figure 3).

In this preliminary experiment, although symptoms were relatively mild for all treatments, they were able to observe that plants co-inoculated with either NAA or PAA showed a slight increase in disease symptom production compared to plants infected with wt DC3000 lacking exogenous auxin. This is indicated by a reduction in leaves that display no chlorosis and an increase in the percent of leaves that display high amounts of chlorosis (60-90%). This data suggests that PAA can promote DC3000 pathogenesis.

For future experiments, I would perform feeding experiments in culture in which DC3000 and the *ald* mutants are supplemented with PAAld. If one or more ALD enzymes are synthesizing PAA, then the *ald* mutants (especially *aldA*) should display decreased PAA levels. Additionally, I would determine if PAAld is a biologically relevant intermediate by measuring PAAld levels in plants. Furthermore, I would examine PAA levels in plants infected with wt DC3000 and *ald* mutants. If PAA levels are reduced in plants infected with the *ald* mutants, then this would indicate that AldA functions to synthesize PAA during infection.

Perhaps the most informative experiment would be to test if PAA and/or PAAld can restore virulence of the *ald* mutants. If we then observe that PAAld can promote virulence of wt DC3000, but not one or more *ald* mutants, then this would indicate that ALDs function to synthesize PAA during infection and that PAA is an important player in DC3000 pathogenesis. This would be an extremely interesting and exciting result and would provide insight regarding the current apparent discrepancies regarding the role of auxin during infection, as these findings would provide an explanation as to why in some situations IAA appears to promote virulence via and SA independent mechanisms (Mutka et al., 2013), and in others appears to function by

inhibiting SA-mediated defenses (Chapter 3, Wang et al., 2006).

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Figure 1. Effects of IAAld on disease symptom production of *A. thaliana*. Disease symptom severity 4 days after dip inoculation with DC3000 supplemented with DMSO, 50uM NAA or 40uM IAAld. Disease symptom severity was quantified as the average percentage of affected leaves per plant exhibiting the indicated amount of chlorosis. 5-7 plants were assayed for each treatment. The results were plotted as the average percentage of leaves from each treatment exhibiting the indicated degree of chlorosis (see S.M. experiment 7-20-14).

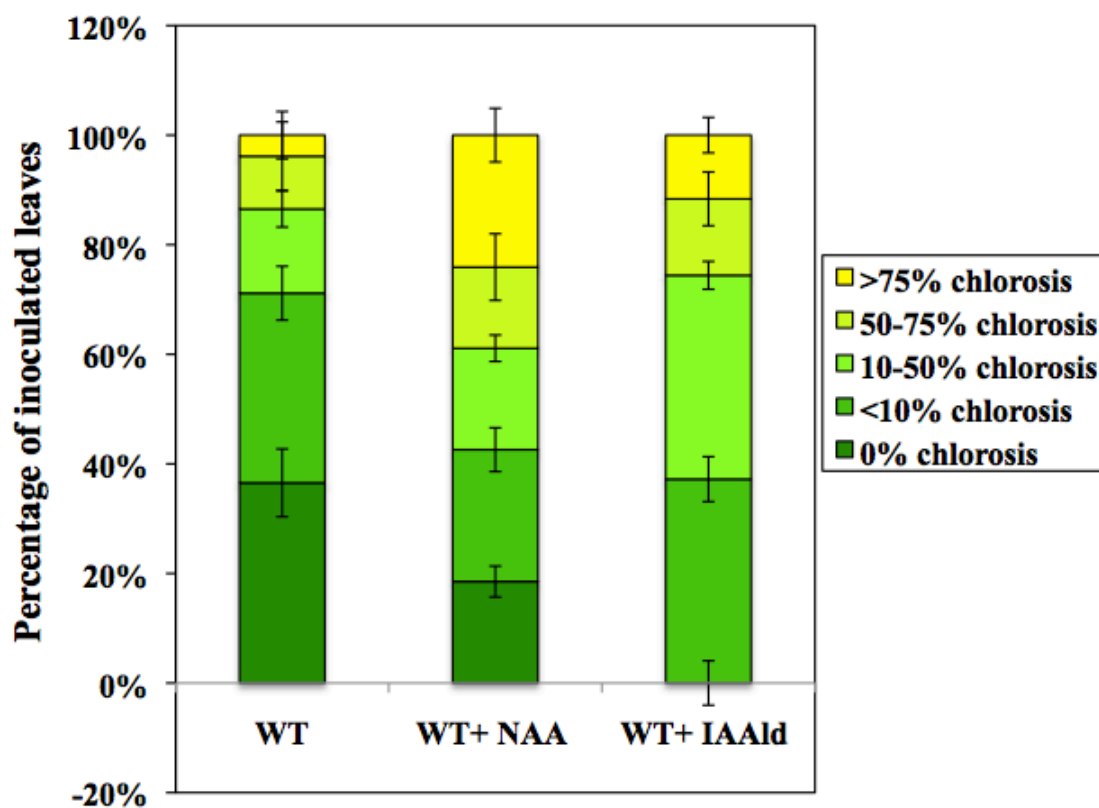


Figure 2. Enzymatic activity of AldA, AldB and AldC. Specific activities were determined using standard assay conditions using IAAld, PAAld, Trp and NAD^+ as substrates. AldA displays significant aldehyde dehydrogenase activity with PAAld. The inset zooms in on the lower specific activities for AldB and AldC, which appear to prefer IAAld to PAAld (Soon Goo Lee).

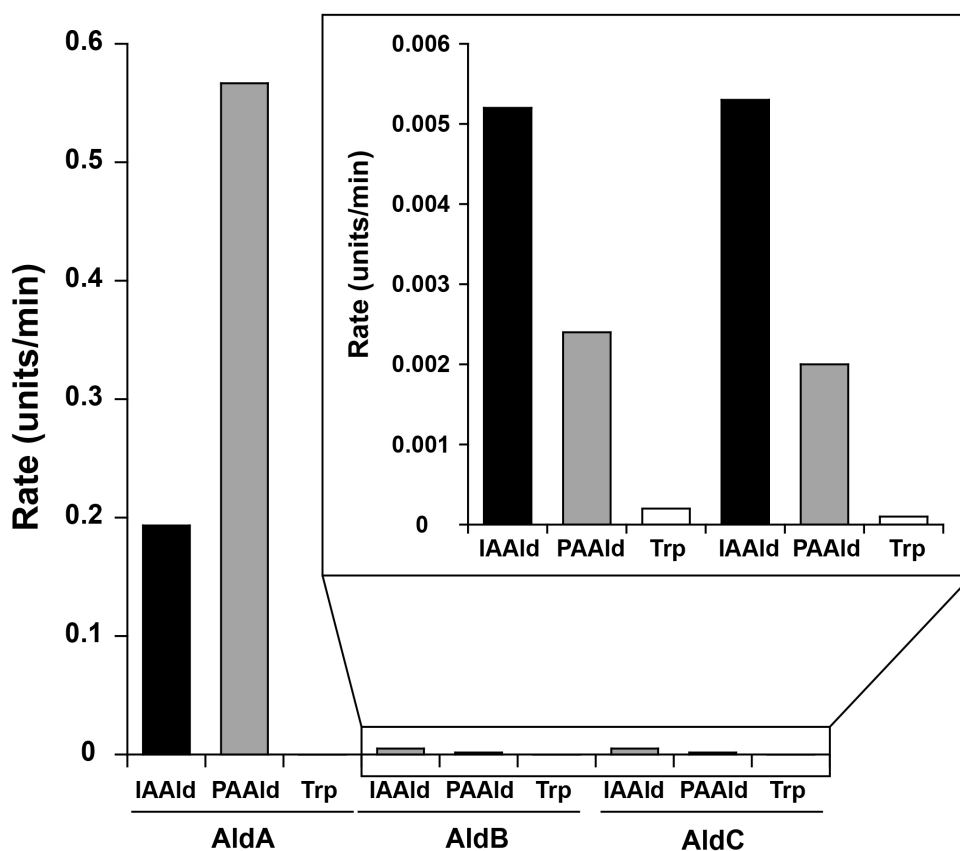
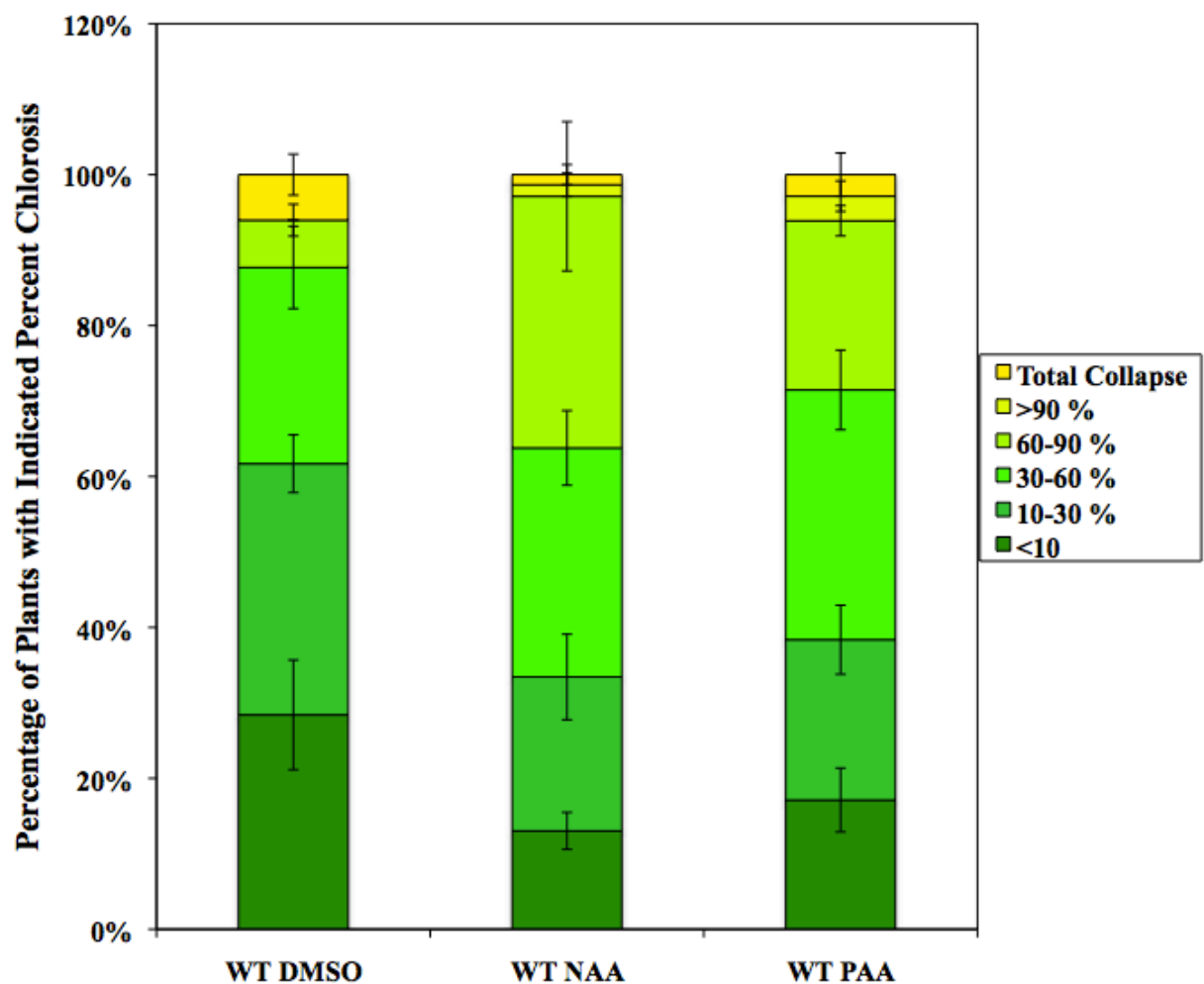


Figure 3. Effects of PAA on disease symptom production of *A. thaliana*. Disease symptom severity 4 days after dip inoculation with DC3000 supplemented with DMSO, 10uM NAA or 10uM PAA. Disease symptom severity was quantified as the average percentage of affected leaves per plant exhibiting the indicated amount of chlorosis. Five plants were assayed for each treatment. The results were plotted as the average percentage of leaves from each treatment exhibiting the indicated degree of chlorosis (see B.N.K notebook 9-2015).



Appendix 2

Examining the role of the cysteine protease activity of AvrRpt2 in the promotion of DC3000 virulence and modulation of *A. thaliana* auxin physiology during infection

AvrRpt2 is one of several effector proteins that are secreted by the type three-secretion system into plants cells as part of *P. syringae*'s strategy to colonize and cause disease in susceptible hosts. AvrRpt2 has been extensively studied in its ability to trigger host immunity when its corresponding resistance (R) gene, *RPS2*, is present in the host. However, AvrRpt2 also promotes *P. syringae* virulence in host plants lacking *RPS2*. The virulence function of AvrRpt2 (studied in *rps2* mutant background) includes promotion of *P. syringae* growth and modulation of host auxin physiology (Chen et al, 2007). Although it is clear that AvrRpt2 has virulence functions, the molecular mechanism underlying this activity remains to be elucidated.

AvrRpt2 is a cysteine protease (Axtell et al 2003; Chisholm et al 2005) in which Cys122 and His208 residues have been shown to be important for this activity (Axtell et al 2003). The cysteine protease activity is required for *RPS2*-mediated gene for gene resistance, however it is unclear whether AvrRpt2's contribution to virulence and modulation of host auxin physiology is dependent on its cysteine protease activity or due to an additional unknown function of AvrRpt2. This appendix presents unpublished data that I collected, using transgenic *A. thaliana* lines that express wild-type AvrRpt2 or two protease-defective mutants with amino acid substitutions C122A or H208A. I generated these lines specifically to test whether the cysteine protease activity is required for AvrRpt2's ability to promote virulence and/or modulate host auxin biology. These experiments were performed using the following transgenic plant lines:

KAT 3036 Col-0; *rps2*

KAT 2308 No-0; *rps2*

KAT 2195A No-0; *rps2* (Chen et al; 2007)

KAT 2192A 35S:AvrRpt2 No-0; *rps2* (Chen et al; 2007)

35S:AvrRpt2; *rps2*

35S:AvrRpt2 C122A; *rps2*

35S:AvrRpt2 H208A; *rps2*

Generating transgenic *A. thaliana* lines expressing AvrRpt2 cysteine protease mutants

Previously it was determined that DC3000 strains expressing AvrRpt2 could be used to analyze its contribution to DC3000 virulence (Chen et al, 2000, Chen et al, 2004). Subsequently AvrRpt2 cysteine protease mutants, in which the C122 and H208 residues were changed to Alanine, were generated on a plasmid-borne copy of *avrRpt2* that could be delivered into plant cells by DC3000 to analyze whether the AvrRpt2 mutant proteins had altered virulence activity. However, DC3000 strains expressing the mutant version of AvrRpt2 did not accumulate protein levels comparable to that of wild-type AvrRpt2 in plant tissue after infection. To try to circumvent any issues with protein levels and stability in plant tissues, I generated transgenic *A. thaliana* plants expressing the wild-type and cysteine protease mutants of AvrRpt2.

The lab of Libo Shan (Texas A&M University) previously generated constructs of wild type, C122A and H208A mutant versions of AvrRpt2 driven by a modified version of the 35S CaMV promoter in an *E. coli* cloning vector (Cui et al., 2013). I obtained these constructs and sub-cloned them into the pCB302 T-DNA binary transformation vector. The pCB302::AvrRpt2 constructs were transferred to *Agrobacterium* and used for subsequent transformation of *A. thaliana*. Transgenic lines were generated in the *rps2* mutant background to avoid *RPS2*-dependent gene-for-gene resistance. Transgenic *A. thaliana* AvrRpt2 constructs include an HA

epitope tag on their C-terminus in order to examine protein levels in plant tissue. *A. thaliana* transformants were screened for BASTA resistance. For details on the genetic analysis of the initial first and second (T1 and T2) generation transgenic lines see my notebooks (S.M. AvrRpt2). Multiple *A. thaliana* T2 lines that carry a single T-DNA insertion site for the transgene were isolated after self crossing Basta^R T2 plants from lines that segregated 3Basta^R:1 Basta^S. T2 lines displaying 100% BASTA^R were identified in both No-0 *rps2* and Col-0 *rps2* backgrounds and used for further analysis (Table 1).

Examining whether the virulence of AvrRpt2 is dependent on its cysteine protease activity

DC3000 strains expressing AvrRpt2 have been shown to promote DC3000 virulence in *A. thaliana* accessions that lack *RPS2* (Chen et al, 2000). Although AvrRpt2's cysteine protease activity is able to trigger host defense responses (Mudgett, 2005), it is unclear whether AvrRpt2's virulence is due to the cysteine protease activity or due to an alternate function of AvrRpt2. In order to test this, with the help of Allen Choi a Bio-500 student working under my supervision, we performed syringe infiltration and dip infections on 35S:AvrRpt2 wild-type and mutant transgenic plants with wild-type DC3000 and monitored bacterial growth and disease symptom production.

A. thaliana plants were infected at approximately 4 weeks of age. For surface inoculations, plants were dipped into a solution containing *P. syringae* at approximately 3×10^8 cells ml⁻¹ (OD₆₀₀ = 0.3), 10 mM MgCl₂ and 0.02% Silwet L-77. For syringe infiltrations, a solution containing 10^4 – 10^5 cells ml⁻¹ (OD₆₀₀ = 10^{-5} – 10^{-4}) in 10 mM MgCl₂ was injected into leaves using a 1-ml needleless syringe. Bacterial growth was monitored 4 days post infection and determined by serial dilutions of bacterial counts from infected plants and normalized by leaf

weight. Quantification of disease symptoms following dip inoculation was carried out 4 days post inoculation. Leaves were categorized based on presence and amount of chlorosis or yellowing of the leaf. For ~ 10 plants per each treatment, each leaf was individually assessed for percent of the leaf exhibiting chlorosis.

The following data summarizes results from transgenic lines in the Col-0 *rps2* background, as I did not observe a reproducible effect of AvrRpt2 in promoting DC3000 virulence in the No-0 *rps2* background (Table 2). In three experiments, multiple independent 35S:AvrRpt2 transgenic lines showed increased disease symptom severity compared to control plants lacking AvrRpt2, consistent with previous reports that AvrRpt2 promotes disease susceptibility (Figure 1)(Chen et al., 2000). In two of three experiments, multiple independent 35S:AvrRpt2 C122A and H208A transgenic lines displayed decreased symptom severity compared to wt 35S:AvrRpt2 plants. More specifically, H208A lines displayed symptoms comparable to control plants lacking AvrRpt2, however C122A lines displayed symptoms intermediate to wt 35S:AvrRpt2 and control plants. In dip infection assays where bacterial growth was also assayed, I did not observe any reproducible difference in growth between empty vector control plants and AvrRpt2 transgenic lines, consistent with previous reports (Chen et al., 2007). Overall these experiments, suggests that the cysteine protease activity of AvrRpt2 is partially required for its ability to promote DC3000 virulence (see Figure 1; Table 2). However, because I was not able to detect HA tagged protein, it is unclear whether transgenic lines have comparable protein levels.

For future experiments I would recommend using transgenic lines with comparable proteins levels. I initially tested AvrRpt2 protein levels in transgenic plants via Western blot however, I was not able to detect any protein using anti-HA antibody. This might require some

trouble-shooting, of both protein isolation and Western blot protocols. Once transgenic lines with comparable expression are identified, I would continue examining virulence effects of AvrRpt2, including monitoring bacterial growth after syringe infiltration.

Examining whether modulation of *A. thaliana* auxin physiology by AvrRpt2 is dependent on its cysteine protease activity

Transgenic *A. thaliana* lines expressing AvrRpt2 were previously shown to have altered auxin physiology (Chen et al., 2007). 35S:AvrRpt2 transgenic lines displayed increased sensitivity to auxin, higher endogenous levels of IAA and increased auxin induced gene expression. The molecular mechanism by which AvrRpt2 is modulating auxin physiology has not been determined, including whether AvrRpt2 modulation of auxin physiology is dependent on its cysteine protease activity. To determine the dependence of AvrRpt2 cysteine protease activity on the modulation of auxin physiology I used the AvrRpt2 transgenic *A. thaliana* lines (described above) and analyzed their auxin sensitivity in root assay experiments.

Auxin root assays were performed by plating transgenic *A. thaliana* lines on ½ MS plates containing a range of auxin concentrations. Plates were placed vertically in growth chamber for 7 days. Seedlings were photographed with a digital camera and root length was measured from images and normalized by the root length of seedlings in the absence of exogenous auxin (Table 3).

Overall these data suggests that the cysteine protease activity of AvrRpt2 is required for its auxin sensitivity phenotype. Predominantly, I observed that wt 35S:AvrRpt2 plants were more sensitive to auxin than control plants. Furthermore, multiple independent C122A and H208A lines displayed auxin sensitivity comparable to control plants. These data suggests that the

cysteine protease activity of AvrRpt2 is required for its ability to modulate auxin physiology (Figure 2; Table 3).

For future experiments, I would recommend identifying transgenic lines with comparable protein expression. Subsequently, auxin root assays could be repeated with additional auxin concentrations. Free IAA levels in transgenic lines and auxin responsive gene expression should be monitored to determine if they are altered in AvrRpt2 mutant lines.

CONCLUSIONS

From initial experiments characterizing the molecular mechanism governing AvrRpt2's ability to promote DC3000 virulence and modulate host auxin physiology, it seems as though there are dual mechanism of the mode of action of AvrRpt2. My results suggests that the cysteine protease activity is required to modulate host auxin physiology, however the cysteine protease activity might only be partially required for AvrRpt2's ability to promote DC3000 pathogenesis. This data is consistent with previous reports in which Cui et al (2013) observed that AvrRpt2 cysteine protease activity is required for AvrRpt2 stimulation of the turn over of Aux/IAA repressor proteins. However, they also observed that the cysteine protease activity is required for AvrRpt2's ability to promote DC3000 virulence. One hypothesis that could explain the discrepancy is that Cui et al (2013) do not directly test the requirement of AvrRpt2's cysteine protease activity on DC3000 pathogenesis. However they make their conclusions based on indirect tests in which they show that axr2 is required for normal DC3000 virulence and AvrRpt2 cysteine mutants no cannot promote axr2 turnover. Further analysis and characterization of the transgenic lines I generated is required to fully understand the molecular mechanisms of AvrRpt2.

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Table 1: 35S: AvrRpt2 wt and mutant transgenic lines*

35S:AvrRpt2 transgenic lines (T2)

AvrRpt2 Col-0 <i>rps2</i>					AvrRpt2 No-0 <i>rps2</i>		
7-4	8-3	9-7	18-2	19-2	6-1	7-2	15-2
7-5	8-7	9-11	18-4	19-5	6-8	7-3	15-8
7-9	8-9		18-6	19-8	6-9	7-4	15-9
7-10			18-8	19-9		7-11	15-11
				19-10			15-12
				19-11			
				19-12			

35S:AvrRpt2 C122A transgenic lines (T2)

AvrRpt2 C122A Col-0 <i>rps2</i>					AvrRpt2 C122A No-0 <i>rps2</i>	
7-1	8-1	13-10	14-9	18-3	3-4	4-3
7-3	8-7	13-12	14-11	18-8	3-6	4-7
7-5	8-8			18-11	3-8	4-10
7-10	8-9				3-9	4-12

35S:AvrRpt2 H208A transgenic lines (T2)

AvrRpt2 H208A Col-0 <i>rps2</i>				AvrRpt2 H208A No-0 <i>rps2</i>			
2-1	5-1	11-5	12-3	4-6	5-1	9-1	10-5
2-3	5-4	11-6	12-10	4-10	5-2	9-2	10-8
2-4	5-7		12-11		5-3	9-4	10-11
2-5	5-8		12-12		5-5		
2-11	5-11				5-6		
2-12					5-7		
					5-9		

* All indicated strains segregated for a single insertion site (3R:1S) according to

Mendelian Genetics and displayed 100% BASTA^R in the T3 generation, indicating a single homozygous insertion.

Transgenic lines indicated in bold were given KAT numbers and stored in KAT box (see Sheri KAT list)

Figure 1. Representative image of disease symptom production on 35S:AvrRpt2 transgenic lines. Disease symptom severity 4 days after dip inoculation with wild-type DC3000. Overall symptoms were mild as shown by KAT 3036 (non-transgenic control). Multiple independent 35S:AvrRpt2 lines displayed increased symptom severity compared to KAT 3036. C122A displayed symptoms intermediate to wt AvrRpt2 lines. H208A displayed symptoms comparable to KAT 3036 (see Allen Choi notebook 9-27-13).

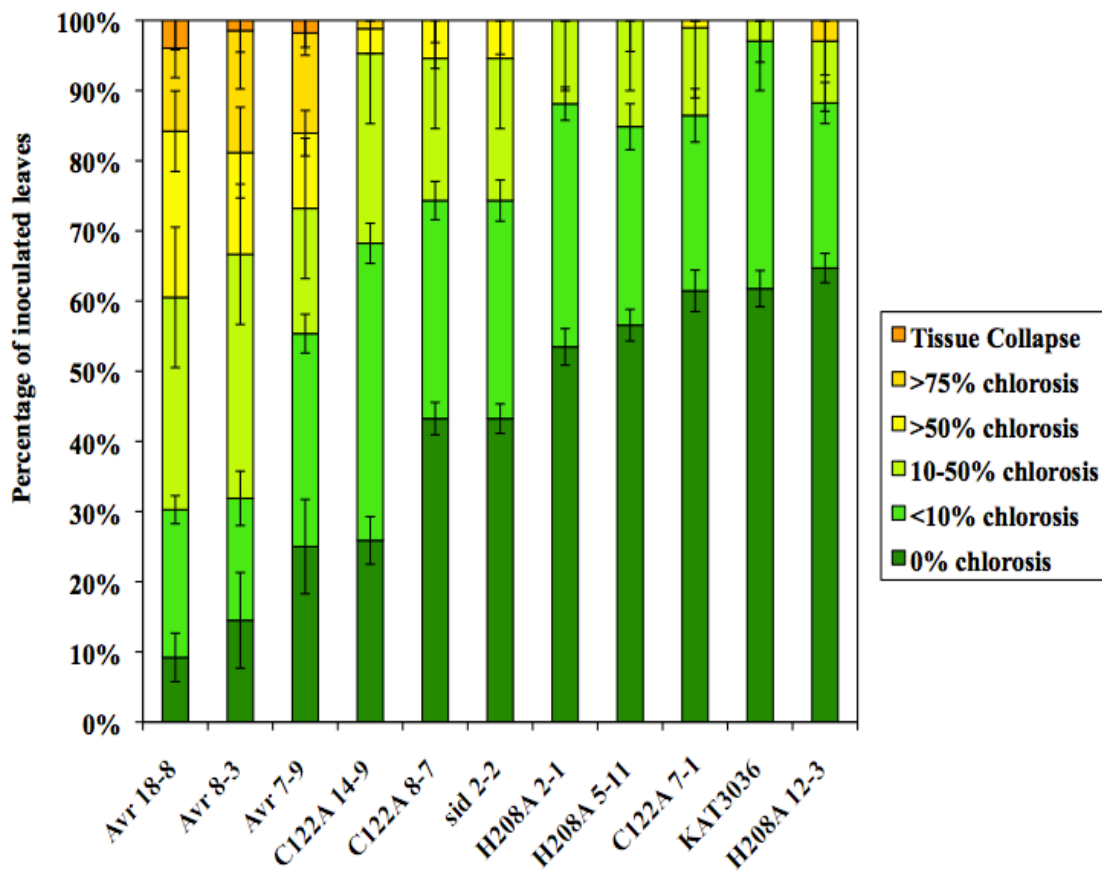


Table 2: Summary of experiments examining virulence of 35S:AvrRpt2 transgenic lines					
Exp. Date	Ecotype	Plant Lines used	Results summary	Exp.	Exp. location
1-30-13	No-0 <i>rps2</i>	KAT 2195A; KAT 2192A; Avr 7-2; Avr 7-3; C122A 4-3; C122A 4-10	No difference in growth; 2192A showed more severe symptoms than 2195A. AvrRpt2 transgenic lines looked like 2195A	Dip infection	S.M. notebook; AvrRpt2 transgenic folder
2-20-13	No-0 <i>rps2</i>	KAT 2308; KAT 2192A; Avr 6-1; Avr 7-2	No difference in growth of plants	Syringe infiltration	S.M. notebook; AvrRpt2 transgenic folder
3-1-13	No-0 <i>rps2</i>	KAT 2308; Avr 6-1; Avr 7-2; C122A 4-3; C122A 4-7	No difference in growth of plants	Syringe infiltration	S.M. notebook; AvrRpt2 transgenic folder
4-3-13	No-0 <i>rps2</i>	KAT 2308; Avr 6-1; Avr 7-2; C122A 4-3; C122A 4-7	AvrRpt2 lines show increased growth compared to control. C122A lines look like control plants.	Dip infection	S.M. notebook; AvrRpt2 transgenic folder
5-29-13	No-0 <i>rps2</i>	KAT 2308; Avr 6-1; Avr 7-2; C122A 4-7; H208A 5-6; H208A 10-5	No difference in growth; Avr wt showed increased symptoms vs. control. H208A lines looked like control	Dip infection	S.M. notebook; AvrRpt2 transgenic folder
7-10-13	Col-0 <i>rps2</i>	KAT 3036;	No difference in	Dip	Allen

		Avr 7-5; Avr 8-7; C122A 8-1; H208A 2-1; H208A 5-1; H208A 11-5	bacterial growth; saw AvrRpt2 effect on disease symptoms. Avr>C122A>H208A	infection	Choi notebook
7-24-13	Col-0 <i>rps2</i>	KAT 3036; Avr 7-5; Avr 8-7; C122A 8-1; H208A 2-1; H208A 5-1; H208A 11-5	wt AvrRpt2 lines show small increase in symptoms vs. control Avr>C122A>H208A	Dip infection	Allen Choi notebook
9-27-13	Col-0 <i>rps2</i>	KAT 3036; Avr 7-9; Avr 8-3; Avr 18-8; C122A 7-1; C122A 8-7; C122A 14-9; H208A 2-1; H208A 5-1; H208A 12-3; <i>sid2-2</i>	wt AvrRpt2 lines show increase in symptoms vs. control Avr>C122A>H208A	Dip infection	Allen Choi notebook
10-22-13	No-0 <i>rps2</i>	KAT 2308; Avr 6-1; Avr 7-2; Avr 15-9; C122A 3-6; C122A 4-7; H208A 4-6; H208A 9-1; H208A 10-5; <i>sid2</i>	Multiple wt Avr show increased growth vs. control. C122A looks like control. H208A are intermediate to wt Avr	Syringe infiltration	S.M. notebook; AvrRpt2 transgenic folder

Figure 2. Representative image of auxin sensitivity of 35S: AvrRpt2 transgenic lines. Seeds of Col-0 *rps2* (KAT 3036) plants and 35S:AvrRpt2 transgenic plants were germinated and grown on 1/2 MS plates containing the indicated concentrations of the auxin analog 2,4-D for 7 days and then measured for root length. A) Multiple 35S:AvrRpt2 lines display increased auxin sensitivity compared to KAT 3036. C122A plants do not display increased sensitivity to auxin and are comparable to KAT 3036. B) Multiple 35S:AvrRpt2 lines display increased auxin sensitivity compared to KAT 3036. H208A plants do not display increased sensitivity to auxin and are comparable to KAT 3036. Data from is expressed as relative root length, normalizing the average length of seedlings grown on 2,4-D to average length of seedlings grown in the absence of 2,4-D. (see S.M. AvrRpt2 transgenic folder; exp 10-23-13/12-1-13).

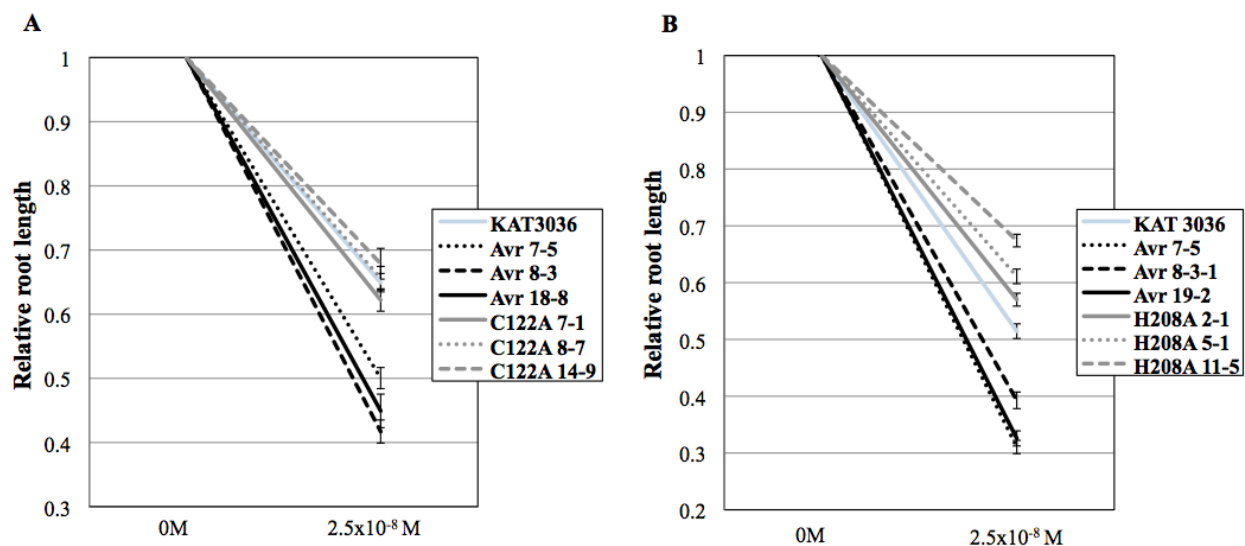


Table 3: Summary of experiments examining auxin sensitivity of 35S:AvrRpt2 transgenic lines				
Exp. Date	Ecotype	Plant Lines used	Results summary	Exp. location
6-14-13	Col-0 <i>rps2</i>	KAT 3036; Avr 8-7 C122A 8-1; H208A 11-5	Wt Avr: like KAT 3036. C122A: less sensitive than KAT 3036. H208A: more sensitive than KAT 3036	Allen Choi notebook
9-23-13	Col-0 <i>rps2</i>	KAT 3036; Avr 7-9; Avr 18-8; Avr 19-2	2 independent wt Avr lines: more sensitive than KAT 3036	S.M. AvrRpt2 transgenic folder
10-23-13	Col-0 <i>rps2</i>	KAT 3036; Avr 7-5; Avr 8-3; Avr 18-8; C122A 7-1; C122A 8-7; C122A 14-9	wt Avr lines: more sensitive than control. C122A lines were like control	S.M. AvrRpt2 transgenic folder
12-1-13	Col-0 <i>rps2</i>	KAT 3036; Avr 7-5; Avr 8-3; Avr 19-2; H208A 2-1; H208A 5-1; H208A 11-5	wt Avr lines were more sensitive than control. H208A lines were like control	S.M. AvrRpt2 transgenic folder

