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Innate Immunity in *Arabidopsis thaliana*: Induction and Suppression by *Pseudomonas syringae*

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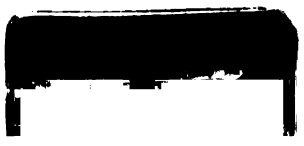

INNATE IMMUNITY IN *ARABIDOPSIS THALIANA:* INDUCTION AND SUPPRESSION BY *PSEUDOMONAS SYRINGAE*

By

William Robert Underwood

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ABSTRACT

INNATE IMMUNITY IN *ARABIDOPSIS THALIANA:*

INDUCTION AND SUPPRESSION BY *PSEUDOMONAS SYRINGAE*

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Innate immunity refers to the ability of eukaryotic organisms to detect the presence of potentially harmful microbes through recognition of highly conserved surface molecules, referred to as pathogen-associated molecular patterns (PAMPs), and to activate defense responses to prevent infection. Like mammals and insects, plants can perceive microbial PAMPs and activate defenses. Microbial pathogens such as bacteria cause significant agricultural losses each year and chemical treatments aimed at abating these losses represent a substantial input cost for agriculture and are damaging to the environment. Therefore, new strategies to control crop losses due to pathogens without the use of chemicals will be highly desirable. To develop such strategies, a thorough understanding of plant defenses and the mechanisms by which pathogens overcome them will be necessary.

My research has contributed to our knowledge of both innate immunity in plants, and the mechanisms used by pathogenic bacteria to overcome innate immune responses. I performed genome-wide microarray analyses in collaboration with Dr. Roger Thilmony to determine global gene expression changes associated with the activation of innate immunity in *Arabidopsis thaliana*. This study provided the first look at the gene expression changes induced by perception of PAMPs present on live bacteria. We also investigated the specific effects of the *Pseudomonas syringae* pv. tomato DC3000 (*Pst*

DC3000) virulence factors coronatine (COR) and the type III secretion system (TTSS) on modulating gene expression in *Arabidopsis*.

My work also contributed to the discovery of bacterium-induced stomatal closure as a novel component of innate immunity in plants. This research was performed in collaboration with Dr. Maeli Melotto and revealed that the guard cells of stomata, pores on the surface of the plant leaf, can sense bacterial PAMPs and close stomata to restrict entry of bacteria into the leaf tissue. Prior to this discovery, stomata were generally regarded as passive ports of bacterial entry. We found that bacterium-induced closure of stomata required the plant hormone abscisic acid (ABA) and an intact ABA signaling pathway. Additionally, we discovered that the phytotoxin COR is required for *Pst* DC3000 to open stomata, explaining the primary virulence function of COR.

Successful phytopathogens such as *Pst* DC3000 can overcome innate immunity and infect plants. The TTSS and effector proteins injected into the plant cells are crucial for the bacteria to overcome defenses and cause disease. I investigated the function of a single TTSS effector, the protein tyrosine phosphatase HopD2, by creating transgenic *Arabidopsis* lines expressing HopD2 or the catalytically-inactive HopD2^{C378S}. I found that HopD2 blocks defense responses associated with PAMP-induced innate immunity, but not the HR. This activity was dependent on an intact phosphatase catalytic site and, interestingly, HopD2^{C378S} had a dominant-negative effect on the function of the wild-type HopD2. Global gene expression profiling revealed that HopD2 does not affect gene expression levels in *Arabidopsis*, suggesting that HopD2 acts at a late stage, downstream or independent of PAMP-induced signaling and gene expression changes, to block activation of innate immunity.

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Introduction

Plants constitute the primary source of energy and food for most terrestrial species, including humans. By converting the energy of the sun into chemical energy stored in the bonds of carbohydrates through photosynthesis, plants provide a readily exploitable source of energy for animals, insects, and other organisms including pathogenic microbes. Collectively, pathogens such as bacteria, fungi, nematodes, protozoa, and viruses infect essentially all species of land plants and, of particular importance, all varieties of crop plants. Crop loss due to disease represents a serious threat to global security of food, fiber, and animal feed (Strange and Scott, 2005). The percentage of crop losses worldwide caused by plant pathogens, insects, and weeds was estimated to be 31-42% of potential production capacity and to account for at least $500 billion in damages (Agrios, 2005; Oerke et al., 1994). In the United States, crop losses due to plant pathogens was estimated at $9.1 billion while worldwide losses as a result of plant disease reduce overall productivity by 12% (Food and Agriculture Organization, 1993).

Set against a backdrop of a steadily increasing human population and stagnating increases in agricultural production, it is clear that novel, cost effective, and environmentally sound strategies aimed at reducing crop losses to pathogens will be needed. Current efforts to control pathogens and pests include widespread use of chemical sprays and pesticides. Large-scale use of such chemicals often results in environmental damage such as soil and groundwater contamination. In addition, chemical sprays and pesticides represent a large input cost for agricultural production. Estimated worldwide costs for pesticide applications totaled $26 billion annually (Food and

Agriculture Organization, 1993). Traditional breeding efforts aimed at generating resistant crop varieties have resulted in varying levels of success. Agricultural practices of planting crops in large, genetically uniform plots place selective pressures on pathogens to overcome resistance conferred by single genes and a lack of basic understanding of the factors that contribute to durable, long-term resistance continues to impair efforts to generate crop varieties that display long-lasting pathogen resistance.

Diseases caused by bacterial pathogens are difficult to control and can sometimes result in severe crop losses. Control of bacterial diseases often involves foliar sprays to control bacterial populations or use of insecticides to kill insects that act as vectors to spread bacteria from plant to plant. Gram-negative bacteria from the genera *Pseudomonas, Ralstonia, Agrobacterium, Xanthomonas,* and *Erwinia*, and gram-positive bacteria from the genera *Clavibacter* and *Streptomyces* are the most common causal agents of bacterial disease in plants. Collectively, these bacteria infect a wide variety of plants and cause numerous types of diseases including leaf specks, wilts, scabs, blights, cankers, and rots. Examples of severe and agronomically important bacterial diseases of plants include fire blight of apples and pears caused by *Erwinia amylovora*, leaf blight of rice caused by *Xanthomonas oryzae*, and wilts and rots of numerous crop plants caused by *Ralstonia solanacearum*.

Life cycles and virulence strategies of phytopathogenic bacteria

Outbreak of bacterial disease in crop plants is often highly variable from year to year depending on environmental conditions such as temperature, rainfall, insect populations, and other factors. Most phytopathogenic bacteria survive through winters in

soil, crop residues and debris, seeds, perennial weeds, stem cankers, and sometimes even insect hosts. Infections and outbreaks are subsequently promoted by favorable environmental conditions and spread from plant to plant by rain drops, oozes, or insect vectors. Although different phytopathogenic bacteria employ a broad range of mechanisms for survival and spread, the disease cycles of most plant pathogenic bacteria are very similar and can be summarized by the following stages: entry into host tissue; suppression of host defenses and acquisition of nutrients to allow establishment of infection; aggressive multiplication within host intercellular spaces; release of bacteria from infected tissue and subsequent further infections of uninfected tissue from the same host plant or spread to other uninfected plants. One common characteristic shared by most phytopathogic bacteria is that, unlike many mammalian pathogens, they infect host tissue and complete the disease cycle without entering plant cells. Interestingly, the opportunistic bacterial pathogen *Pseudomonas aeruginosa* strain PA14 was observed to infect *Arabidopsis thaliana* and to actually perforate mesophyll cell walls and enter mesophyll cells (Plotnikova et al., 2000). More commonly, however, phytopathogenic bacteria remain in the intercellular spaces of plant tissues and must employ strategies to exploit host resources without actually entering host cells.

Common strategies employed by bacteria to exploit plant resources include the secretion of toxins and injection of bacterial proteins into plant cells. These strategies likely promote the release of water and/or nutrients from the host cells into the apoplast, which is generally regarded as a harsh, nutrient poor environment that may not support aggressive bacterial multiplication. Additionally, some bacteria such as *Ralstonia solanacearum* and *Erwinia carotovora* secrete hydrolytic enzymes that degrade the

polysaccharides present in the plant cell walls (Huang and Allen, 2000; Reid and Collmer; 1986). Degradation products of the cell wall may provide nutrients for the bacteria and promote multiplication and such hydrolytic degradation of the cell wall is generally associated with rot and wilt diseases. In the following pages of this chapter, I will focus specifically on virulence mechanisms of the model bacterial plant pathogen *Pseudomonas syringae*.

The *Arabidopsis thaliana-Pseudomonas syringae* model for studying plant disease

During the past 15 years, a model system consisting of the bacterial plant pathogen *Pseudomonas syringae* pathovar tomato and the model host plant *Arabidopsis thaliana* has emerged as a powerful tool to facilitate inquiry into the molecular mechanisms underlying plant disease resistance and bacterial virulence. *Arabidopsis thaliana* is a small, dicotyledonous member of the *Brassicaceae* family and is the most commonly used model organism for the study of plant molecular biology. A large abundance of resources have been developed for the study of the biology of *Arabidopsis* including a completed genome sequence and a collection of T-DNA insertional mutant lines. With the emergence of *Arabidopsis* as a model system for the study of plant biology, Brian Staskawicz and colleagues sought to identify a bacterial pathogen that could infect *Arabidopsis* and potentially serve as a model for the study of bacterial diseases of plants. *Pseudomonas syringae* pathovar tomato (*Pst*), a bacterial pathogen of tomato, was found to infect *Arabidopsis* and one strain of *Pst*, strain DC3000, was found to cause severe disease symptoms (Whalen et al., 1991).

The bacterial pathogen *Pseudomonas syringae*

Pseudomonas syringae is a gram-negative bacterium belonging to the family *Pseudomonadaceae.* This family includes the genera *Pseudomonas, Burkholderia, and Ralstonia* and belongs to the gamma subgroup of proteobacteria, which includes other pathogenic bacteria including enteric mammalian pathogens such as *Yersinia, Shigella, Escherichia,* and *Salmonella* and other plant pathogens including *Xanthomonas, Pantoea,* and *Erwinia.* Pseudomonads are ubiquitous in the environment and inhabit a wide variety of niches. *Pseudomonas syringae* strains collectively infect a wide variety of plants and are classified as pathovars based on their host plant(s). Individual pathovars generally have a very limited host range with the ability to infect only one or a few plant species and often only specific ecotypes of a given plant species. At least 48 pathovars of *Pseudomonas syringae* have been identified based on host range and DNA analysis (Gardan et al., 1999).

Pst DC3000 can infect both *Arabidopsis* and tomato and causes leaf speck disease on these hosts. Prior to infection, *Pseudomonas syringae* can generally live epiphytically on leaf surfaces. *Pst* enters leaf tissues through stomata, the surface apertures that allow plants to conduct gas exchange and regulate water relations, and through accidental openings in the plant cuticle such as wounds. Once inside the leaf intercellular space, *Pst* multiplies aggressively, attaining populations as high as 10^7-10^8 bacteria per square centimeter of leaf tissue in only 3 to 4 days, and causes disease symptoms characterized by necrotic lesions surrounded by chlorotic halos (Hirano and Upper, 2000). At least two well-characterized virulence factors are known to contribute to *Pst* pathogenicity and

development of disease symptoms. These are the phytotoxin coronatine and the type III secretion system and battery of type III effector proteins.

Bacterial type III secretion

Type III secretion apparatuses are employed by a number of gram-negative bacteria to deliver proteins from the bacteria directly into the cytoplasm of the eukaryotic host cell. Type III secretion systems (TTSSs) are often key virulence determinants for pathogenic bacteria of both animals and plants and have been found in enteric mammalian pathogens such as *Yersinia, Escherichia, Salmonella,* and *Shigella* and in plant pathogens including *Pseudomonas, Ralstonia, Xanthomonas, Erwinia*, and others. In addition to pathogenic species, TTSSs are also found in plant and insect symbiotic bacteria which use the TTSS to interact with their hosts (Dale et al., 2001; Marie et al., 2001).

In *Pseudomonas,* components of the TTSS are encoded by *hrp* (for hypersensitive response and pathogenicity) and *hrc* (for *hrp* gene conserved) genes. The *hrp* genes were originally identified as being required for bacteria to elicit the hypersensitive response (HR, a plant defense response) in resistant plant varieties and to cause disease on susceptible plants (Lindgren et al., 1986). It was subsequently discovered that the *hrp* genes play a role in secretion when they were found to be involved in secretion of bacterial proteins in culture (Arlat et al., 1994; He et al., 1993; Wei et al., 1993).

The type III secretion apparatus is composed of a basal body that facilitates transport of proteins across the inner and outer bacterial envelopes, an extracellular needle or pilus structure that likely acts as a conduit for protein transport into the host

cytosol, and accessory proteins that may be involved in translocation of proteins through the host membrane by forming pores and potentially docking the needle or pilus structure (He et al., 2004). Components of the basal body are encoded by 9 *hrc* genes that are conserved among all bacteria with TTSSs and share a high degree of sequence similarity (Hueck, 1998). Interestingly, a number of these conserved genes also share significant sequence similarity with genes encoding components of the flagellar secretion system (Blocker et al., 2003). Structural characterization of the TTSS basal body has been facilitated by the isolation of at least part of the structure from *Salmonella enterica* serovar *typhimurium* (Kubori et al., 1998). The basal body of the TTSS was found to be similar to the flagellar basal body and is made up of outer rings within the bacterial outer membrane and two inner rings interacting with the cytoplasmic membrane. In addition to the basal body, the TTSS apparatus also includes an extracellular appendage that, in mammalian pathogens, consists of a needle-like extension and in plant pathogens, is a longer pilus-like structure. The needle structure has been studied most extensively in mammalian pathogens such as *Salmonella* and *Shigella*. In *Salmonella,* the needle structure is composed of a major structural subunit encoded by the *prgI* gene and was found to be 80nm in length and to have a diameter of 8nm (Kimbrough and Miller, 2000; Kubori et al., 1998). A needle-like structure has not been found to be associated with the TTSS apparatus of plant pathogens. However, plant pathogenic bacteria have been found to assemble a longer, pilus-like extracellular structure associated with the TTSS (He and Jin, 2003). These structures, referred to as Hrp pili, have the same diameter (8nm) as the TTSS needle of mammalian pathogens, but are significantly longer (several μm). This morphological difference between the extracellular components of mammalian and plant

pathogen TTSSs is likely due to the unique cell type encountered by plant pathogens. Plant cells are surrounded by a thick cell wall, which must be penetrated by the phytopathogenic bacterium to reach the plasma membrane and cytosol of the host cell.

The Hrp pilus of *Pseudomonas syringae* is composed of a major subunit encoded by the *hrpA* gene. Purified HrpA protein has been shown to assemble into a pilus-like structure in vitro and to be added to the tip of the nascent pilus during assembly (Li et al., 2002; Roine et al., 1997). Additionally, experimental evidence has demonstrated that type III effector proteins are secreted from the tip of the Hrp pilus, suggesting that the pilus acts as a conduit for the translocation of effectors into the host cell (Jin and He, 2001; Li et al., 2002). Additional extracellular accessory proteins such as harpins are also necessary for function of the TTSS. These proteins may play a role in guiding the growing pilus through the plant cell wall and in forming pores in the host cell plasma membrane to allow docking of the pilus (He et al., 2004).

Type III effector proteins

Proteins secreted into host cells via the TTSS are generally thought to subvert host cell processes to create an environment favorable for bacterial multiplication (Grant et al., 2006). This can include the suppression of host defense responses to avoid detection or destruction by the host or the release of nutrients from the host cell to promote bacterial multiplication. Type III effector proteins from mammalian pathogens are generally less numerous than those from plant pathogens and many studies have shed light on the functions of these proteins. However, this section will focus on type III

effectors from plant pathogens with an emphasis on the effectors encoded by *Pseudomonas syringae.*

In 2003, the completed genome sequence of the model bacterial phytopathogen *Pst* DC3000 became available (Buell et al., 2003). The availability of a complete genome sequence for this bacterium prompted a number of studies aimed at cataloging the type III effector proteins in the *Pst* DC3000 genome. These studies used various bioinformatics and experimental approaches such as identification of a promoter element known as the hrp box which is recognized by the alternative sigma factor HrpL and used to coordinately regulate transcription of type III effectors and structural components of the TTSS (Guttman et al., 2002; Petnicki-Ocwieja et al., 2002; Zwiesler-Vollick et al., 2002). One study suggested that *Pst* DC3000 may encode as many as 40 type III effectors (Schechter et al., 2004). However, more recent evidence gained using a combination or fluorescence-activated cell sorting, *hrpL*-dependent expression, and a reporter fusion system suggests that the number may be closer to 30 (Chang et al., 2005).

Collectively, type III effector proteins are absolutely required for the pathogenicity of *Pst* DC3000 and other phytopathogenic bacteria such as *Xanthomonas, Ralstonia,* and *Erwinia.* Mutant bacteria such as *hrp* mutants that cannot translocate type III effectors into the host cell are rendered non-pathogenic and cannot cause disease on normally susceptible host plants (Lindgren et al., 1986). Because of their importance in pathogenicity, many studies have recently been aimed at discovering their function within the plant host. Type III effectors of phytopathogenic bacteria were originally characterized for their role in imparting avirulence on normally pathogenic bacteria and their ability to elicit the HR in a gene-for-gene manner on plants carrying a corresponding

resistance (R) gene. However, it has become clear that the primary function of type III effector proteins is to act as virulence factors to promote pathogenicity.

Virulence functions of type III effectors

Studying the function of type III effector proteins from plant pathogenic bacteria has proven to be difficult for a number of reasons. First, single pathovars of plant pathogenic bacteria encode large numbers of type III effectors, many of which seem to have overlapping functions in promoting virulence. Because of this, knockout mutants defective in the production of individual type III effectors generally display no effect or only a slight reduction in virulence, rendering the study of bacterial mutants relatively uninformative. Second, although sequence information is available for type III effectors from a variety of plant pathogenic bacteria, the nucleic acid and protein sequences share little or no homology to other genes of known function and, with only a few exceptions, provide little clue as to the virulence function. Recently, to circumvent these problems, a number of groups have employed a strategy of expressing bacterial type III effectors directly in plant cells to study their function. Such studies have begun to reveal clues as to the targets and functions of type III effectors from phytopathogenic bacteria.

A number of type III effectors have been shown to suppress various aspects of plant defense responses. The effector protein AvrPto was originally characterized for its role in eliciting the HR in resistant tomato varieties carrying the Pto kinase (Ronald et al., 1992). Transgenic expression of AvrPto in *Arabidopsis* revealed that AvrPto could suppress the salicylic acid (SA)-independent deposition of callose in response to non-pathogenic type III secretion-deficient mutants of *Pst* DC3000 (Hauck et al., 2003).

Callose, a β-1-3 glucan, is deposited in cell wall reinforcements known as papillae in response to perception of bacteria by the plant (Brown et al., 1995). These cell wall reinforcements are thought to play a role in defense potentially by restricting bacterial access to host cells or by encapsulating bacteria and possibly facilitating their exposure to antimicrobial compounds. In addition to suppression of callose deposition, AvrPto transgenic *Arabidopsis* plants exhibited significant changes in gene expression. Interestingly, it was found that there was a clear bias in AvrPto plants toward the transcriptional repression of genes whose products were predicted to enter the plant cell secretory pathway. Collectively, these results suggest that the function of AvrPto in plant cells may be to block the assembly of defense-associated papillae. More recently, AvrPto was also found to block kinase activation and upregulation of the *flagellin-induced receptor kinase 1* (*frk1*) gene in *Arabidopsis* cell cultures in response to treatment with the flg22 peptide, a synthetic peptide representing a highly-conserved region of the bacterial pathogen-associated molecular pattern (PAMP) flagellin (He et al., 2006). The precise molecular mechanisms by which AvrPto carries out these functions and the host target(s) on which it acts are still unknown.

Recently, a molecular target of the *Pst* DC3000 effector HopM1 was identified. The *hopM1* gene lies within a conserved region of the *Pst* DC3000 genome known as the conserved effector locus (CEL) (Alfano et al., 2000). Partial deletion of the CEL in the ΔCEL mutant of *Pst* DC3000 results in a significant reduction in virulence and it was found that expression of either *hopM1* or the sequence-unrelated *avrE* was sufficient to restore full virulence (Alfano et al., 2000; DebRoy et al., 2004). These results suggest that the CEL-encoded effectors HopM1 and AvrE have overlapping function and that this

function is critical for virulence on host plants. Transgenic expression of HopM1 in *Arabidopsis* revealed that this effector could suppress an SA-dependent pathway leading to deposition of papillae (DebRoy et al., 2004). Subsequently, it was discovered that HopM1 targets a plant ADP ribosylation factor (ARF) guanine nucleotide exchange factor (GEF) named AtMIN7 (*Arabidopsis thaliana* HopM1 interactor 7) for degredation via the plant proteasome (Nomura et al., 2006). ARF GEFs are regulatory components of vesicle trafficking in eukaryotic cells, suggesting that HopM1 may interfere with vesicle trafficking leading to deposition of papillae. Interestingly, AvrE did not target AtMIN7 for degredation, suggesting that AvrE functions in a manner mechanistically distinct from HopM1.

In addition to suppression of papillae-associated basal defenses, a number of *Pst* DC3000 type III effectors have been shown to suppress programmed cell death associated with the HR (Grant et al., 2006). A specific example of this type of effector is AvrPtoB. Like AvrPto, AvrPtoB is capable of triggering HR in resistant tomato varieties carrying Pto (Kim et al., 2002). However, AvrPtoB was found to suppress HR cell death initiated by Pto when both proteins, along with AvrPto, were transiently expressed in *Nicotiana benthamiana* (Abramovitch et al., 2003). Additionally, AvrPtoB was found to suppress the HR initiated by the Cf9 resistance protein in *N. benthamiana* and programmed cell death initiated by the pro-apoptotic mouse Bax protein in both *N. benthamiana* and yeast. It was subsequently discovered that AvrPtoB acts as an E3 ubiquitin ligase and that E3 ligase activity is required for suppression of HR-associated programmed cell death (Abramovitch et al., 2006; Janjusevic et al., 2006). However, other recent publications suggest a role for AvrPtoB in blocking basal defenses triggered

by perception of PAMPs such as flagellin. AvrPtoB was found to block formation of papillae and to promote virulence of the normally non-pathogenic *Pseudomonas syringae* pv phaseolicola strain RW60 on *Arabidopsis* and these functions were not dependent on E3 ligase activity (de Torres et al., 2006). AvrPtoB was also shown to suppress kinase activation and transcriptional upregulation of *Arabidopsis frk1* in response to perception of flg22 (He et al., 2006). In light of these recent results, the function(s) of AvrPtoB in promoting virulence are not clear. It is possible that AvrPtoB has multiple functions in the same plant host conferred by different regions of the protein. Alternatively, AvrPtoB may serve different functions in distinct host plants. Identification of specific host targets for AvrPtoB will likely help to sort out the exact virulence role of this interesting type III effector.

Phytotoxins of plant pathogenic bacteria

In addition to type III effectors, many plant pathogenic bacteria also produce one or more toxins that play a role in promoting virulence on host plants. The various *Pseudomonas syringae* pathovars produce a number of different toxins including coronatine produced by pv tomato and other pathovars, phaseolotoxin produced by pv phaseolicola, tabtoxin of pv tabaci, and syringomycin and syringopeptin of pv *syringae* (Bender et al., 1999). In many cases, phytotoxins have been demonstrated to contribute to the pathogenicity of the toxin-producing strains, however, relatively little is known about the specific mechanisms by which these toxins contribute to virulence.

The phytotoxin coronatine (COR) is produced by at least 5 different pathovars of *P. syringae* including pvs tomato, glycinea, atropurpurea, maculicola, and morsprunorum

(Bender et al., 1999). COR is a polyketide toxin consisting of two distinct structural components, coronofacic acid and coronamic acid (Ichihara et al., 1977). Structurally, COR is closely related to the plant hormone jasmonic acid (JA) and plants such as the COR-insensitive *Arabidopsis coi1* mutants are also resistant to the effects of JA, prompting researchers to hypothesize that COR may act as a molecular mimic of JA (Feys et al., 1994; Greulich et al., 1995; Koda et al., 1996; Weiler et al., 1994). It was observed that a COR-deficient mutant of *Pst* DC3000 could not cause disease when inoculated onto the surface of *Arabidopsis* leaves, but was capable of causing disease symptoms and multiplying to wild-type levels when infiltrated directly into the leaf intercellular spaces (Mittal and Davis, 1995). These results led the authors to speculate that COR may suppress an uncharacterized early defense response in *Arabidopsis*. In chapter 3 I will present evidence that this early defense response is bacterium-induced stomatal closure and that the primary virulence role for COR is the suppression of this stomatal defense response.

The modes of action of several other *P. syringae* toxins have also been determined, however, in most cases the precise mechanisms by which these toxins promote virulence are not understood. For example, phaseolotoxin produced by pathovars phaseolicola and actinidiae has been shown to inhibit ornithine carbamoyl transferase (OCTase). OCTase is involved in the urea cycle in both prokaryotes and eukaryotes and catalyzes the conversion of ornithine and carbamoyl phosphate to citrulline. *P. syringae* pathovars that produce phaseolotoxin also produce resistant OCTase isoforms, rendering them insensitive to the effects of the toxin. The result of inhibition of OCTase is the accumulation of ornithine and a deficiency of intracellular arginine pools and leads to

chlorosis in plant tissues. However, it is not clear how phaseolotoxin contributes to virulence. It has been speculated that phaseolotoxin may actually be involved in direct competition with other epiphytic bacteria for niches in the phyllosphere (Volksch and Weingart, 1998). Syringomycins and syringopeptin are lipodepsipeptide toxins produced by pathovar syringae. These toxins contribute significantly to virulence and have been shown to cause necrosis in plant tissues (Hutchison and Gross, 1997; Quigley et al., 1993). The function of this class of toxins seems to be related to their ability to form pores in the host plasma membrane (Hutchison and Gross, 1997). Pore formation in plant cell membranes may be a mechanism for *P. syringae* pv syringae to acquire nutrients from the host.

Plant defense responses

Although some bacteria have evolved mechanisms to successfully colonize plants and cause disease, most bacteria are unable to infect plants. In addition, most pathovars of phytopathogenic bacteria are only able to infect a very limited number of plant hosts and are non-pathogenic on other plant species or cultivars. Therefore, plants are able to successfully defend themselves against the majority of bacteria to which they are exposed. Plants employ a number of mechanisms to successfully avoid infection by potentially pathogenic bacteria including preformed physical barriers such as the leaf cuticle and cell walls, a suite of basal defense responses including deposition of cell wall reinforcements and synthesis of antimicrobial compounds such as phytoalexins, a form of programmed cell death referred to as the hypersensitive response (HR), and a systemic immunity known as systemic acquired resistance (SAR). During the last 15 years,

significant progress has been made toward understanding the perception and signaling events associated with elicitation of the HR. In contrast, basal defenses have received less attention, and only within the last few years has the importance of basal defenses been recognized. It is likely that both basal defenses and the HR contribute to long-term, durable resistance of plants to a broad spectrum of potential pathogens. Therefore, a thorough understanding of both responses, including perception events, signal transduction, gene expression changes, and ultimately the biochemical processes leading to restriction of multiplication, will be required to develop strategies for engineering durable resistance to agronomically important plant pathogens.

Plant basal defenses

Basal defenses in plants are analogous to innate immunity in animals and insects (Staskawicz et al., 2001). Innate immunity is an evolutionarily ancient form of resistance to microbes and is characterized by the recognition of conserved microbial molecules referred to as pathogen-associated molecular patterns (PAMPs). PAMPs generally contribute to a function that is critical to the lifestyle of the organism and thus, are indispensable and are generally well conserved across a wide range of microbes (Nurnberger et al., 2004). A major breakthrough in the understanding of innate immunity in plants came when plants were found to perceive bacterial flagellin, the proteinaceous subunit that is the main component of the bacterial flagellum (Felix et al., 1999). Perception of flagellin or a 22 amino acid highly elicitor-active peptide, flg22, derived from a well-conserved domain of flagellin, was found to inhibit growth of *Arabidopsis* seedlings and elicit callose deposition in leaf tissue (Gómez-Gómez et al., 1999).

Additionally, treatment of suspension-cultured tobacco cells with flg22 led to alkalinization of the culture medium, suggesting that flg22 triggers production of reactive oxygen species (ROS). The receptor responsible for perception of flagellin by *Arabidopsis* was identified as an LRR receptor-like kinase and named FLS2 (Gómez-Gómez et al., 2000). It was subsequently discovered that signaling mediated by FLS2 proceeds through a mitogen-activated protein kinase (MAPK) cascade involving the *Arabidopsis* MAPKs *At*MPK3 and *At*MPK6 and leads to the rapid transcriptional induction of a number of genes including those encoding the WRKY transcription factors *WRKY22* and *WRKY29* (Asai et al., 2002). *Arabidopsis fls2* mutants lacking the flagellin receptor were found to be slightly more susceptible to *Pst* DC3000 when the bacteria were inoculated on the leaf surface, but not when bacteria were infiltrated directly into the leaf apoplast, suggesting that flagellin perception may be important for triggering a defense active on the leaf surface (Zipfel et al., 2004). Expression profiling studies of *Arabidopsis* seedlings or cell cultures treated with flg22 identified a number of transcriptional changes associated with flagellin perception including the induction of genes whose products are associated with signal transduction, transcriptional regulation, and defense (Navarro et al., 2004; Zipfel et al., 2004). In addition to flagellin, *Arabidopsis* has subsequently been shown to perceive several other bacterial PAMPs including elongation factor Tu (EF-Tu) and lipopolysaccharides (LPS) and the receptor mediating perception of EF-Tu has been identified (Kunze et al., 2004; Zeidler et al., 2004; Zipfel et al., 2006).

The above studies and others have shed significant light on PAMP perception and signaling in plants, however, the subsequent defense responses and their role in

restricting bacterial infection are still poorly understood. Two relatively well-characterized aspects of basal defenses are the deposition of cell wall appositions called papillae and the production of phytoalexins. However, the role of papillae and phytoalexins in restricting bacterial multiplication remains unclear.

The formation of papillae in response to a pathogen was first observed over 100 years ago (reviewed in Aist, 1976). The majority of the literature concerning papilla formation in response to pathogens describes papilla deposition in response to attempted penetration by fungal pathogens. However, formation of papillae or papilla-like structures has also been observed in plants in response to bacteria, viruses, and nematodes (Bestwick et al., 1995; Allison and Shalla, 1974; Riggs et al., 1973). The most prevalent hypothesis for the function of papillae in response to fungal attack is that papillae serve as a structural barrier allowing the plant cell to resist mechanical or enzymatic penetration by the invading fungus. Rapid formation of papillae has been correlated with enhanced resistance to fungal penetration, whereas, delayed papilla formation has been correlated with an increase in penetration frequency (Bayles et al., 1990; Collins et al., 2003). The structural components of papillae seem to vary between plant species, but compounds commonly found associated with papillae include: callose; lignin; phenolics; reactive oxygen species (ROS); peroxidases; and hydroxyproline-rich glycoproteins (Aist, 1976; McLusky et al., 1999). It is thought that these components are cross-linked, possibly through the action of ROS, into a dense, insoluble matrix that serves to strengthen the cell wall locally at the site of interaction with the invading pathogen.

Although the papilla response has been studied for more than a century, very little is known about the elaboration of this response at the molecular level. Studies with

various pharmacological inhibitors suggest that transcription, translation, protein glycosylation, and microfilament polymerization are required for normal papilla deposition (Skalamera et al., 1997). The *Arabidopsis* callose synthase encoded by the *GSL5* gene was shown to be required for deposition of callose in papillae and for wound-induced callose deposition (Nishimura et al., 2003; Jacobs et al., 2003). However, *gsl5* mutant *Arabidopsis* plants were able to form papillae, which, although lacking callose, did not differ significantly in size and shape from papillae formed by wild type plants. Interestingly, *gsl5* mutants showed enhanced resistance to several normally virulent species of powdery mildew. This enhanced resistance was found to be dependent on a functional salicylic acid (SA) defense signaling pathway, as double mutants impaired in SA defense signaling regained susceptibility. Additionally, the *Arabidopsis* SNARE (soluble NSF attachment protein receptor) protein PEN1 (also known as SYP121) was found to be associated with deposition of papillae in response to perception of non-host fungal pathogens (Assaad et al., 2004; Collins et al., 2003; Lipka et al., 2005).

Deposition of papillae has been observed in response to strains of several plant-associated bacteria including *Pseudomonas syringae* and *Xanthomonas campestris* in a variety of plant species such as lettuce, pepper, bean, and *Arabidopsis* (Brown et al., 1995; Bestwick et al., 1995; Brown et al., 1998). Whether components mediating the deposition of papillae in response to fungi, such as PEN1, are also involved in formation of papillae in response to bacteria is currently not known. Of particular significance is the observation that saprophytic strains, avirulent strains and non-pathogenic mutants such as *hrp* mutants elicit deposition of papillae, whereas pathogenic strains do not elicit this response (Brown et al., 1995). This observation suggests that pathogenic strains have the

ability to suppress this defense response, and that this suppression is dependent on a functional type III secretion system, implicating the involvement of type III-secreted effector proteins in suppressing this response. As discussed previously, several type III effectors have already been implicated in suppressing this defense response. In chapter 4, I will present evidence that the *Pst* DC3000 effector HopD2 can suppress deposition of papillae.

Gene-for-gene resistance

In addition to recognition of PAMPs, plants have also evolved the ability to detect the presence of virulence factors such as type III effectors secreted directly into the host cell. Recognition of specific type III effectors is mediated by host resistance (R) proteins, most of which belong to a family of leucine-rich repeat (LRR) and nucleotide-binding (NB) domain containing proteins with either a coiled-coil (CC) or Toll-interleukin-1 receptor (TIR) domain at their N terminus. Activation of R proteins leads to a defense known as the hypersensitive response (HR), which involves the death of the affected cell and is thought to be a form of programmed cell death. The requirement of a specific single gene from the pathogen (generally referred to as an avirulence or *avr* gene) and a specific single gene from the host (the *R* gene) to confer resistance led H.H. Flor to describe this phenomenon as gene-for-gene resistance over 50 years ago (Flor, 1955).

The most straightforward and simple model for recognition of bacterial virulence proteins and activation of plant R proteins is one in which the R protein acts as a receptor for the bacterial virulence protein and, through direct interaction, becomes activated and subsequently promotes initiation of the HR. In the case of several R-avr genetic

interactions, a direct physical interaction between R and Avr proteins has been demonstrated experimentally. Specific examples include recognition of the AVR-Pita protein from the rice blast fungus *Magnaporthe grisea* by the corresponding rice R protein Pi-Ta (Jia et al., 2000); recognition of Avr proteins of the flax rust (*Melampsora lini*) *AvrL567* locus by the flax (*Linum usitatissimum*) R proteins L5, L6, and L7 (Dodds et al., 2006); and recognition of the PopP2 Avr protein of *Ralstonia solanacearum* by the *Arabidopsis* RRS1-R protein (Deslandes et al., 2003). However, for many other R-Avr pairs, a direct interaction has not been demonstrated and strong experimental evidence instead points to an alternative model referred to as the "guard hypothesis." The guard hypothesis proposes that R proteins act as sentries within the plant cell to guard virulence targets of bacterial effector proteins by detecting the activity of the virulence proteins or the perturbation of the host target, thus indirectly detecting the virulence factors (Nimchuck et al., 2001). Recently, the guard hypothesis has gained significant experimental support. The best characterized example of indirect recognition of bacterial effector proteins is that of the *Arabidopsis* protein RIN4 (RPM1-interacting protein 4). Activation of at least two *Arabidopsis* R proteins, RPS2 and RPM1, has been shown to be associated with modification of RIN4. The R protein RPM1 is activated in response to phosphorylation of RIN4 by either of two unrelated *P. syringae* effectors, AvrB or AvrRpm1 (Mackey et al., 2002). RPS2, on the other hand, is activated by the absence of RIN4 caused by its proteolytic degradation by the *P. syringae* effector AvrRpt2 (Axtell and Staskawicz, 2003; Mackey et al., 2003). These results suggest that RIN4 may be an important virulence target of these effectors, however, the precise role of RIN4 in the plant response to bacteria is still not known. Recent work suggests that RIN4 may play a

role in the plant basal defense system activated in response to PAMP perception (Kim et al., 2005). Another example of an *Arabidopsis* R protein guarding a potential host virulence target is that of RPS5. Similar to RPS2, RPS5 detects the proteolytic cleavage of a host protein, the PBS1 kinase, by the *P. syringae* type III effector AvrPphB (Shao et al., 2003). The role of PBS1 as a host virulence target is also currently unknown.

Project summary and rationale

One of the most important long-term goals underlying the study of plant-microbe interactions is to develop new strategies for reducing the impact of microbial pathogens on agricultural production. In order to successfully formulate and institute such strategies, it is necessary to understand the mechanisms employed successfully by plants to defend themselves against most potential pathogens and the strategies employed by successful pathogens to overcome these defenses. While gene-for-gene resistance and defenses associated with the hypersensitive response have received significant attention over the past two decades, resistance conferred by a single R gene is not likely to be durable and long-lasting due to significant selection pressure placed on the pathogen. Emerging evidence suggests that stable and durable resistance is likely conferred by a combination of basal defenses associated with PAMP perception and R gene-mediated defenses. In comparison to R gene-mediated defenses, relatively little was known about plant basal defenses at the onset of this study. One of the major goals of this work was to improve our understanding of plant basal defenses associated with perception of PAMPs.

Successful pathogens have evolved mechanisms to overcome plant defenses. To achieve effective pathogen resistance in crop plants, it will also be important to understand the ways in which pathogens are able to block, break down, or circumvent normally effective host defenses. A second goal of this work was to investigate mechanisms by which the bacterial pathogen *Pst* DC3000 overcomes the basal defenses of the host plant *Arabidopsis thaliana.* It was known that two major virulence factors, COR and TTSS effectors, contribute to the ability of *Pst* DC3000 to overcome host defenses. However, the mechanism of action of COR or any specific effector protein was not known at the onset of this study. The work described here investigates aspects of both virulence systems.

Chapter 2 describes microarray analyses of *Arabidopsis* gene expression changes associated with activation of basal defenses and with the activities of each of the two virulence factors of *Pst* DC3000, COR and the TTSS. Prior to the initiation of this study expression profiling of the interaction of *Pst* DC3000 with *Arabidopsis* had been reported (Tao et al., 2003). This study addressed the compatible interaction between *Pst* DC3000 and *Arabidopsis* and the incompatible interaction associated with *Pst* DC3000 carrying an avirulence gene. However, gene expression changes associated with the activation of basal defense were not addressed in this study. Additionally, the specific roles of COR and the TTSS in modulating gene expression also were not addressed. To identify gene expression changes associated with the activation of basal defenses, I analyzed the expression profiles of *Arabidopsis* plants inoculated with non-pathogenic *hrp* mutants of *Pst* DC3000 or with the human pathogen *E. coli* O157:H7. Additionally, I sought to determine the role of flagellin perception in mediating the gene expression changes

associated with basal defenses. At the onset of this study, flagellin was the only bacterial PAMP known to be perceived by plants and it was conceivable that perception of flagellin may contribute specifically and uniquely to gene expression changes associated with the activation of basal defenses. To address this question, I compared the expression profiles of *Arabidopsis* plants inoculated with *fliC* mutant bacteria lacking flagella to those of plants inoculated with the wild-type, flagella producing strains. To address the specific roles of TTSS effectors and COR in modulating host gene expression, I collaborated with Dr. Roger Thilmony to analyze the expression profiles of plants inoculated with defined *Pst* DC3000 mutants defective specifically in COR biosynthesis or secretion of TTSS effectors.

Chapter 3 reports the discovery of a novel component of plant basal defenses, closure of stomata. At the time this study was initiated, it was known that mutants of *Pst* DC3000 defective in COR biosynthesis were significantly compromised in virulence when inoculated onto the surface of host leaves, but were normally virulent when infiltrated directly into the leaf apoplast, suggesting the possible existence of a defense mechanism active on the leaf surface and a role for COR in overcoming this hypothetical defense (Mittal and Davis, 1995). Additionally, *fls2* mutant *Arabidopsis* plants lacking the flagellin receptor were more susceptible to *Pst* DC3000 when inoculated on the leaf surface, but not when the bacteria were infiltrated directly into the leaf apoplast (Zipfel et al., 2004) This result suggested that flagellin perception may contribute to a defense mechanism active on the leaf surface. These results led us to hypothesize that stomata may play a role in basal defenses of plants against bacteria. To address this hypothesis, I collaborated with Dr. Maeli Melotto to investigate the behavior of plant stomata in

response to perception of bacteria and PAMPs and to investigate a potential role for COR in modulating stomatal behavior.

Chapter 4 describes my research on the virulence function of a specific *Pst* DC3000 type III effector, HopD2. HopD2 shares some sequence similarity with protein tyrosine phosphatases, including a conserved active domain (Bretz et al., 2003; Espinoza et al., 2003). This potential enzyme activity provided a starting point for the study of this type III effector. To study the function of HopD2 in *Arabidopsis,* I created transgenic plant lines expressing wild-type HopD2 or a phosphatase-inactive mutant derivative under the control of an inducible promotor. Transgenic *Arabidopsis* plants expressing HopD2 allow enhanced multiplication of normally non-pathogenic *hrp* mutants of *Pst* DC3000. Additionally, these transgenic plants display reduced papillae-associated callose deposition in response to non-pathogens, suggesting that HopD2 may play a role in blocking basal defenses. This defense-suppressing activity of HopD2 is dependent on its tyrosine phosphatase activity as plants expressing the catalytically inactive mutant protein did not show these phenotypes. To investigate the possibility that HopD2 might exert its virulence function by inactivating *Arabidopsis* MAP kinases known to be involved in PAMP-triggered signaling leading to activation of basal defense, I collaborated with Dr. Shuqun Zhang to assess the activation of *At*MPK3 and *At*MPK6 in the HopD2 transgenic plants. I performed microarray and lipid profiling analyses to address the phosphatase-specific changes associated with HopD2 in host plants. These experiments provide evidence of a role for HopD2 in suppressing basal defenses in the host plant *Arabidopsis thaliana.*

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Chapter 2

Genome-wide transcriptional analysis of the *Arabidopsis thaliana* interaction with the plant pathogen *Pseudomonas syringae* pv. *tomato* DC3000 and the human pathogen *Escherichia coli* O157:H7.

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Abstract

Pseudomonas syringae pv. tomato DC3000 (*Pst)* is a virulent pathogen, which causes disease on tomato and *Arabidopsis*. The type III secretion system (TTSS) plays a key role in pathogenesis by translocating virulence effectors from the bacteria into the plant host cell, while the phytotoxin coronatine (COR) contributes to virulence and disease symptom development. Recent studies suggest that both the TTSS and COR are involved in the suppression of host basal defenses. However, little is known about the interplay between the host gene expression changes associated with basal defenses and the virulence activities of the TTSS and COR during infection. In this study, we used the Affymetrix full genome chip to determine the *Arabidopsis* transcriptome associated with basal defense to *Pst* DC3000 *hrp* mutants and the human pathogenic bacterium *Escherichia coli* O157:H7. We then used *Pst* DC3000 virulence mutants to characterize *Arabidopsis* transcriptional responses to the action of *hrp*-regulated virulence factors (e.g., TTSS and COR) during bacterial infection. Additionally, we used bacterial *fliC* mutants to assess the role of the PAMP flagellin in induction of basal defense-associated transcriptional responses. In total, our global gene expression analysis identified 2,800 *Arabidopsis* genes that are reproducibly regulated in response to bacterial pathogen inoculation. Regulation of these genes provides a molecular signature for *Arabidopsis* basal defense to plant and human pathogenic bacteria, and illustrates both common and distinct global virulence effects of the TTSS, COR, and possibly other *hrp*-regulated virulence factors during *Pst* DC3000 infection.

Introduction

Pseudomonas syringae strains collectively infect hundreds of taxonomically diverse plant species and cause disease symptoms ranging from leaf spots to stem cankers (Hirano and Upper, 2000). *P. syringae* pv. tomato *(Pst)* strain DC3000 used in this study cause necrotic lesions that are often surrounded by chlorotic halos in susceptible tomato and *Arabidopsis* plants (Katagiri et al., 2002; Ma et al., 1991; Whalen et al., 1991). To successfully colonize plants, *P. syringae* strains have evolved a variety of virulence factors to subvert host defenses or to obtain nutrients. One common virulence mechanism is the *hrp*-gene-encoded type III protein secretion system (TTSS; He et al., 2004; Jin et al., 2003). The TTSS is used by *P. syringae* to inject > 40 virulence effector proteins into the host cell (Chang et al., 2005; Collmer et al., 2002; Greenberg and Vinatzer, 2003; Nomura and He, 2005). Different *P. syringae* strains also produce a variety of phytotoxins (Bender et al., 1999). Athough phytotoxins are generally not required for bacterial pathogenicity, they do enhance pathogen virulence in host plants (Bender et al., 1999). *Pst* DC3000, for example, produces a polyketide toxin, coronatine (COR), which is required for full virulence in *Arabidopsis* and tomato plants (Brooks et al., 2004; Ma et al., 1991; Mittal and Davis, 1995; Zhao et al., 2003). Emerging evidence suggests that the production of the TTSS and COR is coordinately regulated in *Pst* DC3000 (Fouts et al., 2002; Penaloza-Vazquez *et al.*, 2000) so that mutations in the regulatory *hrp* genes (e.g., *hrpS*, *hrpL*, and *hrpA*) could affect the expression of both the TTSS and COR biosynthetic enzymes.

How TTSS effectors promote bacterial pathogenesis is poorly understood and remains an outstanding question in biology. In a previous study, we conducted

microarray analysis examining the effects of wild-type *Pst* DC3000 and its *hrpS* mutant (defective in hrp regulon expression producing a nonfunctional TTSS), *Pst* DC3118 COR$^-$ mutant (defective in COR toxin production), or the COR$^-$*hrpS* double mutant (defective in the TTSS, COR toxin and potentially other *hrp* regulon controlled virulence factors) on the expression of about 7,200 *Arabidopsis* genes in the compatible *Arabidopsis-Pst* DC3000 interaction (Hauck et al., 2003). That study revealed that the TTSS is involved in biased suppression of *Arabidopsis* genes that encode putatively secreted proteins (Hauck et al., 2003). The TTSS-mediated suppression of these *Arabidopsis* genes is correlated with the ability of the TTSS and its effector AvrPto to suppress callose deposition (Hauck et al., 2003) and presumably other host responses that are associated with plant basal defense to *hrp* mutant bacteria (Bestwick et al., 1995) or to "pathogen-associated molecular patterns" (PAMPs) such as bacterial flagellin or EF-Tu (Gomez-Gomez et al., 1999; Kunze et al., 2004). However, it remains to be determined what role Type III secreted effectors play in the transcriptional regulation of other host physiological processes important for pathogenesis.

The molecular mechanism by which COR facilitates *Pst* DC3000 virulence is also not well understood. COR shows a remarkable structural similarity to the plant hormone methyl jasmonic acid (MeJA), which is involved in defense and wound response signaling (Bender et al., 1999; Benedetti et al., 1995; Feys et al., 1994; Uppalapati et al., 2005). Identification of the *Arabidopsis* JA/COR perception mutant *coi1*, which is insensitive to both MeJA and COR, further suggests a similar mode of action of COR and MeJA (Xie et al., 1998; Kloek et al., 2001). The *COI1* gene encodes a subunit of an SCF^{COI1} complex (E3 type ubiquitin ligase) presumably involved in proteasome-mediated

protein degradation (Devoto et al., 2002; Turner et al., 2002; Xu et al., 2002). Kloek et al., (2001) and Zhao et al., (2003) observed hyperexpression of a defense gene, *PR-1,* in *P. syringae*-inoculated *coi1 Arabidopsis* and tomato plants, respectively. Resistance in *coi1 Arabidopsis* plants is apparently caused by elevated SA-mediated defense, because a *coi1 Arabidopsis* mutant expressing the *nahG* gene, which encodes an SA-degrading enzyme, fails to restrict bacterial growth (Kloek et al., 2001). These observations suggest that COR is involved in the suppression of an SA-dependent host defense. However, the precise nature of the COR-targeted host defense and the contribution of COR to global host gene expression during bacterial infection have not yet been determined.

Recently, gene expression studies were performed using *Arabidopsis* suspension-cultured cells or seedlings treated with purified flagellin peptide (flg22), providing the first glimpse into the global gene expression changes during basal defense (Navarro et al., 2004; Zipfel et al., 2004). Whether the same set of *Arabidopsis* genes are regulated by flagellin and other PAMPs during bacterial infection of intact plant leaves remains to be determined. Recent studies suggest that the TTSS and COR have overlapping functions in the suppression of some PR genes and induction of some wounding response genes (He et al., 2004; Zhao et al., 2003). The extent of such overlap, however, has not yet been determined at the whole genome scale. Nor is it known how many of the PAMP-induced genes are subjected to transcriptional suppression by the TTSS and/or COR, respectively.

In this study, we conducted genome-wide gene expression analysis of *Arabidopsis* plants treated with defined *Pst* DC3000 and *E. coli* O157:H7 mutants to gain molecular insights into 1) the transcriptional changes associated with basal defense to live bacteria, which carry multiple PAMPs, 2) the contribution of flagellin to the regulation of the basal

defense transcriptome during infection, and 3) the global effects of *hrp*-regulated virulence factors, primarily the TTSS and COR, on the basal defense transcriptome and other host physiological processes. Previous global gene expression analyses have been performed in resistant *Arabidopsis-Pst* interactions (Tao et al., 2003) and other defense-related treatments (reviewed in Wan et al., 2002). We have conducted genome-wide analyses aimed at understanding the transcriptional responses of *Arabidopsis* plants to PAMPs, TTSS effectors, and COR from the same bacterium during infection. The results should complement other studies and provide a useful resource for future study of *Pst* DC3000 pathogenesis in plants.

Experimental Procedures

Plant Growth, Bacterial Strains and Bacteria Enumeration.

Arabidopsis thaliana accession Col-0 *gl1* plants were grown in soil in growth chambers with a day/night cycle of 12 h/12 h, a light intensity of 100 μE, and a constant temperature of 20°C. Four-week-old plants were used for experiments. Each biological replicate contains plants that were grown, inoculated and harvested side-by-side in the growth chamber entirely independent of the other replicates (typically separated in time by several weeks or months). Bacteria for inoculation were grown in low-salt Luria–Bertani broth (Katagiri et al., 2002) to the mid-logarithmic phase at 30°C. Bacterial cultures were centrifuged to recover bacteria, which were resuspended in sterile water to a final OD_{600} of 0.002 (equivalent to $1x10^6$ bacteria/mL), 0.1 (equivalent to $5x10^7$ bacteria/mL) or 0.2 (equivalent to $1x10^8$ bacteria/mL). Dilution plating was used to

confirm the number of bacteria present in the inoculum. The mock inoculum was water containing 0.004% Silwet L-77 surfactant (Osi Specialties, Friendship, WV). Fully expanded leaves were vacuum-infiltrated with bacterial suspensions, and *in planta* bacteria were enumerated as described by Katagiri et al., 2002. The mean values of the bacterial populations are plotted with the standard deviation displayed as error. The leaves of 10-15 plants grown in several different pots were harvested for each RNA sample used for microarray and RNA blot analysis. The regulation/secretion-defective *Pst hrpS* mutant, the *Pst* DC3118 COR$^-$*hrpS* double mutant, the secretion-defective *Pst hrpA* mutant, *E. coli* O157:H7 and the *E. coli* TUV86-2 *fliC* mutant have been previously described (Berin et al., 2002; Hauck et al., 2003; Hayashi et al., 2001; Yuan and He, 1996). Inoculation with *E. coli* strains was staggered with the 10^8 dataset such that replicate number 1 of inoculations with *E. coli* O157:H7 and TUV86-2 *fliC* was performed alongside replicate number 2 of the other 10^8 inoculations. To complete 3 replicates for each *E. coli* strain, we performed a final set of inoculations consisting of *E. coli* O157:H7, *E. coli* TUV86-2 *fliC*, and mock inoculum. Therefore, in the publicly available dataset in the NASCArrays database, the *E. coli* inoculations are annotated as replicates 2, 3, and 4 to correspond with the other 10^8 inoculation data to which they are directly compared.

RNA Isolation and Labeling

Total RNA was isolated from *Arabidopsis* leaves using the Promega (Madison, WI) RNAgents total RNA isolation system. The RNA concentration was determined by absorbance at 260 nm and its quality was evaluated by separation on 2% formaldehyde

denaturing agarose gels. The total RNA was further purified using Qiagen (Valencia, CA) RNeasy minicolumns. Biotinylated target complementary RNA (cRNA) was prepared from 16μg of total RNA using the procedure described by the Affymetrix (Santa Clara, CA). Briefly, a primer encoding a T7 RNA polymerase promoter sequence fused to (dT)24 (Genset Oligos, La Jolla, CA) was used to prime double-stranded cDNA synthesis using the Invitrogen (Carlsbad, CA) SuperScript II Reverse Transcriptase. The resulting cDNA was transcribed *in vitro* using the BioArray High-Yield RNA Transcript Labeling Kit (Enzo Biochem, New York, NY) in the presence of biotinylated UTP and CTP to produce biotinylated target cRNA.

Affymetrix GeneChip Hybridization and Data Collection

The labeled target cRNA was purified, fragmented, and hybridized to *Arabidopsis* ATH1 GeneChip arrays according to protocols provided by the manufacturer (Affymetrix) in a Hybridization Oven model 640 (Affymetrix). The GeneChips were washed and stained with streptavidin-phycoerythrin using a GeneChip Fluidics Station model 400 and then scanned with a Gene Array Scanner (Hewlett-Packard, Palo Alto, CA).

Affymetrix GeneChip data analysis

The gene expression data was normalized and analyzed using the Affymetrix Microarray Program Suite (MAS 5.0 statistical algorithms and the Data Mining Tool (version 2.0)). Output from all GeneChip hybridizations was scaled globally such that its average intensity (TGT value) was equal to an arbitrary target intensity of 500 allowing

comparisons between GeneChips. Data was then compared between sample chips from the same biological replicate producing a Signal Log_2 Ratio (SLR) calculated from the GeneChip fluorescence signal intensity data. The software was used to determine whether there was a genuine change in mRNA accumulation (Change Call, D for Decrease, I for Increase) and Change Call p-value. SLRs, Change Calls and p-values were determined for each bacterially inoculated sample compared with its corresponding mock control or bacterial mutant treated sample.

Six pair wise comparisons were made within the $1x10^6$ bacteria/mL dataset, 3 pair wise comparisons for the $5x10^7$ bacteria/mL dataset and 8 pairwise comparisons for the $1x10^8$ bacteria/mL dataset (see Table 1). We used Significance Analysis of Microarrays (SAM; Tusher et al. 2001) to identify significant genes based on their differential expression between our set of samples. The SAM analysis feature contained in the TIGR Microarray Experiment Viewer v3.1 (Saeed et al., 2003; http://www.tm4.org/mev.htmL) was used to conduct the analysis. The normalized signal log_2 ratios for each replicate of each comparison were used for the analysis. Each inoculation level dataset (7 HPI $1x10^8$, 10 HPI $5x10^7$ and 24 HPI $1x10^6$) was independently analyzed using the multiclass design, with the default settings. The delta value was set at level such that the median False Discovery Rate was less than 5%. This analysis identified a nonredundant list of 3,864 probesets. Microsoft (Redmond, WA) Access database management software was used to further filter and query the data. Reproducibly differentially expressed probe sets were selected from this list of 3,864, based on signal log_2 ratio of at least -1.0, a gene expression change call of D (decrease) and p-value >0.995 or signal log_2 ratio of at least 1.0, change call of I (increase) and p-value of <0.005 for all 3 biological replicates for at

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least one type of comparison. Probe sets that meet these rigorous selection criteria were further analyzed in detail. To our surprise, not a single probeset was reproducibly differentially regulated in the $1x10^6$ bacteria/mL *Pst* COR$^-$*hrpS* verses mock comparison (Table 1). This result illustrates the stringency of our selection criteria, because one would expect that a few probesets might be reproducibly differentially expressed just by chance. Our failure to identify *Pst* COR$^-$*hrpS* (PAMP) differentially regulated genes following inoculation with $1x10^6$ bacteria, suggest that the level of bacteria in this treatment is insufficient to elicit robust, detectable changes in host gene expression 24 HPI and supports our rationale to use higher doses of bacteria to identify PAMP-regulated gene expression in these comparisons.

Hierarchical clustering and viewing was performed using either the Cluster v3.0 and TreeView programs (Eisen et al., 1998; http://rana.lbl.gov/EisenSoftware.htm) or the TIGR Microarray Experiment Viewer v3.1. The hierarchical clusters displayed in Figures 1 and 2 were analyzed using the Microarray Experiment Viewer with the default similarity metric settings (Euclidean Distance) with the Complete Linkage Clustering method selected. MapMan v1.6.0 (Thimm et al., 2004; Usadel et al., 2005) was used for analysis of the functional classes and metabolic pathways impacted following bacterial inoculation. The 944 COR toxin regulated genes were identified from the $1x10^6$ bacteria/mL *Pst* DC3000 vs *Pst* COR$^-$ comparison, while the 791 *hrp*-regulated genes were identified from the $5x10^7$ bacteria/mL *Pst* COR$^-$ vs *Pst* COR$^-$*hrpS* comparison using the criteria listed above. The SLRs for the 3 biological replicates in the selected comparison were averaged and displayed using the MapMan software onto the Metabolism Overview, Secondary Metabolism and Photosynthesis displays. The

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Nottingham *Arabidopsis* Stock Centre (NASC) web site (http://nasc.nott.ac.uk/) contains the raw microarray data in a MIAME compliant format for all of the experiments described, reference NASCArrays-340.

RNA isolation and blot analysis

Total RNA was isolated from *Arabidopsis* leaves using the Promega (Madison, WI) RNAgents Total RNA isolation system. The RNA concentration was determined by absorbance at 260 nm and separated on 2% formaldehyde denaturing agarose gels. The RNA was blotted onto Amersham (Piscataway, NJ) Hybond N+ nylon membranes using 10xSSC and UV crosslinked using a Stratagene (La Jolla, CA) Statalinker using the autocrosslink setting. The cDNA sequences of 15 differentially expressed genes identified by microarray analysis were used for RNA blot hybridization. Fourteen of the sequences were derived from ESTs acquired from the *Arabidopsis* Biological Resource Center stock center. Upon receipt of the ESTs, they were sequenced to confirm their identity. The cDNA probe sequences were liberated from the cloning vector using restriction digestion or PCR amplification with vector primers. The following cDNA sequences were used for labeling and RNA blot hybridization: At3g16370 from EST 175O18T7, At1g12090 from EST 135D13T7, At2g38540 from EST 135H16T7, At1g29670 from EST 240F18T7, At2g19860 from EST E1C7T7, At1g03870 from EST 172M22T7, At3g16240 from EST 206H6T7, At1g33240 from EST 88L16XP, At2g41940 from EST 163F20T7, At5g61590 from EST 215C17T7, At1g72610 from EST 85C7T7, At2g10940 from EST 40D7T7, At5g24780 from EST 114D3XP, At1g52890 from EST 165P18T7, At1g43160 from EST 132C14T7, At5g26340 from

EST 181G24T7, At2g24850 from EST 245M18T7, At3g45140 from EST 304E6T7, At2g17500 from EST 178E2T7, At1g62660 from EST 145A20T7, At4g27410 from EST 99D19T7, At4g02380 from EST E5E10T7, At4g02940 from EST 251H23T7, At3g16390 from EST 215J18T7. A 1.15 kb cDNA sequence for *CLH1/CORI1* (At1g19670) was amplified with RT-PCR using the following primers: 5'-CAGAATTCAACACAACTCTTTAATTATC-3' and 5'-ATCTCGAGTAACAAATGTTTTGATCGAG-3'. The amplified product was cloned into the EcoRI and XhoI sites of pBluescript SK+ and sequenced . cDNA sequences from the above listed clones were labeled with ^{32}P-dCTP using the Stratagene Prime-It II Random Primer Labeling kit. RNA blot hybridizations were performed using Sigma (St. Louis, MO) PerfectHyb Plus hybridization buffer following the manufacturers protocol and then washed to 0.5x SCC and exposed to film.

Results and Discussion

Rationale and Experimental design

All the experiments described were conducted using the Affymetrix ATH1 GeneChip and four-week-old *Arabidopsis* leaves. For discovery of PAMP- TTSS- and COR-regulated genes, we used *Pst* DC3000, *Pst* DC3118 COR$^-$ mutant, and the *Pst* DC3118 COR$^-$*hrpS* double mutant bacteria, as described previously (Hauck et al., 2003). Because some of the strains have different growth potentials in plants, we used three different levels of bacterial inocula for gene expression profiling: low ($1x10^6$ bacteria/mL), moderately high ($5x10^7$ bacteria/mL) and high ($1x10^8$ bacteria/mL) (Table

2-1). At $1x10^6$ bacteria/mL, *Pst* DC3000 reliably causes disease within 3 to 4 days and is used in this and other laboratories as a standard disease assay (Katagiri et al., 2002). This inoculum level has also previously been used to identify hundreds of *Pst*-regulated genes (Hauck et al., 2003). Gene expression changes at this inoculum level were examined after a 24 hour infection period (Figure A-1). During this time period, *Pst* DC3000 and the *Pst* COR$^-$ mutant multiply similarly to levels 40-to-50-fold higher than that of the *Pst hrpA*, *hrpS* and COR$^-$*hrpS* mutants (data not shown). Therefore, to minimize the effects the different bacterial population numbers have on host gene expression, and to examine samples with early, synchronous responses, we also used inoculum concentrations of $1x10^8$ and $5x10^7$ bacteria/mL. In these cases, the tissues could be harvested earlier (at 7 hours and 10 hours after inoculation respectively) when development of basal defense-associated papillae is occurring (Hauck et al., 2003). At the inocula and time points chosen for various strains, high and comparable expression levels of a known PAMP-induced gene, *Flagellin-Induced Receptor Kinase 1* (*FRK1*), were observed (Figure A-2).

In total, we conducted eight pair wise comparisons for the $1x10^8$ dataset, three for the $5x10^7$ dataset, and six for the $1x10^6$ dataset. A summary of the number of genes that passed our selection criteria (see Experimental Procedures) and were reproducibly differentially regulated in all 3 biological replicates for each comparison is shown in Table 2-1. These comparisons identified genes that are responsive to bacterial PAMPS of *Pst hrp* mutants and *E. coli* O157:H7 as well as those that are regulated by *Pst* DC3000 TTSS effectors and/or COR toxin (Table 2-1). Our global transcriptional profiling identified 2800 genes that were reproducibly differentially regulated.

Table 2-1: *Arabidopsis* genes reproducibly regulated by bacterial inoculation.

Comparison	Treatment[a]	Repressed Genes[b]	Induced Genes[b]	Total	Expression Pattern Identified
1. *Pst hrpA* vs. mock	10^8, 7 hrs	224	253	477	PAMP regulation
2. *Pst hrpA fliC* vs. mock	10^8, 7 hrs	5	95	100	PAMP regulation
3. *Pst hrpA* vs. *Pst hrpA fliC*	10^8, 7 hrs	11	9	20	Flagellin-specific regulation
4. *E. coli* O157:H7 vs. mock	10^8, 7 hrs	96	186	282	PAMP regulation
5. *E. coli* TUV-86-2 *fliC* vs. mock	10^8, 7 hrs	240	218	458	PAMP regulation
6. *E. coli* O157:H7 vs. *E. coli* TUV-86-2 *fliC*	10^8, 7 hrs	0	0	0	Flagellin-specific regulation
7. *Pst* DC3000 vs. mock	10^8, 7 hrs	901	495	1396	*Pseudomonas* regulation
8. *Pst* DC3000 vs. *Pst hrpA*	10^8, 7 hrs	904	415	1319	*Pst* virulence factor regulation
		1270	**797**	**1901**	**Non-redundant Totals**
9. *Pst* COR$^-$*hrpS* vs. mock	$5x10^7$, 10 hrs	197	154	351	PAMP regulation
10. *Pst* COR$^-$ vs. mock	$5x10^7$, 10 hrs	829	399	1228	*Pseudomonas* regulation
11. *Pst* COR$^-$ vs. *Pst* COR-*hrpS*	$5x10^7$, 10 hrs	516	275	791	Type III effector regulation
		1018	**549**	**1537**	**Non-redundant Totals**
12. *Pst* COR$^-$*hrpS* vs. mock	10^6, 24 hrs	0	0	0	PAMP regulation
13. *Pst* COR$^-$ vs. mock	10^6, 24 hrs	212	297	509	*Pseudomonas* regulation
14. *Pst* COR$^-$ vs. *Pst* COR$^-$*hrpS*	10^6, 24 hrs	175	253	428	Type III effector regulation
15. *Pst* DC3000 vs. *Pst* COR$^-$	10^6, 24 hrs	347	597	944	COR toxin regulation
16. *Pst* DC3000 vs. mock	10^6, 24 hrs	685	680	1365	*Pseudomonas* regulation
17. *Pst* DC3000 vs. *Pst* COR-*hrpS*	10^6, 24 hrs	777	700	1477	*Pst* virulence factor regulation
		955	**1018**	**1929**	**Non-redundant Totals**
		1724	**1430**	**2800**	**Overall Non-redundant Totals**

[a]Summary data from plants 7 hours after inoculation with $1x10^8$ bacteria/mL (rows 1-8), 10 hours after inoculation with $5x10^7$ bacteria/mL (rows 9-11), and 24 hours after inoculation with $1x10^6$ bacteria/mL *Pst* bacteria (rows 12-17) are shown.
[b]Criteria for gene selection is described in the text.

PAMP-induced transcriptional changes

In order to identify the transcriptional changes resulting from PAMP perception, we inoculated *Arabidopsis* with three different bacteria: *Pst* DC3000 *hrpA* mutant, *Pst* DC3000 COR$^-$*hrpS* mutants, and human pathogenic *E. coli* O157:H7. The *Pst hrpA* and COR$^-$*hrpS* mutants lack the ability to secrete TTSS effectors into the host and are unable to multiply significantly in plant host leaves or produce disease symptoms. Furthermore, the COR-*hrpS* mutant is unable to produce COR toxin and similarly, the *hrpA* mutant is also likely defective in COR toxin production via a feedback mechanism. The *hrpA* mutant strain has repressed expression of the *hrpS* and *hrpL* genes, which globally control the expression of genes involved in the production of the TTSS, COR toxin, and possibly other virulence factors (Fouts et al., 2002; Wei et al., 2000). Therefore, in the *hrpA* and COR$^-$*hrpS* mutants, many, if not all, plant-specific virulence factors are not produced. *E. coli* O157:H7, a human pathogen, is also unable to multiply or cause any symptoms on *Arabidopsis* leaf tissue (data not shown) and is not expected to carry plant-specific virulence factors. We hypothesized that inclusion of these three bacterial strains in the analysis should give us a global view of common and distinct plant transcriptional responses through the perception of PAMPs displayed by human and plant pathogenic bacteria. To assess the contribution of flagellin perception in host gene regulation during bacterial infection, we examined *Arabidopsis* gene expression regulated by the *hrpA fliC* double mutant of *Pst* DC3000 (Hu et al., 2001) and a *fliC* mutant derivative of *E. coli* TUV86-2 (Gunzer et al., 1998).

We identified a set of 736 PAMP-responsive genes that were differentially regulated at least 2-fold in 3 biological replicates for at least 1 out of the following 3

treatments with non-pathogenic bacteria: *Pst hrpA* vs mock 10^8 bacteria/mL, *E. coli* O157:H7 vs mock 10^8 bacteria/mL, and *Pst* COR$^-$*hrpS* vs mock $5x10^7$ bacteria/mL. A tally of the number of *Arabidopsis* genes regulated in response to each of the three bacterial treatments is presented in Table 2-1. Of the 736 reproducibly regulated genes, 347 were induced and 389 were repressed. Because we are particularly interested in genes whose products may be involved in the induction or elaboration of basal defenses, we chose to focus our analysis primarily on the set of 347 PAMP-induced genes.

Hierarchical cluster analysis of PAMP-induced genes

To provide an overview of the regulation of the 347 PAMP-induced genes across our set of treatments and biological replicates and to visualize the regulation of these genes in response to inoculation with virulent *Pst* DC3000, we performed hierarchical clustering. The resulting cluster is displayed in Figure 2-1. The induction of *Arabidopsis* genes after inoculation with *E. coli* was strikingly similar to the induction elicited by the *Pst hrp* mutants. The correlation coefficients based on the PAMP induced genes for each of the $1x10^8$ and $5x10^7$ comparisons are shown in Table A-3. Although *Pst hrpA* inoculation resulted in the reproducible regulation of 195 more genes (that matched or exceeded our selection criteria) than inoculation with *E. coli* O157:H7 (Table 2-1), the expression profiles were remarkably similar for the two treatments as shown in Figure 2-1 and their correlation coefficient was 0.79. We are particularly interested in identifying genes whose products may play a role in the plant basal defense response. Virulent *Pst* DC3000 is able to overcome or evade this host basal defense response and may do so by repressing or blocking the induction of host genes whose products are involved in the

elaboration of these defenses. Therefore, we expected that some PAMP-induced genes would be either repressed or simply not reproducibly induced upon inoculation with *Pst* DC3000. The blue bar in Figure 2-1 illustrates a cluster of genes that exhibit an expression pattern consistent with a role in basal defense. A total of 201 of the 347 PAMP-induced genes were either repressed or not significantly regulated (average fold change of <1.5) in response to *Pst* DC3000 inoculation. We propose that these genes may have a role in the *Arabidopsis* basal defense response to bacteria.

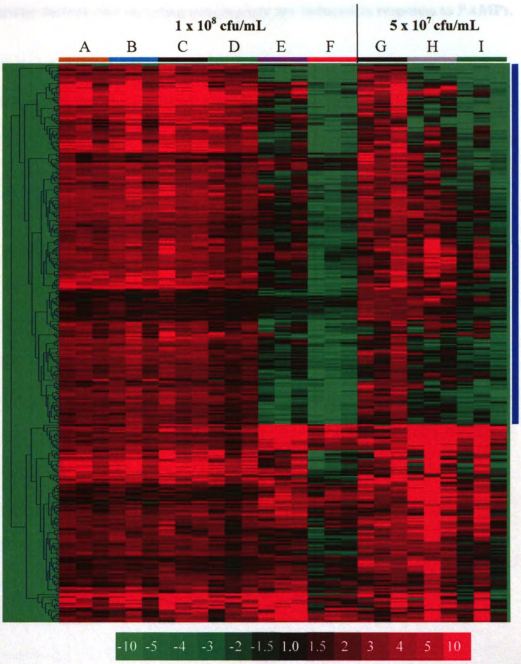

Figure 2-1: Expression profile of 347 PAMP-induced *Arabidopsis* genes. Treatments: A- *E. coli* v mock; B- *E. col fliC* v mock; C- *hrpA* v mock; D- *hrpAfliC* v mock; E- DC3000 v mock; F- DC3000 v *hrpA*; G- *corhrpS* v mock; H- *cor* v mock; I- *cor* v *corhrpS*. Genes highlighted by the blue bar on the right are of particular interest as these genes are induced by PAMP perception, but are not reproducibly induced or are repressed by *Pst* DC3000 and/or *Pst* COR$^-$.

Transcription factors and signaling components are induced in response to PAMPs.

Of the 347 PAMP-induced *Arabidopsis* genes, 86 encode known or predicted transcription factors and kinases. Thirty transcription factors are reproducibly induced in response to PAMP perception, 17 of which are repressed or not significantly regulated in response to DC3000 (Table 2-2A). Of these 30 PAMP-induced transcription factors, 12 belong to the WRKY family and 8 of these are induced by PAMPs but not by *Pst* DC3000. WRKY22 (At4g01250) was previously shown to participate in the FLS2-mediated signaling cascade triggered by perception of the synthetic flagellin peptide flg22 (Asai et al., 2002). Additionally, 6 of these 12 WRKY transcription factor genes (shaded gray in Table 2-2A) were shown to be induced in *Arabidopsis* seedlings in response to treatment with flg22 (Zipfel et al., 2004). These data suggest that WRKY family transcription factors play an important role in regulating the *Arabidopsis* basal response to bacteria. Determining the target genes regulated by these PAMP-induced WRKY transcription factors should further our understanding of the processes involved in the plant basal defense response.

In addition to WRKY transcription factors, 9 known or putative zinc finger transcription factors and 4 ERF/AP2 family transcription factors were induced by PAMPs. Two of the four ERF/AP2 family transcription factors and 6 of the 9 zinc finger proteins were previously shown to be induced by flg22 (Zipfel et al., 2004). Interestingly, the ethylene responsive *AtERF1* transcription factor was found to be induced by PAMPs. The plant hormone ethylene has been shown to play a role in plant defense against pathogens, and *AtERF1* was also found to be induced in response to flg22 treatment. However, our study shows that *AtERF1*, while reproducibly induced approximately 2-

fold by PAMP perception, is even more strongly induced (~10-fold) by the virulent pathogen *Pst* DC3000. These data suggest that ethylene signaling may play a role in the induction of basal defenses, but that the basal defense responses triggered by ethylene signaling are either insufficient to prevent *Pst* DC3000 pathogenesis or that *Pst* DC3000 blocks ethylene signaling or responses downstream of *AtERF1* induction. This example illustrates the value of determining the expression profile of these genes in response to *Pst* DC3000 in addition to PAMPs rather than simply identifying genes that are induced by PAMPs.

In addition to transcription factors, a number of other signaling components were induced by PAMP perception. Particularly abundant among PAMP-induced genes were genes known or predicted to encode kinases. Fifty-six known or putative kinases were induced by PAMP perception (Table A-1). Of these 56 PAMP-induced kinases, 31 were found to be induced by flg22 treatment (shaded gray in Table A-1). Receptor-like kinases, including lectin family kinases, and wall-associated kinase-like proteins are the most highly represented classes of kinases induced by PAMPs. Wall-associated kinases (WAKs) and WAK-like kinases (WAKLs) have recently been implicated in the *Arabidopsis* response to pathogens. *AtWak1* was shown to be induced by *P. syringae* and SA (He et al., 1998) and WAK1 was recently shown to interact with the glycine rich protein AtGRP3 and to phosphorylate oxygen evolving enhancer protein 2 (OEE2; Yang et al., 2003). Treatment of *Arabidopsis* protoplasts with purified AtGRP3 induced *PR-1* gene expression and inoculation of plants with avirulent *Pst* DC3000 enhanced WAK1-dependent phosphorylation of OEE2, suggesting that WAK1 and GRP3 may play a role in the response to pathogens. Additionally, the WAK-like kinase WAKL22 was recently

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found to be identical to the resistance gene *Rfo1* which confers to resistance to a number of isolates of the fungal pathogen *Fusarium oxysporum* (Diener and Ausubel, 2005). Although neither *WAK1* nor *WAKL22* were specifically induced by PAMPs in our study, the prevalence of WAK-like kinases among PAMP-induced genes taken together with the recent results highlighted above suggest that this class of proteins may play an important role in the plant response to pathogens.

Also of particular interest are a number of leucine-rich repeat (LRR)-containing receptor-like kinases. These receptor-like kinases may be candidates for Pattern Recognition Receptors (PRRs) in *Arabidopsis*. Although *Arabidopsis* is known to perceive a number of bacterial PAMPs including flagellin, LPS, and EF-Tu, only a single PRR for bacterial PAMPs has been identified (Felix et al., 1999; Kunze et al., 2004; Zeidler et al., 2004). FLS2, the PRR which mediates flagellin perception in *Arabidopsis,* is an LRR receptor kinase (Gómez-Gómez and Boller, 2000). Identifying additional PRRs in *Arabidopsis* is a high priority and should lead to new insights into the signaling pathways initiated by perception of bacterial PAMPs and the downstream basal defense responses that protect plants against a wide array of bacteria. These PAMP-induced LRR receptor-like kinases provide a starting point in the search for other *Arabidopsis* PRRs.

PAMPs induce genes that may be involved in secretion and cell wall modification.

One of the most well characterized aspects of plant basal defense against pathogens is modification of the cell wall. Plants challenged with bacteria or fungi typically develop cell wall appositions referred to as papillae at the point of interaction with the invading pathogen. Formation of papillae is characterized by the deposition of

electron dense materials such as phenolic compounds, callose, and cell wall proteins such as hydroxyproline-rich glycoproteins. Callose is synthesized at the plasma membrane, but the rest of the components are synthesized within the cell and must be delivered to the proper location at the wall for deposition into papillae. Because of this, components of the plant secretory system are likely to play an essential role in basal defense against pathogens. Consistent with the predicted importance of cell wall modification and polarized secretion to basal defense, we found that a number of PAMP-induced genes are potentially involved in these processes. Table 2-2B contains 28 PAMP-induced genes that may have a role in cell wall modification or polarized secretion. Among these 28 genes are a large number of genes encoding putative hydrolase enzymes that may potentially play a role in reorganizing the structure of the cell wall during papilla formation. Additionally, 4 genes encoding proline-rich and hydroxyproline-rich glycoproteins are induced by PAMPs. Seven putative peroxidases are induced which may be involved in the oxidative cross-linking of proteins and polymers into the papilla structure. The peroxidase *ATP24a* (At5g39580) was previously shown to be upregulated during the *RPP4* and *RPP8*-mediated resistance responses to the oomycete pathogen *Peronospora parasitica* (Eulgem et al., 2004). Consistent with a role in oxidative cross-linking in the cell wall, all of these peroxidases are predicted to enter the secretory pathway by TargetP (www.cbs.dtu.dk/services/TargetP).

Strikingly, 3 genes encoding potential components of the secretory pathway are upregulated by PAMP perception. Two members of the RabA family of small GTPases, *RABA1a* and *RABA1e*, were induced by PAMPs. Rab proteins are a class of small GTPases that also function in vesicle trafficking and regulate vesicle docking. Based on

work in mammalian and yeast systems, this class of Rabs is predicted to participate in post-golgi vesicle trafficking (Vernoud et al., 2003). Additionally, two RabA isoforms from pea were suggested to have a role in delivery of cell wall components to the plasma membrane (Nagano et al., 1995). Also, a member of the EXO70 family of proteins (At3g55150) was upregulated in response to PAMPs. EXO70 family proteins are subunits of the exocyst protein complex which is required for exocytosis in yeast and is also suggested to play a role in docking of exocytotic vesicles (Cole et al., 2005). The EXO70 family member was previously found to be upregulated in response to flg22 treatment (Zipfel et al., 2004), however, *RABA1a* and *RABA1e* represent novel PAMP-induced genes with functions related to secretion. Taken together, the upregulation of these genes supports a role for targeted vesicle trafficking in the *Arabidopsis* basal defense response to bacteria and identifies candidate secretory pathway components that may play a role in secretion of papilla components or other defense-related compounds.

Table 2-2: Selected PAMP-induced *Arabidopsis* genes. The average fold changes of each treatment compared to the mock control are shown. The *hrpA*, *E. coli* and DC3000 treatments were 7 hours post inoculation (HPI) with $1x10^8$ bacteria/mL, while the COR$^-$ *hrpS* treatment was at 10 HPI with $5x10^7$ bacteria/mL. **(A)** Transcription factors induced by PAMP perception. **(B)** Genes encoding proteins known or predicted to be involved in cell wall modification or secretion. Lines within tables denote cutoff for PAMP-induced genes that are repressed or not significantly induced (less than 1.5-fold) by *Pst* DC3000. Genes below the line are also induced by DC3000. Grey shading indicates genes previously identified as induced by the bacterial PAMP flagellin.

A

			Average Fold Change			
Array Element	AGI	Description	*hrpA*	COR$^-$*hrpS*	*E. coli*	DC3000
254231_at	At4g23810	AtWRKY53	2.09	2.64	2.00	-10.56
267385_at	At2g44380	CHP-rich zinc finger protein, putative	4.39	2.70	4.00	-6.06
246993_at	At5g67450	AZF1 Cys2/His2-type zinc finger protein 1	2.96	4.49	2.58	-4.70
254159_at	At4g24240	AtWRKY7	2.70	2.58	2.46	-3.25
255596_at	At4g01720	AtWRKY47	2.30	3.03	2.09	-2.58
255547_at	At4g01920	DC1 domain protein, similar to CHP-rich proteins	1.55	2.41	1.38	-2.46
260037_at	At1g68840	AtRAV2 AP2 domain-containing protein	1.95	1.18	2.14	-2.09
266010_at	At2g37430	putative C2H2-type zinc finger protein	3.73	3.17	3.65	-2.00
251745_at	At3g55980	zinc finger (CCCH-type) family protein	2.00	2.24	2.30	-1.59
267384_at	At2g44370	CHP-rich zinc finger protein, putative	21.61	7.64	11.58	-1.52
253535_at	At4g31550	AtWRKY11	2.46	1.74	2.64	-1.38
264616_at	At2g17740	CHP-rich zinc finger protein, putative	9.40	4.81	5.79	-1.38
255568_at	At4g01250	AtWRKY22	5.92	2.52	5.40	-1.07
263797_at	At2g24570	AtWRKY17	2.76	3.03	3.25	1.00
248306_at	At5g52830	AtWRKY27	4.49	2.46	4.59	1.10
260783_at	At1g06160	ERF subfamily ERF/AP2 transcription factor family.	3.91	1.32	1.38	1.41
249890_at	At5g22570	AtWRKY38	1.41	4.19	1.82	1.48
255753_at	At1g18570	AtMYB51 myb family transcription factor	2.46	2.14	2.09	1.55
257919_at	At3g23250	AtMYB15 myb family transcription factor	5.66	2.64	2.96	1.74
253485_at	At4g31800	AtWRKY18	1.78	3.17	1.62	2.00
250153_at	At5g15130	AtWRKY72	4.29	4.92	5.79	2.52
267588_at	At2g42060	CHP-rich zinc finger protein, putative	6.81	7.46	4.81	2.58
260432_at	At1g68150	AtWRKY9	7.13	3.65	4.39	3.25
261648_at	At1g27730	AtZAT10 salt-tolerance zinc finger protein	2.00	2.30	2.46	3.82
246214_at	At4g36990	AtHSF4 putative heat shock transcription factor	3.17	3.17	2.70	6.81
245252_at	At4g17500	AtERF-1 ethylene response factor 1	2.58	1.29	2.00	10.08
245976_at	At5g13080	AtWRKY75	7.29	3.17	3.73	11.58
252214_at	**At3g50260**	**DREB subfamily A-5 of ERF/AP2 transcription factor family.**	**1.87**	**2.58**	**1.78**	**13.30**
267140_at	**At2g38250**	**putative GT-1-like transcription factor**	**3.17**	**2.05**	**2.52**	**13.93**
247264_at	**At5g64530**	**no apical meristem (NAM) family protein**	**2.70**	**6.20**	**3.56**	**30.55**

Table 2-2 (cont'd).

B

Array Element	AGI	Description	Average Fold Change *hrpA*	COR⁻*hrpS*	*E. coli*	DC3000
255524_at	At4g02330	hypothetical protein, similar to pectinesterase	3.25	3.73	2.00	-5.40
245052_at	At2g26440	putative pectinesterase	2.14	2.96	1.74	-2.41
251895_at	At3g54420	AtEP3 class IV chitinase	2.70	4.92	3.03	-2.24
247954_at	At5g56870	beta-galactosidase, putative / lactase, putative	2.24	1.23	2.70	-2.19
255595_at	At4g01700	chitinase, putative, similar to peanut type II chitinase	3.03	6.06	2.64	-1.59
264669_at	At1g09630	AtRABA1a Rab small GTPase	1.70	2.24	1.52	-1.52
259173_at	At3g03640	AtGLUC beta glucosidase	1.95	3.91	1.74	-1.48
259443_at	At1g02360	chitinase, putative	3.25	4.49	3.17	-1.45
253099_at	At4g37530	peroxidase, putative	1.91	2.19	2.14	-1.05
251832_at	At3g55150	exocyst subunit EXO70 family protein	14.25	5.53	9.19	-1.02
254543_at	At4g19810	glycosyl hydrolase family 18; similar to chitinase/lysozyme	3.48	3.73	2.58	1.07
260556_at	At2g43620	glycosyl hydrolase family 19 (chitinase)	1.29	6.35	1.55	1.07
246532_at	At5g15870	glycosyl hydrolase family 81; similar to beta-glucan-elicitor receptor	2.35	2.46	2.30	1.10
265499_at	At2g15480	putative glucosyltransferase	2.24	1.91	1.62	1.15
254468_at	At4g20460	UDP-glucose 4-epimerase-like protein	3.73	2.30	3.25	1.18
247327_at	At5g64120	peroxidase, putative	3.03	3.40	3.56	1.87
254673_at	At4g18430	AtRABA1e RAB GTPase	4.81	4.49	2.76	1.70
254262_at	At4g23470	hydroxyproline-rich glycoprotein family protein	1.32	2.52	1.35	2.14
264960_at	At1g76930	AtEXT1/4 Extensin hydroxyproline-rich glycoprotein	1.59	2.76	1.38	2.14
255111_at	At4g08780	peroxidase, putative	3.32	3.82	1.91	2.30
254314_at	At4g22470	putative hydroxyproline-rich glycoprotein	4.81	3.03	4.00	2.89
259553_at	At1g21310	proline-rich extensin-like family protein contains extensin-like region	3.65	3.73	3.73	3.25
261474_at	At1g14540	anionic peroxidase similar to lignin forming peroxidase	6.35	5.66	6.35	3.32
249459_at	At5g39580	ATP24a peroxidase	11.85	9.19	8.77	4.39
245148_at	At2g45220	pectinesterase family protein	6.20	3.40	4.70	4.92
247487_at	At5g62150	peptidoglycan-binding LysM domain-containing protein	3.25	2.76	3.03	8.19
246228_at	At4g36430	peroxidase, putative	11.06	3.40	11.5	8.98
255110_at	At4g08770	peroxidase, putative	54.44	9.40	14.5	24.82

Flagellin does not contribute uniquely to PAMP-induced transcriptional changes.

Given the substantial overlap of the PAMP-induced genes identified by our microarray analysis with genes previously identified as induced by the synthetic flagellin peptide flg22 (131 of 347 PAMP-induced genes), we sought to determine the specific contribution of flagellin to the induction of these genes. To determine if perception of flagellin makes a specific contribution to the transcriptional changes observed in *Arabidopsis* after bacterial inoculation, we compared the expression profiles of *Arabidopsis* plants inoculated with *fliC* mutant derivatives of *Pst hrpA* and *E. coli* O157:H7 to those of plants inoculated with the parent strains. *fliC* mutants are impaired in the production of the flagellin protein. Therefore, this comparison allows us to determine the specific contribution of flagellin perception to PAMP-induced transcriptional changes.

A *fliC* mutant of *E. coli* O157:H7 was not available; therefore, a *fliC* mutant of the O157:H7 derivative strain TUV86-2 was used. To validate the use of this strain, we performed microarray analysis to compare the expression profiles of plants inoculated with *E. coli* O157:H7 or *E. coli* TUV86-2 and found that the expression profiles of these plants were highly similar (data not shown). The expression profiles of the 347 PAMP-induced genes from plants inoculated with *fliC* mutant bacteria were similar to those inoculated with the parental, flagellin-producing strains (Figure 2-1). The correlation values for the *Pst hrpA* vs mock and *Pst hrpA fliC* vs mock comparisons is 0.93, and for the *E. coli* vs mock and *E. coli fliC* vs mock comparisons it is 0.97. Consistent with the similarity apparent in the clustering analysis and the calculated correlation, we found that only 20 genes were reproducibly regulated more strongly by the flagellin-producing

strains than by the *fliC* mutant derivatives (Table 2-1). No genes were regulated more strongly by *E. coli* O157:H7 than by the *fliC* mutant derivative. However, when we compared the expression profile of plants inoculated with *Pst hrpA fliC* to mock inoculation, we found that using our selection criteria 377 fewer genes were reproducibly regulated by *hrpA fliC* than by *hrpA* (Table 2-1). This is a result of our stringent selection criteria as genes were regulated similarly, but not as robustly, by *Pst hrpA fliC.* This suggests that, at least in the case of *hrpA*, flagellin perception may make a quantitative contribution to the PAMP-induced regulation of transcription. However, this quantitative contribution is relatively minor as only 9 genes were reproducibly induced at least 2-fold more strongly by the flagellin-producing *hrpA* strain than by *hrpA fliC* (Table 2-1). These results suggest that although perception of flagellin induces transcriptional changes in *Arabidopsis*, flagellin does not make a substantial unique contribution to the PAMP-induced transcriptional changes observed after bacterial inoculation. *Arabidopsis* can perceive other PAMPs such as LPS and EF-Tu and our results suggest that perception of flagellin induces transcriptional changes that overlap almost completely with those induced by other PAMPs. Consistent with these results, multiplication of *fliC* mutants of *Pst* DC3000 and *hrpA* in *Arabidopsis* after vacuum-infiltration is similar to that of the flagellin-producing parent strains (Figure A-3). These results suggest that although flagellin may make a minor quantitative contribution to PAMP-induced transcriptional changes, it is only one of multiple PAMPs recognized by plants and that different PAMPs induce overlapping plant responses to bacteria within leaf tissue. The recent finding from Zipfel *et al.* that multiplication of *Pst* DC3000 is enhanced after inoculation by dipping, but not after inoculation by vacuum-infiltration suggests that perception of bacterial

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flagellin may make a unique contribution to defenses effective on the surface of the leaf, but does not make a unique contribution to elicitation of defenses when bacteria are infiltrated directly into the leaf intercellular spaces. Our data support this notion.

Analysis of the TTSS- and COR-regulated genes

To put the results of the PAMP gene regulation in perspective relative to a virulent pathogen capable of causing disease, and to identify host genes regulated by *Pst* *hrp*-dependent TTSS effectors and COR toxin, we examined host gene expression using wild-type *Pst* DC3000 and the virulence compromised mutants *Pst* COR$^-$ and *Pst* COR$^-$ *hrpS* at both low ($1x10^6$ bacteria/mL) and moderately high ($5x10^7$ bacteria/mL) inoculum levels (Table 2-1). Overall, we observed that relatively equal numbers of repressed and induced genes were identified in each comparison examined, with the exception of the $5x10^7$ bacteria/mL *Pst* COR- vs mock and *Pst* COR$^-$ vs *Pst* COR$^-$ *hrpS* comparisons which identified approximately twice as many repressed genes as induced genes. Together the $5x10^7$ and $1x10^6$ datasets identified 2,439 host genes with *Pst*-responsive gene expression. Hierarchical cluster analysis of these 2,439 reproducibly differentially regulated genes is shown in Figure 2-2. Of these 2,439 genes, 1,027 (42%) were reproducibly differentially expressed in both datasets (Table 2-1; Figure 2-2). Inspection of Figure 2-2 illustrates that even though 1,412 of the genes were not contained in the lists of differentially regulated genes for both datasets (because they failed to surpass the selection criteria in both cases), many were regulated in a similar way. The overall reproducibility between the independent biological replicates is evident as indicated by the uniformity of the gene expression changes within the 3 biological replicates of each

comparison (Figure 2-2). COR toxin was a potent regulator, inducing or repressing the expression of 944 genes 24 HPI (Table 2-1 Row 4). The COR-induced genes comprise approximately 60% (597/1018) of the reproducibly induced genes (Figure 2-2 clusters A1 and A2, Table 2-1), while only 347 genes are COR toxin repressed (Table 2-1). By comparing the samples inoculated with $5x10^7$ *Pst* COR$^-$ and COR$^-$*hrpS* bacteria/mL (to minimize bacterial population differences), a total of 791 genes exhibiting *hrp*/TTSS effector-dependent regulation were identified (Table 2-1, Row 11). These two strains lack COR and allow the identification of *hrp*/TTSS effector-dependent regulated genes, in the absence of the effect of COR toxin. Two hundred and seventy five of these genes exhibited *hrp*/TTSS effector-dependent induction (Table 2-1; Figure 2-2, cluster B). Upon visual inspection of Figure 2-2, it is clear that many of the differentially expressed genes are coordinately regulated in response to *Pst* DC3000 and the *Pst* COR$^-$ mutant (repressed by both strains or induced by both strains compared to the mock control), while other sets of genes are regulated in opposing directions. This result illustrates the importance of using both *hrp* and COR$^-$ mutant bacterial strains in discerning the specific effects of TTSS effectors and COR on host gene expression.

Independent validation of the microarray expression data was pursued using RNA blot analysis. A total of 12 repressed and 13 induced genes were selected and cDNA probes for each gene were used for RNA blot hybridization. The results are shown in Figure A-1. The RNA samples used for blot analysis were from independent biological samples (different from those used for Affymetrix chip hybridization) and included a time-course following inoculation with $1x10^6$ bacteria/mL. The results confirmed the expression patterns observed with the microarray and illustrate well that genes

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differentially expressed 24 HPI in our microarray experiments may exhibit changes as early as 6 hours and/or continue to be differentially expressed until at least 36 hours after treatment.

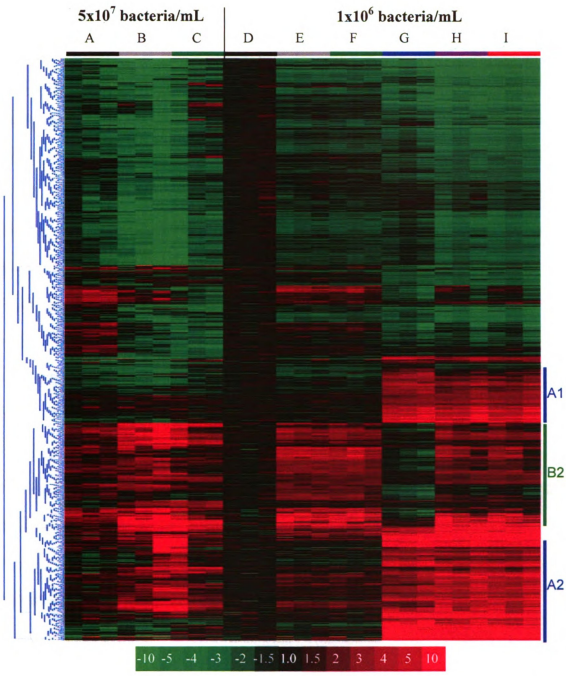

Figure 2-2: The expression profile of the 2439 *Arabidopsis* genes reproducibly regulated by 1 x10^6 and/or 5x10^7 *Pst* bacteria/mL is shown. Treatments: A- *corhrpS* v mock; B- *cor* v mock; C- *cor* v *corhrpS*; D- *corhrpS* v mock; E- *cor* v mock; F- *cor* v *corhrpS*; G- DC3000 v *cor*; H- DC3000 v mock; G- DC3000 v *corhrpS*.

Impact of *Pst* pathogenesis on host metabolism

An advantage of global gene expression profiling is the comprehensive view it can provide concerning the transcriptional regulation of genes involved in all aspects of plant physiology. In order to take advantage of this large dataset, we utilized MapMan to visualize how *Pst* pathogenesis impacted the expression of genes involved in various host metabolic pathways and processes (Thimm et al., 2004; Usadel et al., 2005). Some of the most dramatic global differences are illustrated in MapMan's metabolism overview map displaying the 944 COR toxin regulated genes and 791 *hrp*-regulated genes. The most obvious bias within the COR-regulated genes is the induction of many genes involved in secondary metabolism (Figure 2-3A). Genes that encode enzymes in the phenylpropanoid, terpanoid, anthocyanin and glucosinolate pathways are all COR-induced (Figure 2-3A, Figure A-4). This result is consistent with recent data demonstrating that JA and other jasmonates induce the expression of numerous genes which encode enzymes involved in secondary metabolism (Sasaki-Sekimoto *et al.*, 2005; Taki *et al.*, 2005). In contrast, *hrp*-dependent TTSS effectors are responsible for the repression of cell wall genes, particularly pectin esterases, as well as fatty acid biosynthesis (in the chloroplast) and a dramatic overall repression of photosynthesis (Figure 2-3B). Many genes encoding chloroplast localized proteins involved in photosynthesis and the Calvin cycle as well as genes involved in photorespiration and tetrapyrrole synthesis are repressed by TTSS effectors (Figure 2-3B).

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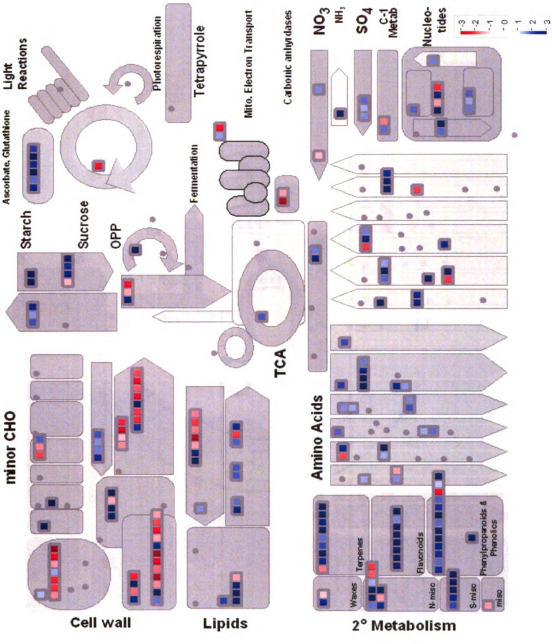
Figure 2-3: COR toxin and type III effectors target different aspects of plant metabolism. **(A)** The MapMan 'Metabolism Overview' display created using the 944 COR toxin-regulated genes identified from the *Pst* DC3000 vs *Pst* COR$^-$ comparison (24 HPI, 1x10^6 bacteria/mL) is shown. **(B)** The display created using the 791 *hrp*-regulated genes identified from the *Pst* COR$^-$ vs *Pst* COR$^-$ *hrpS* comparison (10 HPI, 5x10^7 bacteria/mL) is shown. The average fold change of the 3 biological replicates is displayed as illustrated in the fold change color bar in the lower right of each panel (note red is repressed, blue is induced).

Fig

A

Figure 2-3

A


Light Reactions
Ascorbate, Glutathione
Starch
Sucrose
OPP
Photorespiration
Fermentation
Tetrapyrrole
Mito. Electron Transport
Carbonic anhydrases
TCA
minor CHO
Amino Acids
NO_3
NH_4
SO_4
C-1 Metab
Nucleo-tides
-3
-2
-1
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1
2
3
Cell wall
Lipids
2° Metabolism
Waxes
Terpenes
Flavonoids
N-miso
S-miso
Phenylpropanoids & Pharolics
misc

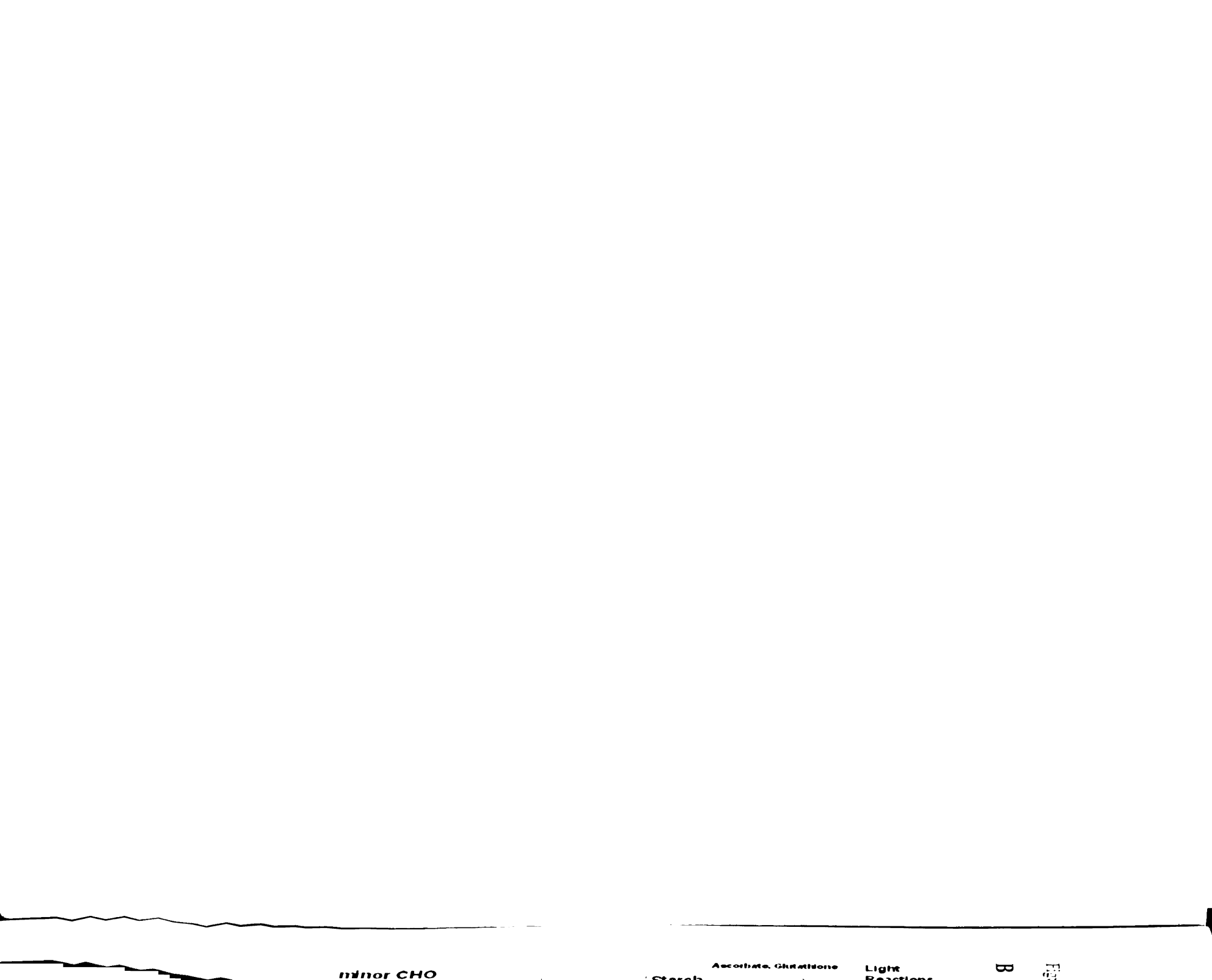
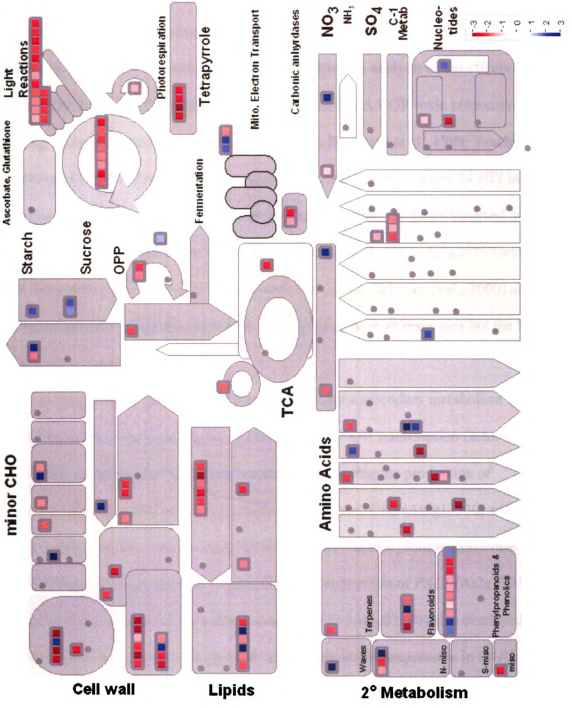

minor CHO
Ascorbate, Glutathione
Light
Reactions

Figure 2-3 (cont'd).

B



Coronatine activates JA responses and secondary metabolism

Analysis of the data has also uncovered that *Pst* challenge significantly targets several plant hormone signaling and stress-response pathways. The expression patterns observed for some genes involved in plant hormone signaling and stress responses are displayed in Figure 2-4. Consistent with the results from an analysis of the effects of purified COR in tomato (Uppalapati *et al.*, 2005), known JA/COR toxin responsive genes including chlorophyllase (*CLH1/CORI1* At1g19670; Benedetti et al., 1998; Tsuchiya et al., 1999) and *CORI3* (At4g23600; Lopukhina et al., 2001) were induced 24 HPI in a coronatine-dependent manner. Interestingly, 4 genes encoding enzymes involved in jasmonate biosynthesis (*LOX2* At3g45140, *AOS* At5g42650, *AOC1* At3g25760 and *OPR3* At2g06050; Bell et al., 1995; von Malek et al., 2002; Schaller et al., 2000) are also COR toxin induced, suggesting activation of not only jasmonate responses but the host biosynthesis of jasmonates as well.

As suggested by the analysis using MapMan, host secondary metabolism is activated by COR toxin including the induction of the *PAP1* transcription factor (At1g56650), a regulator of secondary metabolism including the production of anthocyanin pigments (Borevitz et al., 2000). As expected, *CHS* (At5g13930) and a putative anthocyanidin synthase (At2g38240) and UDP- anthocyanidin transferase (At4g27570) are strongly induced (Figure 2-4). The expression of *PAL1* (At2g37040) involved in phenylpropanoid biosynthesis and *GSTF11* (At3g03190) also displayed COR-dependent induction, consistent with their observed overexpression in *PAP1* activation-tagged plants (Borevitz et al., 2000). Several other genes involved in phenylpropanoid metabolism also exhibited reproducible induction (*F5H2* At5g04330,

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4CL9 At1g20510 and *Eli3-2/CAD-B2* At4g37990), but somewhat surprisingly, numerous others were repressed (*PAL3* At5g04230, *F5H1* At4g36220, *4CL1* At1g51680, *COMT1* At5g54160 and *CAD1* At4g39330; Gachon et al., 2005). The physiological impact of this conflicting differential regulation on lignin biosynthesis is unclear.

Two genes encoding enzymes putatively involved in terpenoid biosynthesis (S-linalool synthase At1g61120 and *TPS03* At4g16740) and *DHS1*, the first enzyme of the shikimate pathway (At4g39980; Keith et al., 1991) are induced by COR toxin (Figure 2-4). The shikimate pathway produces precursors for the synthesis of numerous secondary metabolites as well as chorismate for the production of aromatic amino acids. One metabolic pathway coordinately regulated is that of tryptophan (Trp) biosynthesis. *Pst* coordinately induces three genes that encode enzymes that convert chorismate to Trp (Figure A-5). Phosphoribosyl anthranilate isomerase (*PAI1/PAI2* At1g07780/At5g05590, both represented by probe set 259770_s_at; He and Li 2001) is induced in a COR toxin-dependent way, while *ASA1* (At5g05730; Niyogi and Fink 1992) and *ASB1/ASB2* (At1g25220/At5g57890; Niyogi et al., 1993) are induced by both COR toxin and TTSS effectors (Figure A-5). The coordinated induction of these genes is consistent with the induced expression of *ATR1* (*AtMyb34* At5g60890) and *ATR2* (At5g46760) that are known to activate Trp and glucosinolate production in *Arabidopsis* (Celenza et al., 2005; Smolen et al., 2002). Since the tryptophan pathway activators *ATR1* and *ATR2* are COR toxin induced, we examined whether other genes which encode enzymes in this pathway were differentially regulated, but did not meet our stringent selection criteria. Indeed, 5 genes, *PAT* (At5g17990) *TSA1* (At3g54640) *TSB1/TSB2* (At5g54810/At4g27070; Radwanski et al., 1995) *IGPS* (At2g04400; Li et al.,1995) and a tryptophan sysnthase

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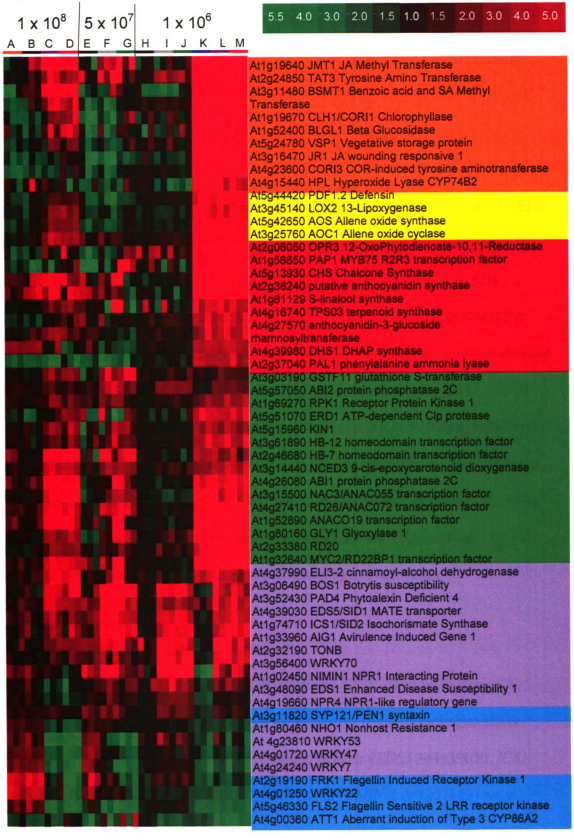
beta-like gene (At5g38530) were also induced following bacterial inoculation (Figure A-5). Tryptophan is likely being used as a substrate for the production of glucosinolates, and IAA, since *CYP79B2* (At4g39950; Zhao et al., 2002), *CYP79B3* (At2g22330; Zhao et al., 2002), and *NIT3* (At3g44320; Vorwerk et al., 2001) are reproducibly induced following inoculation with $1x10^6$ bacteria/mL *Pst* DC3000 bacteria. The tryptophan pathway may also potentially contribute to the accumulation of the phytoalexin camalexin since *PAD3* is a known *Pst* induced gene (At3g26830; Zhou et al., 1999; Figure A-5). The synthesis of methionine-derived glucosinolates is also implied since *CYP79F1/CYP79F2* (At1g16400/At1g16410; Chen et al., 2003; Reintanz et al., 2001; Tantikanjana et al., 2004), and *AOP2* (At4g03060; Kliebenstein et al., 2001) are induced by COR toxin. The expression pattern displayed in Figure A-5 also illustrates how COR toxin and TTSS effectors can both synergistically induce the expression of some genes while antagonistically regulating the expression of other genes.

Figure 2-4: *Pst* coordinately regulates hormone and stress response genes. The expression profile for 57 genes following *Pst* inoculation is shown. See Figure 1 legend for a description of the gene expression display. Gene annotation is listed on the right with background shading delineating a relationship to known pathways or responses. Orange- JA or COR toxin inducible, yellow-JA biosynthesis, red-secondary metabolism, green-ABA and abiotic stress response, violet-SA and defense responses, and blue-basal defense.

Figure 2-4


1 x 10^8
5 x 10^7
1 x 10^6
A B C D E F G H I J K L M
5.5 4.0 3.0 2.0 1.5 1.0 1.5 2.0 3.0 4.0 5.0
At1g19640 JMT1 JA Methyl Transferase
At2g24850 TAT3 Tyrosine Amino Transferase
At3g11480 BSMT1 Benzoic acid and SA Methyl Transferase
At1g19670 CLH1/CORI1 Chlorophyllase
At1g52400 BLGL1 Beta Glucosidase
At5g24780 VSP1 Vegetative storage protein
At3g16470 JR1 JA wounding responsive 1
At4g23600 CORI3 COR-induced tyrosine aminotransferase
At4g15440 HPL Hyperoxide Lyase CYP74B2
At5g44420 PDF1.2 Defensin
At3g45140 LOX2 13-Lipoxygenase
At5g42650 AOS Allene oxide synthase
At3g25760 AOC1 Allene oxide cyclase
At2g06050 OPR3 12-OxoPhytodienoate-10,11-Reductase
At1g56650 PAP1 MYB75 R2R3 transcription factor
At5g13930 CHS Chalcone Synthase
At2g38240 putative anthocyanidin synthase
At1g61120 S-linalool synthase
At4g16740 TPS03 terpenoid synthase
At4g27570 anthocyanidin-3-glucoside rhamnosyltransferase
At4g39980 DHS1 DHAP synthase
At2g37040 PAL1 phenylalanine ammonia lyase
At3g03190 GSTF11 glutathione S-transferase
At5g57050 ABI2 protein phosphatase 2C
At1g69270 RPK1 Receptor Protein Kinase 1
At5g51070 ERD1 ATP-dependent Clp protease
At5g15960 KIN1
At3g61890 HB-12 homeodomain transcription factor
At2g46680 HB-7 homeodomain transcription factor
At3g14440 NCED3 9-cis-epoxycarotenoid dioxygenase
At4g26080 ABI1 protein phosphatase 2C
At3g15500 NAC3/ANAC055 transcription factor
At4g27410 RD26/ANAC072 transcription factor
At1g52890 ANAC019 transcription factor
At1g80160 GLY1 Glyoxylase 1
At2g33380 RD20
At1g32640 MYC2/RD22BP1 transcription factor
At4g37990 ELI3-2 cinnamoyl-alcohol dehydrogenase
At3g06490 BOS1 Botrytis susceptibility
At3g52430 PAD4 Phytoalexin Deficient 4
At4g39030 EDS5/SID1 MATE transporter
At1g74710 ICS1/SID2 Isochorismate Synthase
At1g33960 AIG1 Avirulence Induced Gene 1
At2g32190 TONB
At3g56400 WRKY70
At1g02450 NIMIN1 NPR1 Interacting Protein
At3g48090 EDS1 Enhanced Disease Susceptibility 1
At4g19660 NPR4 NPR1-like regulatory gene
At3g11820 SYP121/PEN1 syntaxin
At1g80460 NHO1 Nonhost Resistance 1
At 4g23810 WRKY53
At4g01720 WRKY47
At4g24240 WRKY7
At2g19190 FRK1 Flagellin Induced Receptor Kinase 1
At4g01250 WRKY22
At5g46330 FLS2 Flagellin Sensitive 2 LRR receptor kinase
At4g00360 ATT1 Aberrant induction of Type 3 CYP86A2

Pst induces ABA/abiotic stress response genes

COR toxin and, to some degree, TTSS effectors induce numerous ABA responsive genes, including a significant number of genes implicated in ABA/abiotic stress-responsive signaling (Figure 2-4; Bray 2002; Finkelstein and Rock 2002). *ABI1* (At4g26080; Leung et al., 1994) and *ABI2* (At5g57050; Leung et al., 1997) are both induced in the absence of COR toxin at the $5x10^7$ bacteria/mL inoculum level, suggesting that their expression is at least partly regulated by TTSS effectors (Figure 2-4). *NCED3* (At3g14440; Iuchi et al., 2001), a 9-cis-epoxycarotenoid dioxygenase crucial for ABA synthesis, is also induced following *Pst* COR$^-$ inoculation (Figure 2-4). *NCED4* (At4g19170), a related family member, is repressed by virulent *Pst* DC3000 and *Pst* COR$^-$ bacteria at the higher inoculum levels. A total of 6 transcription factors associated with ABA and abiotic stress responses (*HB-12* At3g61890, *HB-7* At2g46680, *NAC3/ANAC055* At3g15500, *RD26 ANAC072* At4g27410, *ANAC019* At1g52890 and *MYC2/RD22BP1* At1g32640) are also induced by *Pst* infection (Figure 2-4) (Abe et al., 2003; Fujita et al., 2004; Greve et al., 2003; Olsson et al., 2004; Soderman et al., 1996; Tran et al., 2004). These results imply that ABA responses are being activated by *Pst* and that both TTSS effectors and COR toxin are involved in this activation.

Defense response-related gene expression

Of particular interest is the role COR toxin and TTSS effectors have on SA-mediated defenses since those responses are necessary for resisting pathogen attack. Several SA-responsive genes involved in pathogen defense (*EDS5* At4g39030, *ICS1* At1g74710, *PAD4* At3g33960, *EDS1* At3g48090, and *NIMIN-1* At1g02450) were

induced in a *hrp*-dependent manner (Figure 2-4) (Falk et al., 1999; Jirage et al., 1999; Nawrath et al., 2002; Wildermuth et al., 2001; Weigel et al., 2005). The *Pst*-induced expression of these genes is consistent with previous published results, but it was somewhat surprising that their induction is enhanced between 1.5- and 20-fold by TTSS effectors. The expression of these genes is either not reliably induced or induced 2-to-5-fold by *Pst* COR$^-$*hrpS* bacteria relative to the mock inoculated control in the $5x10^7$ dataset. This suggests that the host plant is recognizing PAMPs present on the *Pst* COR$^-$ *hrpS* mutant and potentially also recognizing specific TTSS effectors and activating defense gene expression (Figure 2-4). Interestingly, COR toxin actually dampens by approximately 4-fold the induction of at least one SA-related defense gene (*NIMIN-1*). In the samples examined, the defense response genes *AIG1* (At1g33960; Reuber and Ausubel 1996), and *TONB* (At2g32190; de Torres et al., 2003) are induced 6-to-20-fold.

In contrast to the induction observed for the previously discussed SA/defense-related genes, virulent *Pst* bacteria repress three genes associated with basal defense responses. *Flagellin Sensitive 2* (*FLS2* At5g46330) which is required for the perception of the PAMP flagellin is repressed by both COR toxin and TTSS effectors at high inoculum concentrations (Figure 2-4) (Gomez-Gomez et al., 1999; Zipfel et al., 2004). The *Flagellin induced Receptor Kinase 1* (*FRK1* At1g19190; Asai et al., 2002; de Torres et al., 2003) and *WRKY22* (At4g01250; Asai et al., 2002) are induced by *E. coli*, *Pst hrpA* and *Pst* COR$^-$*hrpS* mutant bacteria at high inoculum levels and that induction is blocked by TTSS effectors in the *Pst* COR$^-$ mutant (Figure 2-4). COR toxin produced by *Pst* DC3000 also contributes to the repression of *FRK1*, *WRKY22* and *FLS2* expression at the

$1x10^6$ inoculum level. These results are consistent with the idea that TTSS effectors and COR toxin contribute to pathogenesis by suppressing basal defense responses.

Expression of auxin and cytokinin-related genes are altered during *Pst* infection

It is clear that *Pst* virulence systems are modulating stress hormone response pathways (JA, SA and ABA), but plant host physiology also appears to be experiencing specific shifts in auxin and cytokinin responses as well. Thirty-one auxin-related genes are repressed while 13 other genes are induced following *Pst* inoculation (Figure A-6). In most cases the gene regulation appears to be partially dependent on both COR toxin and TTSS effectors. A few genes are also reproducibly differentially regulated in response to bacterial PAMPs present on *E. coli* and nonpathogenic strains of *Pst* as well. The 31 repressed genes include 4 *AUX/IAA* and 18 *SAUR* (Small Auxin Up-Regulated) genes, 6 auxin transporters (*PIN3* At1g70940, *PIN4* At2g01420, *PIN7* At1g23080, *AUX1* At2g38120, *LAX1* At5g01240, and *LAX3* At1g77690) and *ARF18* (At3g61830) (Hagen and Guilfoyle, 2002; Okushima et al., 2005; Swarup et al., 2004). *AUX/IAA* genes are rapidly induced by auxin and are hypothesized to act in a feedback loop repressing the auxin response signaling pathway (Tiwari et al., 2004). Five *SAUR* genes, three IAA-amino acid hydrolases (*ILR1* At3g02875, *ILL5* At1g51780 and *ILL6* At1g44350), 3 IAA-amido synthases (DFL2 At4g03400, GH3.3 At2g23170 and GH3.12 At5g13320), *IAA18* (At3g16500) and *NIT3*, are induced. NIT3 will synthesize IAA (Figure A-5B) while IAA-amino acid hydrolases and amido synthases convert IAA between an amino acid conjugate and free form (Staswick et al., 2005). Taken together, these results suggest that *Pst* is impacting host auxin signaling, potentially de-repressing the pathway, altering

auxin movement and activating biosynthesis of the hormone. These results are consistent with the observation that *Pseudomonas syringae* strains express biosynthetic enzymes that can produce IAA from tryptophan (Buell et al., 2003; Glickmann *et al.*, 1998). *Pst* DC3000 has been shown to elicit the accumulation of IAA in *Arabidopsis* leaves during infection and that activation of this pathway contributes to disease susceptibility (O'Donnell et al., 2003).

Auxin and cytokinin are involved in many important aspects of plant growth and development. *Pst* pathogenesis appears to not only target auxin signaling but also genes involved in cytokinin accumulation and signaling. Seven type A response regulators (*ARR4, 5, 6, 7, 9, 15* and *ARR16*; D'Agostino et al., 2000; To et al., 2004) are repressed by COR toxin following inoculation with $1x10^6$ *Pst* DC3000 bacteria/mL (Figure A-7). The other type A family members had low expression in the samples tested and in most cases were scored as absent (data not shown). One type B response regulator (*ARR14* At2g01760) was repressed by *Pst*, but in this case repression appears to be dependent on TTSS effectors and not COR toxin. None of the other type B family members were reproducibly differentially regulated. Cytokinin synthesis is mediated by the isopentenyl transferase gene family, one member of that family; *IPT3* (At3g63110) is COR toxin repressed (Kakimoto 2003a, Miyawaki et al., 2004). Two cytokinin oxidase genes (*CKX4* At4g29740, *CKX5* At1g75450; Werner et al., 2003) are induced by TTSS effectors and COR toxin respectively. The repression of *IPT3* together with *CKX4* and *CKX5* induction suggests a reduction in the levels of cytokinin within the leaves following inoculation. The type A response regulators are known to be rapidly induced by cytokinin and their repression would be consistent with a fall in the cytokinin levels. Members of the type A

response regulator family appear to be negative regulators of cytokinin signaling and may be part of feedback loop repressing induced responses (Kakimoto 2003b; To et al., 2004). The coordinated repression of this gene family suggests that cytokinin signaling may be de-repressed by *Pst* during infection.

Conclusion

Although the *Arabidopsis-Pst* DC3000 interaction was initially developed as a model for studying disease resistance mechanisms (Whalen et al., 1991), this pathosystem is now also used widely for studying susceptible plant-pathogen interactions (Nomura et al., 2005). A comprehensive transcriptional analysis of this interaction using defined *Pst* DC3000 virulence mutants, we thought, would provide the community a valuable resource for future understanding of this interaction at whole plant, tissue, cellular, and molecular levels. Indeed, this analysis revealed several interesting results. First, it appears that *Arabidopsis* plants respond similarly to PAMPs presented on live human and plant pathogenic bacteria, and that flagellin perception is not necessary for the regulation of PAMP-induced genes or bacterial multiplication in the context of *Pst* DC3000 infection of *Arabidopsis* under our experimental conditions in which bacteria are infiltrated directly into the leaf intercellular spaces. We found that an impressive number of PAMP-induced genes belong to transcription factors, signaling components, and cell wall- and/or secretion-associated proteins, suggesting coordinate regulation of genes involved in signaling and secretion with those that encode secreted products during basal defense. Second, use of *Pst* DC3000 mutant strains deficient in COR toxin (*Pst* DC3118) or COR and the TTSS (the *hrpS* COR^- mutant) enabled us to obtain genome-wide

knowledge of both distinct and overlapping effects of these two important virulence factors in modulating host gene expression during actual bacterial infection. There is clear evidence for TTSS effector-mediated suppression of basal defense-associated genes. In contrast, many of the SA-regulated defense genes, including some of those associated with basal defense, are induced by TTSS effectors. The most dramatic effect of COR is the induction of JA-regulated genes, as expected, but COR also appears to exert a major impact on the expression of genes involved in secondary metabolism. Finally, both TTSS effectors and COR contribute to the dramatic regulation of genes that are responsive to plant hormones, such as auxin, ABA, and cytokinin.

We hope that the results described in this paper and the online supplemental materials will facilitate future study of *Pst* DC3000 pathogenesis in *Arabidopsis* using a variety of genomic, cell biology, and biochemical approaches.

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Chapter 3

A novel function for plant stomata in innate immunity against bacteria

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Abstract

Stomata are microscopic pores on the surface of plant leaves that allow the plant to conduct gas exchange and regulate water relations. Plants regulate the opening and closing of these pores through changes in turgor pressure within the guard cells, the pair of cells that creates a single stomata. Stomata have been instrumental in the evolution of land plants; however, such openings on the surface of the leaf can potentially render the plant vulnerable to invasion by pathogenic microbes. This is particularly relevant for bacterial pathogens that must enter the plant tissue to initiate pathogenesis and, unlike many fungal plant pathogens, are generally unable to directly penetrate the cuticle and cell wall. It has been assumed that stomata serve as passive ports of entry for bacteria, allowing potential pathogens to freely enter the leaf intercellular spaces. However, in this chapter I report that stomata play a role in plant innate immunity by closing in response to perception of bacteria on the leaf surface. Specifically, stomata of *Arabidopsis* epidermal peels were found to close in response to both plant and human pathogenic bacteria, and in response to purified PAMPs. Bacteria- and PAMP-induced stomatal closure required ABA biosynthesis and components of the ABA signaling pathway. Unlike human pathogenic bacteria, the plant pathogen *Pseudomonas syringae* pv. tomato strain DC3000 was able to re-open *Arabidopsis* stomata after approximately three hours of incubation with epidermal peels. Re-opening of stomata by *Pst* DC3000 was dependent on production of the phytotoxin coronatine.

Introduction

Entry into host tissue is often a critical first step in the establishment of infection by microbial pathogens. Foliar pathogens of plants face unique challenges in gaining entry into the host tissue. The epidermis and cuticle of the plant leaf must be traversed by microbial pathogens in order to gain access to the intercellular spaces and internal leaf tissues. Many fungal plant pathogens have the ability to directly penetrate the epidermis using cell wall-degrading enzymes, mechanical force, or both. Bacterial pathogens, on the other hand, generally cannot directly penetrate the leaf epidermis and are thought to enter leaf tissues through natural openings or accidental wounds on the leaf surface.

Stomata, microscopic pores on the surface of the plant leaf that allow the plant to conduct gas exchange and regulate water relations, provide such natural openings into the leaf intercellular spaces through which bacteria may potentially enter. It is generally believed that openings on the leaf surface such as stomata serve as passive ports of entry for bacteria. However, emerging evidence suggests that plants may have a defense mechanism that acts at an early stage of pathogenesis such as entry into the host tissue. Recent studies have demonstrated the ability of plants to perceive pathogen-associated molecular patterns (PAMPs) such as bacterial flagellin and lipopolysaccharide (LPS) and that perception of PAMPs contributes to resistance to the bacterial phytopathogen *Pseudomonas syringae* pv. tomato strain DC3000 (*Pst* DC3000; Felix et al., 1999; Kunze et al., 2004; Zeidler et al., 2004; Zipfel et al., 2004). Interestingly, resistance to *Pst* DC3000 mediated by perception of flagellin by the FLS2 receptor was found to be effective against bacteria that had been inoculated onto the leaf surface, mimicking a natural infection, but not against bacteria that were artificially delivered into the leaf

tissue by vacuum-infiltration, bypassing the leaf surface (Zipfel et al., 2004). However, the mechanism by which PAMP perception contributes to limiting bacterial infection on the leaf surface is not yet known.

Pst DC3000 infects both tomato and *Arabidopsis* and is widely used a model to study bacterial diseases of plants. Plant pathogenic bacteria such as *Pst* DC3000 have evolved a variety of virulence factors that contribute to their ability to colonize their hosts through subversion of host defenses or release of nutrients (Abramovitch and Martin, 2004; Nomura et al., 2005). The type III secretion system (TTSS) encoded by the *hrp* genes of *Pst* DC3000 is one such virulence factor (Alfano and Collmer, 2004; Buttner and Bonas, 2002; He et al., 2004; Mudgett, 2005; Staskawicz et al., 2001). *Pst* DC3000 uses the TTSS to inject a number of virulence effector proteins into host cells (Chang et al., 2005; Collmer et al., 2003; Greenberg and Vinatzer, 2003; Nomura and He, 2005). In addition to TTSS effector proteins, another virulence factor produced by *Pst* DC3000, the phytotoxin coronatine (COR), seems to be required for full virulence. COR is a polyketide toxin that is structurally similar to the plant hormone jasmonic acid (JA). Interestingly, bacterial mutants impaired in COR biosynthesis were found to be significantly reduced in virulence when inoculated onto the surface of *Arabidopsis* leaves, but fully virulent when infiltrated directly into the leaf intercellular spaces (Mittal and Davis, 1995). This observation prompted the authors to speculate that COR may suppress an "early" defense in *Arabidopsis*. The nature of this early defense, however, has remained elusive.

In this study, plant stomata were found to play an active role in innate immunity against bacteria. Stomata of intact *Arabidopsis* leaves and epidermal peals were found to

close in response to incubation with both plant- and human-pathogenic bacteria. Interestingly, the closure was transient after incubation with the plant pathogen *Pst* DC3000, but persistent after incubation with the human pathogen *E. coli* O157:H7. This result suggests that plant pathogens, but not human pathogens, have evolved mechanisms to overcome stomatal closure. We found that closure of stomata in response to bacteria or purified PAMPs required ABA synthesis and components of the ABA signaling pathway and that re-opening of stomata by *Pst* DC3000 was dependent on COR biosynthesis. These results suggest that stomata function as innate immunity gates to restrict entry of bacteria into plant tissues and that overcoming stomatal closure may be a key step required for bacterial pathogenicity on plants.

Experimental Procedures

Plant Materials

Arabidopsis plants (ecotypes Col-0, Col-5, WS, Landsberg *erecta* [Ler] and mutant lines derived from these ecotypes) were grown in controlled growth chambers at 22°C with a 12hr photoperiod under light intensities of 100 $\mu E/m^2/s$. For all experiments, 5- to 6-week-old plants were used. Two independent T-DNA lines of the *fls2* mutant (SAIL_691C4 and SALK_93905) were used for the experiments and they showed similar responses to treatments.

Chemicals

Purified chemicals were used at the following concentrations: 10 μM abscisic acid (ABA, Sigma), 0.2 mM L-NNA (Nω-nitro-L-arginine, Sigma), 0.5 ng/μl coronatine (COR, purchased from C. Bender, Oklahoma State University), 100 ng/μl lipopolysaccharide (LPS from *P. aeruginosa*, Sigma), 5 μM flg22 peptide (Alpha Diagnostics, Inc. and EZBiolab) Chemicals were diluted in MES (2-(*N*-morpholino)-ethanesulfonic acid) buffer (25 mM MES-KOH [pH 6.15] and 10 mM KCl), except for LPS solution, which also contained 0.25 mM $MgCl_2$ and 0.1 mM $CaCl_2$. Ultrapure LPS preparations from *E. coli* O55:B5 (Sigma) and *Salmonella Minnesota* R595 (Re, Calbiochem) were also tested with similar results. Concentrations of LPS, flg22, and COR were chosen based on previous studies (Zeidler et al., 2004; Zhao et al., 2003; Zipfel et al., 2004) and dose-response experiments.

Assessment of Response of Stomata to Treatments

To assure that most stomata were open before beginning experiments, we kept plants under light (100 $\mu mol.m^{-2}.s^{-1}$) for at least three hours. Fully expanded, young leaves were immersed in water or bacterial suspension [10^8 colony forming units $(CFU).ml^{-1}$ in water]. At various time points, epidermis of three leaves was peeled off and immediately observed under a microscope (Zeiss Axiophot D-7082 photomicroscope with A3 fluorescence cube or laser scanning confocal microscope). Alternatively, epidermis was peeled from fully expanded leaves and placed on glass slides with the cuticle side in contact with water, MES buffer (25 mM MES-KOH, pH 6.15, and 10 mM KCl), chemical solutions in MES buffer or bacterial suspensions in water. At various

time points, pictures were taken of random regions. The width of the stomatal aperture was measured using the software Image-Pro, version 4.5 for windows (Cybernetics, Inc. Silver Spring, MD, USA). We found that stomata in intact leaves and epidermal peels responded similarly to various treatments. All stomatal aperture results reported here were from blind experiments, in which genotypes and treatments were unknown to the experimenters who measured stomatal responses until the completion of experiments. All experiments reported here were performed at least 3 times with similar results.

Detection of Nitric Oxide Production in Guard Cells

Epidermal peels of Arabidopsis plants were pre-incubated for 3 hr in MES buffer (25 mM MES-KOH, pH 6.15, and 10 mM KCl), soaked in 15 μM DAF-2 DA (4,5-diaminofluorescein diacetate, Sigma) diluted in MES buffer for 20 min, washed three times in MES buffer, and then incubated with chemicals or bacterial suspensions. To assess the COR effect on PAMP-induced NO production, peels were incubated with COR for 30 min prior to addition of purified PAMPs. MES buffer was used as control for purified chemicals and water was the control for bacterial suspensions. Photographs of guard cells were taken with a digital camera attached to a fluorescence microscope equipped with a 502-530 band pass filter.

Bacterial Growth Assay

Pst DC3000 and mutant derivatives were cultured at 30°C in Luria-Bertani (LB; Sambrook et al., 1989) medium supplemented with appropriate antibiotics until an OD_{600} of 0.8 was reached. Bacteria were collected by centrifugation and resuspended in water

to the final concentration of 10^8 CFU.ml^{-1} containing 0.05% Silwet L-77 (Osi Specialties, Friendship, WV). Arabidopsis plants were dipped in bacterial suspension and kept under high humidity until disease symptoms developed. Some plants were vacuum-infiltrated with bacterial suspension at a concentration of 10^6 CFU.ml^{-1} containing 0.004% Silwet L-77. Infiltrated plants were allowed to dry until leaves were no longer water soaked and then covered with humidity domes until completion of the experiment. Bacterial population in the plant apoplast was determined as previously described (Katagiri et al., 2002). All bacterial multiplication assays reported here were performed at least 3 times with similar results.

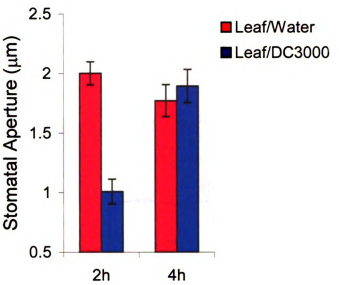
Results

Plant- and human-pathogenic bacteria induce closure of stomata

Plant stomata have the ability to respond to a variety of environmental stimuli including light, humidity, and CO_2 concentration (Schroeder et al., 2001; Fan et al., 2004). However, the presence of pores on the surface of the plant leaf could potentially leave the plant vulnerable to invasion by bacteria. To determine if plant stomata can also respond to the presence of live bacteria, we first treated intact *Arabidopsis* leaves with the plant pathogen *Pst* DC3000. Prior to treatment, *Arabidopsis* leaves generally contained 70%-80% open stomata and 20%-30% closed stomata under our growth conditions. After treatment of detached leaves with 1 x 10^8 cfu/ml *Pst* DC3000, we observed a marked reduction in the number of open stomata, to ~30% after 2 hours of treatment (data not shown). This reduction in the percentage of open stomata was correlated with a significant decrease in the average width of the stomatal apertures (Figure 3-1A). Observations and measurements of stomatal behavior are commonly performed using epidermal peels rather than intact leaves. Epidermal peels allow stomata to be more easily viewed and recorded microscopically. We therefore tested the ability of stomata in *Arabidopsis* epidermal peels to respond to *Pst* DC3000 in a manner similar to those in intact leaves. After 1 hour of incubation with 1 x 10^8 cfu/ml *Pst* DC3000, stomata in epidermal peels of *Arabidopsis* leaves were found to behave similarly to those in intact leaves (Figure 3-1B), therefore, epidermal peels were used for all subsequent experiments.

In nature, plants are exposed to a variety of bacteria including highly adapted pathogens such as *Pst* DC3000, but also epiphytes that survive on the leaf surface and even human or animal pathogens. To determine whether closure of *Arabidopsis* occurs as a general response to a wide variety of bacteria or is limited to highly adapted plant-pathogenic bacteria such as *Pst* DC3000, epidermal peels of *Arabidopsis* leaves were incubated with *Escherichia coli* O157:H7. *E. coli* O157:H7 is an enteric human pathogen that is often a causal agent of food poisoning associated with fresh fruits and vegetables. Despite this association, *E. coli* O157:H7 is not a pathogen of plants and is unable to multiply in plant tissues under normal growth conditions (Figure 3-2A). Stomata in *Arabidopsis* epidermal peels closed after incubation with 1 x 10^8 cfu/ml *E. coli* O157:H7, similar to the response to *Pst* DC3000 (Figure 3-2B). This result suggests that closure of stomata is likely to be a generalized response to perception of a wide variety of bacteria. Interestingly, however, unlike stomata in epidermal peels incubated with *Pst* DC3000, stomata incubated with *E. coli* O157:H7 remained closed throughout the entire duration of the experiment (8hrs; Figure 3-2B). This result suggests that the adapted plant pathogen *Pst* DC3000 may have evolved a mechanism (or mechanisms) to overcome the closure of stomata and gain access to the leaf intercellular spaces, whereas the human pathogen lacks this ability.

A



B

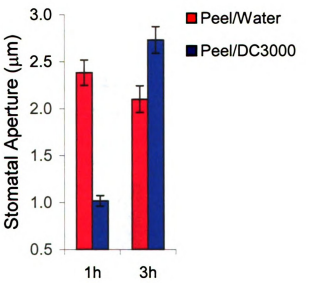
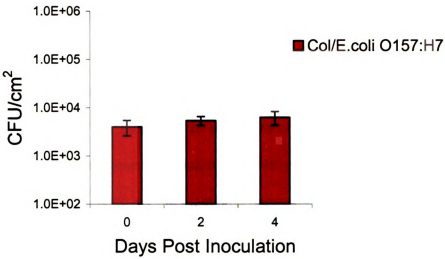


Figure 3-1: *Pst* DC3000 triggers closure of *Arabidopsis* stomata. Stomatal aperture in (**A**) intact leaves or (**B**) epidermal peels of Col-0 plants exposed to water (red bars) or 1 x 10^8 cfu/ml *Pst* DC3000 (blue bars). Results in panel A are shown as mean (n = 120 stomata) ± SEM. In panel B and all subsequent experiments, results are shown as mean (n = 60 stomata) ± SEM.

A



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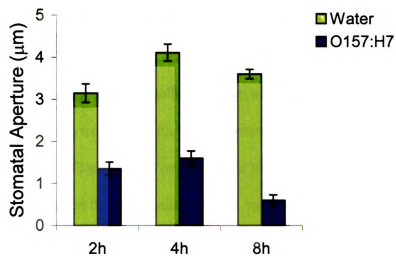
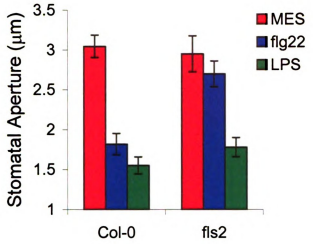


Figure 3-2: (A) Multiplication of *E. coli* O157:H7 in *Arabidopsis* Col *gl1* plants after inoculation at 1 x 10^6 cfu/ml of bacteria. Error bars represent standard deviation. **(B)** *E. coli* O157:H7 triggers persistent closure of *Arabidopsis* stomata. Stomatal apertures in epidermal peels of Col-0 after incubation with water (green bars) or 1 x 10^8 cfu/ml *E. coli* O157:H7 (blue bars).

Involvement of PAMPs in triggering stomatal closure

Pathogen-associated molecular patterns (PAMPs) such as flagellin, lipopolysaccharides and elongation factor Tu (EF-Tu) are perceived by plants and trigger the plant's innate immune response (Felix et al., 1999; Kunze et al., 2004; Zeidler et al., 2004). Recognition of PAMPs has been shown to contribute to disease resistance (Kim et al., 2005; Zipfel et al., 2004). However, the precise mechanisms triggered by PAMP perception that lead to resistance to infection have not yet been discovered. The ability of stomatal guard cells to respond to both plant- and human-pathogenic bacteria suggests that these cells can sense conserved bacterial molecules such as PAMPs. To test the involvement of PAMPs in bacteria-triggered stomatal closure, we incubated epidermal peels from *Arabidopsis* Col-0 plants with the purified PAMPs flg22 (a biologically active 22 amino acid peptide derived from a highly conserved domain of eubacterial flagellin) or LPS. We consistently observed a reduction in stomatal aperture after 1 hour of incubation with purified PAMPs (data not shown) and the closure was persistent for at least 4 hours of treatment (Figure 3-3A). Closure of stomata in response to the flg22 peptide was dependent on the FLS2 flagellin receptor, as stomata of *fls2* mutant plants did not close in response to treatment with flg22. However, LPS (Figure 3-3A) and live *E. coli* O157:H7 bacteria (Figure 3-3B) were still able to induce stomatal closure on *fls2* leaves. These results suggest that perception of flg22 by guard cells requires the flagellin receptor FLS2, but that FLS2 is likely only one of a number of pattern recognition receptors that allow guard cells to perceive PAMPs and initiate closure. The involvement of PAMPs and FLS2 in stomatal closure suggests that closure of stomata may be an integral component of the *Arabidopsis* innate immune response to bacteria.

A



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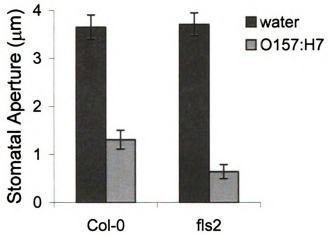


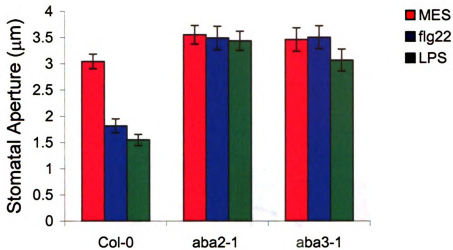
Figure 3-3: (A) Purified PAMPs trigger closure of *Arabidopsis* stomata. Stomatal apertures in epidermal peels of *Arabidopsis* Col-0 or *fls2* mutant (SAIL_691C4) leaves after 4 hours of incubation with MES buffer (red bars), 5 µM flg22 (blue bars), or 100 ng/µl LPS (green bars). **(B)** Stomatal responses of wild type (Col-0) and *fls2* mutant (SALK_93905) plants to 1 x 10^8 cfu/ml *E. coli* O157:H7. Stomatal aperture measurements were determined after 4 hours of exposure to bacteria.

ABA synthesis and signaling components are required for bacteria- and PAMP-induced stomatal closure.

Closure of stomata in response to environmental cues and abiotic stresses has been studied extensively and is dependent on the plant hormone abscisic acid (ABA). The ABA signal in guard cells is transduced through a network of signaling components including the protein phosphatase 2Cs ABI1 and ABI2, a guard cell-specific kinase, OST1, nitric oxide (NO), and H_2O_2 (Fan et al., 2004; Schroeder et al., 2001). The signaling events triggered by ABA ultimately lead to regulation of ion channels, such as the guard cell outwardly rectifying potassium channel GORK1, that modulate ion efflux from the guard cells (Hosy et al., 2003). Ion efflux from the guard cells drives the efflux of water and results in a change in guard cell turgor that causes the closure of the stomatal pore. To determine if ABA synthesis and/or signaling are required for PAMP-induced closure of stomata we tested a number of mutants defective in ABA synthesis or signaling for their ability to close stomata in response to PAMP perception. The *Arabidopsis aba2-1* and *aba3-1* mutants are defective in ABA biosynthesis and impaired in stomatal closure in response to abiotic stresses (Leon-Kloosterziel et al., 1996; Merlot et al., 2002). Neither flg22 nor LPS could induce closure of stomata in epidermal peels from either *aba2-1* or *aba3-1* plants (Figure 3-4A), demonstrating that PAMP-induced closure of stomata requires ABA. To determine if PAMP-induced ABA signaling in guard cells leading to stomatal closure proceeds through known components of ABA signaling, we tested mutant plants defective in components of guard cell ABA signaling pathways for their response to flg22 and LPS. The protein phosphatase 2C proteins encoded by *ABA Insensitive 1 (ABI1)* and *ABI2* are required for stomatal closure in

response to ABA (Leung et al., 1997; Pei et al., 1997). Stomata of *Arabidopsis abi1* and *abi2* mutant plants failed to respond to purified flg22 or LPS (Figure 3-4B). Additionally, the *Arabidopsis* mutants *ost1* (Mustilli et al., 2002), lacking a guard cell-specific kinase, and *gork1* (Hosy et al., 2003), lacking a guard cell outwardly rectifying potassium channel, both failed to close stomata in response to LPS and *ost1* failed to close in response to flg22 (Figure 3-5). The response of *gork1* to flg22 was not tested as the *gork1* mutation is in the WS genetic background that naturally lacks the FLS2 flagellin receptor. Production of the small signaling molecules nitric oxide (NO) and hydrogen peroxide (H_2O_2) has also been associated with ABA signal transduction leading to closure of stomata (Fan et al., 2004; Schroeder et al., 2001). The NADPH oxidase double mutant *rbohD/F* was previously shown to be partially impaired in ABA-induced stomatal closure (Kwak et al., 2003). Similarly, this mutant showed an intermediate response to both flg22 and LPS (Figure 3-5). To test the involvement of NO in PAMP-induced stomatal closure, we performed DAF-2 DA staining on epidermal peels treated with flg22, LPS, or MES buffer to detect production of NO in guard cells. Treatment of epidermal peels with flg22 and LPS rapidly (within 10 min) induced production of NO in guard cells of stomata that eventually closed (Figures 3-6B and 3-6C). Additionally, treatment of epidermal peels with Nω-nitro-L-arginine (L-NNA), an inhibitor of nitric oxide synthase (NOS), prevented closure of stomata in response to flg22, LPS, and *E. coli* O157:H7. Taken together, these results clearly demonstrate that ABA synthesis and downstream signaling components of the ABA signal transduction network in guard cells are required for bacteria- and PAMP-induced stomatal closure.

A



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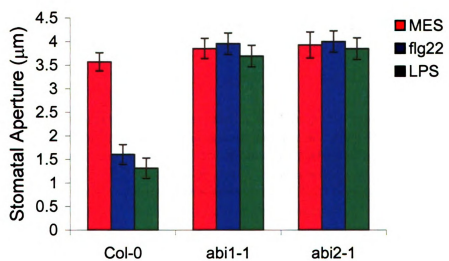


Figure 3-4: Stomatal responses in *Arabidopsis* ABA **(A)** biosynthetic mutants *aba2* and *aba3*, and **(B)** signaling mutants *abi1-1* and *abi2-1*. Stomatal aperture measurements were determined after 4 hours of incubation with MES buffer (red bars), 5 µM flg22 (blue bars), or 100 ng/µl LPS (green bars).

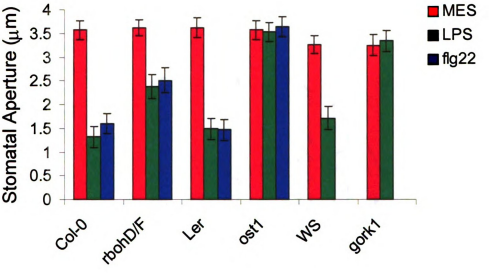
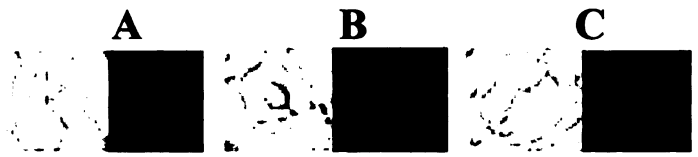


Figure 3-5: Stomatal responses of *Arabidopsis* ABA response mutants *ost1, gork1,* and *rbohD/F*. Stomatal aperture measurements were determined after 4 hours of incubation with MES buffer (red bars), 5 μM flg22 (blue bars), or 100 ng/μl LPS (green bars). Col-0 was wild-type for *rbohD/F*, Landsberg *eracta* (Ler) was wild-type for *ost1*, and WS was wild-type for *gork1*. The response of WS and *gork1* to flg22 was not tested as the WS genetic background naturally lacks the FLS2 flagellin receptor.



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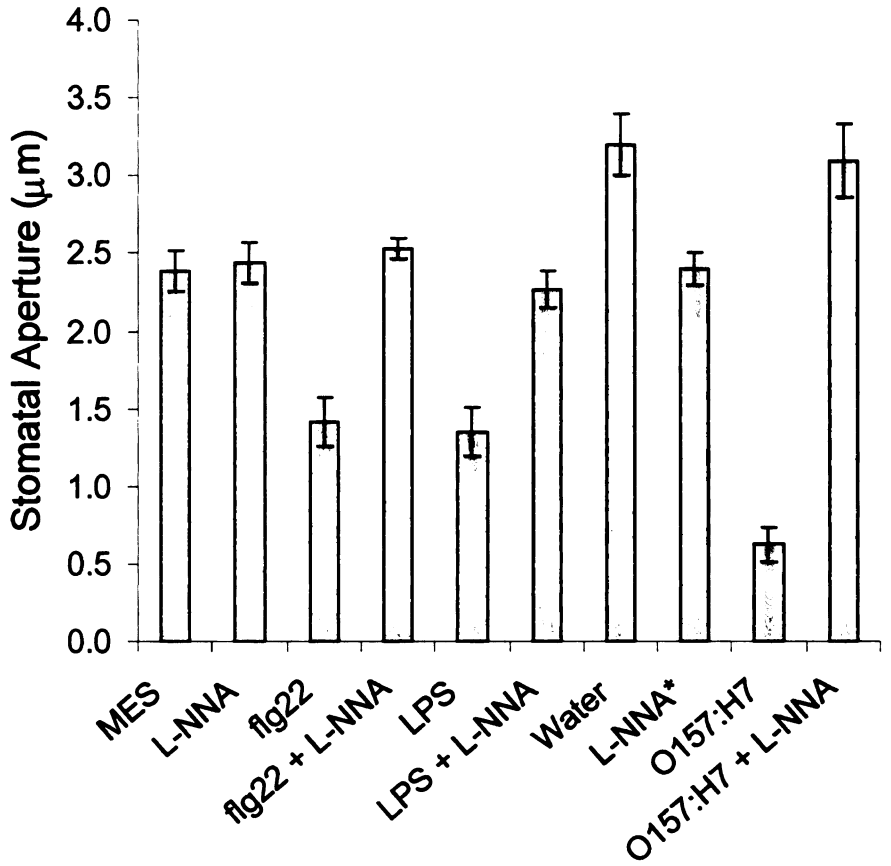

Figure 3-6: Involvement of nitric oxide (NO) in PAMP-induced stomatal closure. **(A-C)** NO production in guard cells of Col-0 treated with MES buffer (A), 5 μM flg22 (B), or 100 ng/μl LPS (C). Brightfield images are displayed on the left, with fluorescent images displayed on the right. NO production was not observed in the control treatment; therefore, only a black screen was seen under the fluorescent microscope. **(D)** Effect of the NOS inhibitor L-NNA (0.2mM) on stomatal closure induced by PAMPs (5 μM flg22 or 100 ng/μl LPS) or 1 x 10^8 cfu/ml *E. coli* O157:H7. L-NNA represents the control treatment of L-NNA dissolved in MES for treatment with purified PAMPs whereas L-NNA* represents the control treatment of L-NNA dissolved in water for treatment with *E. coli.* Stomatal aperture measurements were determined after 2 hours of treatment.

***Pst* DC3000 overcomes stomatal closure by production of the phytotoxin coronatine.**

The observation that *Pst* DC3000 causes a transient stomatal closure (Figure 3-1), whereas *E. coli* O157:H7 causes persistant closure of stomata (Figure 3-2B) suggests that *Pst* DC3000 may have evolved a mechanism (or mechanisms) to overcome PAMP-induced stomatal closure and that the non-plant-pathogen *E. coli* O157:H7 lacks this ability. It is likely that *Pst* DC3000 encodes a virulence factor that can promote re-opening of stomata that initially closed in response to PAMP perception. *Pst* DC3000 is known to encode two well-characterized virulence factors: a TTSS that secretes a battery of effector proteins, and the phytotoxin COR. To determine if either the TTSS or COR are required for re-opening of stomata by *Pst* DC3000 we examined stomatal responses to mutants defective either in the TTSS (nonpolar *hrcC*⁻; Penaloza-Vazquez et al., 2000) or in COR synthesis (*cor*⁻; Ma et al., 1991). The TTSS-deficient *hrcC*⁻ mutant was not impaired in the ability to re-open closed stomata, behaving similarly to the wild-type *Pst* DC3000 (Figure 3-7B). The *cor*⁻ mutant, however, was unable to re-open stomata, similar to *E. coli* O157:H7 (Figure 3-7A). Furthermore, purified COR at a concentration of 0.5 ng/µl could interfere with stomatal closure elicited by both flg22 and LPS (Figure 3-8A). To determine if COR acts downstream of ABA or at an earlier stage in guard cell signaling such as PAMP perception or initial signaling from PAMP receptors, we tested the ability of COR to interfere with stomatal closure induced by exogenous ABA. Stomata of epidermal peels pretreated with 0.5 ng/µl COR and subsequently incubated with 0.5 ng/µl COR + 10 µM ABA failed to close (Figure 3-8B), suggesting that COR acts downstream of ABA to interfere with stomatal closure. COR interference of ABA-induced stomatal closure was not observed in the COR-insensitive *Arabidopsis* mutant

coi1. COR did not effect production of NO in guard cells elicited either by ABA or PAMPs (Figure 3-9), suggesting that COR acts downstream or independent of NO synthesis.

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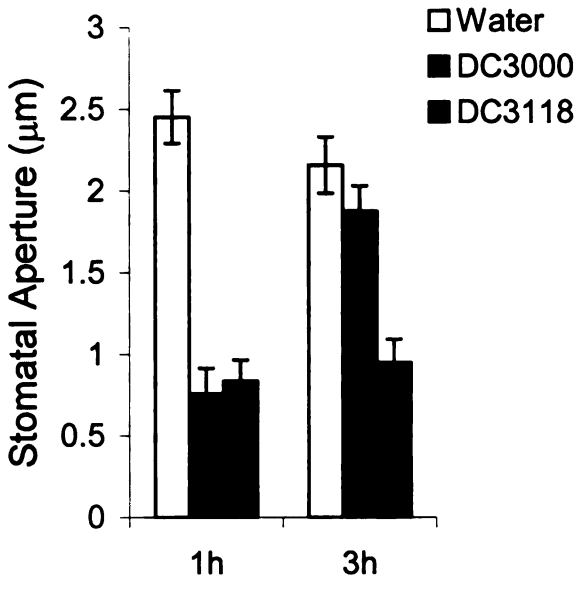

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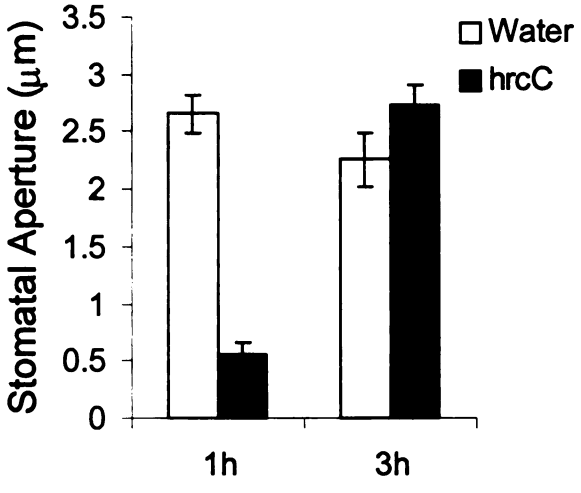
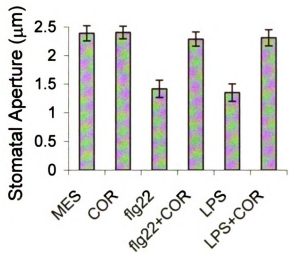


Figure 3-7: Stomatal responses to **(A)** *cor*$^-$ (DC3118) and **(B)** *hrcC*$^-$ mutants of *Pst* DC3000. Epidermal peels of Col-0 leaves were exposed to water (white bars in both figures), or 1 x 10^8 cfu/ml *Pst* DC3000 (red bars), *cor*$^-$ mutant *Pst* DC3118 (blue bars), or TTSS-deficient *hrcC*$^-$. Stomatal aperture measurements were determined after 3 hours of treatment.

A



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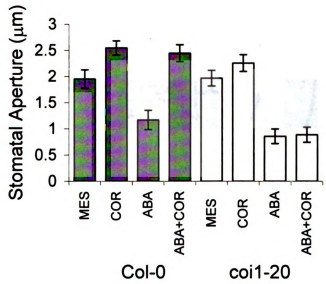


Figure 3-8: Purified COR blocks both ABA- and PAMP-induced closure of stomata. **(A)** Epidermal peels of Col-0 leaves were incubated with MES buffer, 5 µM flg22, or 100 ng/µl LPS with our without 0.5 ng/µl COR. Apertures were measured after 3 hours of treatment. **(B)** Epidermal peels of Col-0 or *coi1-20* mutant leaves were incubated with MES buffer, 0.5 ng/µl COR, 10 µM ABA, or 0.5 ng/µl COR + 10 µM ABA. For COR + ABA experiments, peels were pre-incubated with COR for 30 min. The COR solution was then replaced with COR + ABA. Apertures were measured after 1 hour of treatment.

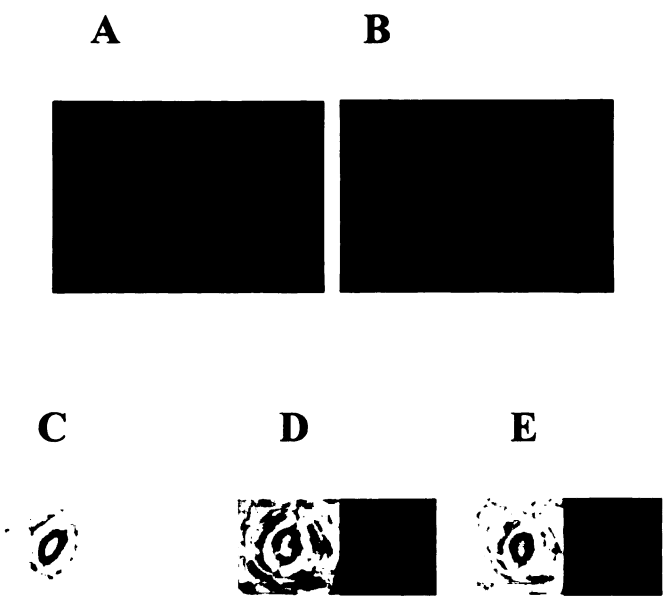


Figure 3-9: COR does not effect NO production in guard cells in response to ABA or PAMPs. **(A-B)** Fluorescence micrographs of Col-0 epidermal peels incubated with 10 μM ABA (A) or 10 μM ABA and 0.5 ng/μl COR (B). **(C-E)** Brightfield (left) and fluorescence (right) images NO production in guard cells of Col-0 epidermal peels incubated with 0.5 ng/μl COR (C), 0.5 ng/μl COR + 100 ng/μl LPS (D), or 0.5 ng/μl COR + 5 μM flg22 (E).

Biological relevance of stomatal closure in bacterial infection.

The observation that plant stomata close in response to live bacteria and purified PAMPs compellingly suggests that closure of stomata may be an important component of the plant innate immune response and provide a defense barrier against bacterial ingress. However, for this to be the case, stomata must effectively restrict entry of bacteria into the host tissue. Thus, it is important to demonstrate that closure of stomata plays a biologically relevant role in restricting bacterial entry into the leaf and protecting the plant from potential infection. To address this important question, we placed GFP-labeled *Pst* DC3000 or *cor*$^-$ *Pst* DC3118 directly underneath an epidermal peel (with the cuticle side in contact with the bacterial suspension) and examined the ability of the bacteria to move through stomata and reach the upper surface of the peel. After 3 hours of incubation, numerous GFP-labeled *Pst* DC3000 were observed in clusters on the upper surface of Col-0 epidermal peels (Figure 3-10A). In contrast, very few *cor*$^-$ mutant bacteria were observed on the upper surface of epidermal peels (Figure 3-10B). This result directly demonstrates that PAMP-induced stomatal closure can effectively restrict the movement of *cor*$^-$ mutant bacteria through stomata and that COR-producing *Pst* DC3000 can overcome stomatal closure and move through stomata.

To further test the relevance of PAMP-induced stomatal closure in the context of bacterial infection of intact plants, we examined the ability of the *cor*$^-$ mutant to infect *Arabidopsis* mutants defective in stomatal closure. When vacuum-infiltrated directly into the apoplast of *Arabidopsis* Col-0 leaves, *cor*$^-$ mutants achieved a population level similar to DC3000 after 3 days (Figure 3-11A). However, when inoculated on the surface of leaves by dipping, multiplication of *cor*$^-$ mutants in wild-type Col-0 or Landsberg *erecta*

(Ler) leaves was significantly reduced compared to wild-type *Pst* DC3000 (Figure 3-11B). These results suggest that the *cor*⁻ mutant is defective in entering the leaf tissue, but is fully virulent once inside the apoplast. Remarkably, *cor*⁻ mutant bacteria were able to achieve a population level similar to that of wild-type *Pst* DC3000 after surface inoculation onto leaves of *aba3* or *ost1 Arabidopsis* mutants, which are impaired in PAMP-induced stomatal closure (Figure 3-11B). Taken together , these results suggest that the primary virulence function of COR is to promote opening of stomata to allow bacterial entry into the leaf intercellular spaces and that this COR-mediated suppression of plant stomatal defense is critical for pathogenicity of *Pst* DC3000.

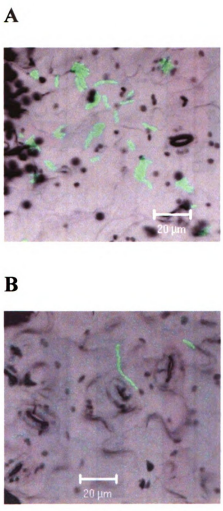
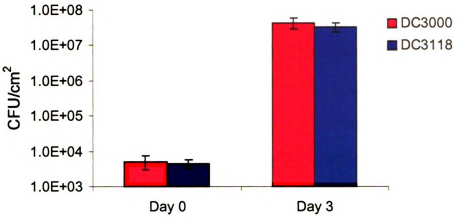


Figure 3-10: Movement of GFP-labeled **(A)** *Pst* DC3000 and **(B)** *cor*$^{-}$ *Pst* DC3118 through stomata of *Arabidopsis* Col-0 epidermal peels. Bacterial suspensions (1 x 10^{7} cfu/ml) were placed in contact with the cuticle surface of epidermal peels. Microscopic images show the relative number of bacteria (green) that moved through stomata to reach the upper side of the peel after 3 hours of incubation.

A



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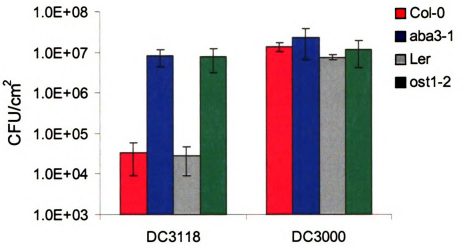

Figure 3-11: (A) Multiplication of *Pst* DC3000 (red bars) and *cor⁻ Pst* DC3118 (blue bars) in Col-0 after vacuum-infiltration of 1 x 10^6 CFU/ml bacteria. **(B)** Multiplication of *Pst* DC3000 and *cor⁻ Pst* DC3118 in *Arabidopsis* Col-0 (red bars), ABA-deficient *aba3-1* (blue bars), Landsberg *erecta* (grey bars), and *ost1-2* (green bars), after dipping in 1 x 10^8 CFU/ml bacterial suspensions. Col-0 is the wild-type control for *aba3*-1, Ler is the control for *ost1*-2. Multiplication was assessed 3 days after inoculation. Error bars represent standard deviation.

Discussion

In nature, plants encounter and interact with a wide variety of microbes. Many of these microbes may be potential pathogens against which the plant must defend itself. In recent years, a new view of plant defenses against microbes as a multi-tiered system consisting of several layers of defense responses has begun to emerge. The defenses associated with the hypersensitive response (HR) have received the most attention over the past decade, however, the discovery that plants perceive PAMPs has led to an increased appreciation for so-called innate immunity. In this chapter I have presented evidence for a new layer of innate immunity in plants. We found that stomatal guard cells present on the surface of *Arabidopsis* leaves could sense the presence of live bacteria and close to prevent bacterial entry into the leaf intercellular spaces. Prior to this discovery, stomata were widely regarded as passive ports of bacterial entry into the host, allowing bacteria to indiscriminately enter the plant. The emerging picture is that of a gauntlet of layered plant defenses that must be overcome in order for bacteria to infect a host plant and cause disease.

Pathogenic bacteria must live epiphytically on the leaf surface prior to entering the leaf through wounds or stomata, and during this period, potential pathogens are likely to encounter competition from other epiphytic microbes normally present on the surface of the leaf. Relatively little is known about the initial stages of infection, when bacteria transition from living epiphytically on the leaf surface to a pathogenic lifestyle within the host plant. This is largely due to the use of artificial inoculation procedures in the laboratory, such as vacuum-infiltration, that bypass this transition and introduce bacteria directly into the leaf tissue. The discovery that stomata play a role in defense against

bacteria is likely to open up many new areas of inquiry. It is likely that potential bacterial pathogens face challenges before and directly after entry into the host through stomata. A recent discovery suggests that immediately upon entry through stomata, pathogenic bacteria may be challenged with a battery of antimicrobial compounds in the substomatal cavity. The *Arabidopsis* ATP-binding cassette (ABC) transporter encoded by *PEN3/PDR8* has been shown to contribute to penetration resistance against a number of non-host fungi (Stein et al., 2006). Unexpectedly, *Arabidopsis pen3* mutant plants were found to be resistant to the normally virulent powdery mildew *Erisyphe cichoracearum.* This resistance was correlated with chlorosis, cell death, and activation of the SA pathway and was partially suppressed by a mutation at another locus called *PEN2. PEN2* encodes a peroxisome-localized glycosyl hydrolase that the authors speculate is involved in the synthesis of an antimicrobial compound that is subsequently exported by the ABC transporter encoded by *PEN3.* A recent study suggests that *PEN3* may also participate in the response to bacterial pathogens and, strikingly, found that *PEN3* was strongly and constitutively expressed in the cells of the substomatal cavity (Kobae et al., 2006). This result suggests that plants may have a second line of defense, in the form of secreted antimicrobial compounds, waiting for those bacteria that can overcome stomatal defenses.

In addition to the novel discovery of stomata as a relevant battleground for plant-bacteria interactions, our results have also shed light on the mystery of the role of coronatine in bacterial virulence. Our results demonstrate that COR is required for *Pst* DC3000 to overcome stomatal defenses, as the *cor*$^-$ mutant was unable to re-open closed stomata. Additionally, purified COR was sufficient to interfere with stomatal closure

induced by PAMPs and ABA. The observation that *Arabidopsis* mutants defective in closure of stomata, such as *aba3* and *ost1*, allow *cor*⁻ mutant bacteria to achieve population levels similar to wild-type *Pst* DC3000 suggests that the primary function of COR in bacterial virulence is to overcome stomatal defenses. This finding provides an explanation for the observation of Mittal and Davis (1995) over a decade ago that the *cor*⁻ mutant was defective in virulence when inoculated on the leaf surface, but not when infiltrated directly into the leaf tissue. Our results demonstrate that the "early defense" postulated by Mittal and Davis is stomatal closure. We did not observe a role for the TTSS and associated effectors in re-opening stomata. Whether the TTSS contributes to bacterial fitness and virulence prior to entering the leaf tissue or is only involved in manipulating host cells after the bacteria have entered through stomata is not yet clear. It will be interesting to determine if the genes encoding TTSS components are expressed on the leaf surface and if the TTSS plays a role in virulence prior to bacterial entry.

Only five pathovars of *P. syringae* are known to produce COR. This raises the question of whether stomatal closure is a general defense response conserved in a broad range of plants, and if so, how other *P. syringae* pathovars overcome stomatal defense. To begin to address these questions, Dr. Maeli Melotto tested the stomatal response of tomato to *Pst* DC3000 and LPS and the response of tobacco to *P. syringae* pv. tabaci. The response of tomato to both LPS and *Pst* DC3000 was similar to that of *Arabidopsis* (Melotto et al., 2006). Additionally, *P. syringae* pv. *tabaci*, which does not produce COR, induced closure of stomata initially but was able to re-open stomata at later timepoints similar to *Pst* DC3000. These results suggest that stomatal closure may be a common plant defense response initiated by perception of bacterial PAMPs and that coronatine is

only one of a number of virulence factors used by bacteria to overcome stomatal defenses. Collectively, *P. syringae* pathovars produce a variety of phytotoxins and it will be interesting to determine if any of these other toxins are also involved in the suppression of stomatal defenses.

During the course of our observations, we noticed that in experiments where leaves and epidermal peels were incubated with bacteria or purified PAMPs, a population of stomata (generally 25-35%) remained open regardless of the treatment. The concentrations of bacteria used were clearly high enough to coat the entire leaf surface with bacteria and we would expect that the leaf surface was evenly exposed to the purified PAMPs, therefore, it is somewhat unexpected that some stomata would remain open in the presence of a stimulus that should elicit closure. Stomata are required to respond to a number of stimuli including light, humidity, CO_2 concentration, bacteria, and the circadian clock. How these inputs are prioritized by guard cells and the mechanisms of prioritization are unknown but are interesting and important questions for future study. Plant leaves are generally colonized by epiphytes and potential pathogens, therefore, stomata are likely to perceive bacteria on a regular basis. However, it is likely that only a proportion of stomata are interacting with bacteria at any given time, allowing other stomata to normally carry out their roles in regulating water relations and gas exchange. It is interesting to note that most severe outbreaks of bacterial disease in crop plants are associated with periods of heavy rain or high humidity. It is possible that, under these environmental conditions, stomatal defenses are compromised due to the plant's need to prioritize stomatal responses to other stimuli, therefore allowing more bacteria to enter the leaf tissue and promoting infection. Further experiments will be

needed to determine how plants prioritize guard cell signaling events initiated by multiple external stimuli and whether stomatal defense against bacteria is compromised under conditions of rain or high humidity.

Our results demonstrate that stomata close initially in response to PAMPs present on the surface of *Pst* DC3000 and that *Pst* DC3000 can re-open stomata after approximately 3 hours. This observation clearly demonstrates a role for stomata in the early stages of bacterial infection. However, the movements of stomata throughout the entire course of infection have not yet been investigated. It is possible that stomata may also play a role later in the infection process, permitting exit of bacteria from infected leaves and spread to uninfected tissue or to other plants. It will be interesting to determine the role of stomata throughout the entire disease cycle and to determine if COR or TTSS effectors potentially play a role in modulating stomatal movements at later stages of infection.

The results presented in this chapter reveal a novel aspect of plant innate immunity against bacteria. Closure of stomata is likely to be an important, and potentially first line of defense against foliar bacterial pathogens and may be a widespread defense mechanism in vascular plants. Therefore, to be a successful foliar pathogen of plants, bacteria must either possess mechanisms to counteract stomatal defenses, or rely on environmental conditions under which stomatal defenses may be compromised in order to enter plant leaves. The discovery of PAMP-induced stomatal closure as a defense mechanism effective against bacterial plant pathogens and of COR as a suppressor of stomatal defense represents a significant advance in our understanding of bacterial

pathogenesis of plants and opens up many new avenues for potential future research on bacterial pathogenesis, guard cell signaling, and microbial ecology.

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Chapter 4

Characterization of the virulence function of HopD2, a type III effector tyrosine phosphatase of *Pst* DC3000 that suppresses basal defenses in *Arabidopsis thaliana*

Abstract

The bacterial pathogen *Pseudomonas syringae* infects plants by colonizing leaf tissue and multiplying aggressively in the leaf intercellular spaces. To promote multiplication, *Pst* DC3000 secretes a battery of effector proteins into host cells via a type III secretion system. Type III effectors are critical for virulence of *Pst* DC3000 as mutants that cannot secrete effectors are no longer virulent on normally susceptible hosts. However, little is currently known about the molecular mechanisms by which individual type III effectors promote virulence.

Most type III effectors identified in the *Pst* DC3000 genome have little or no similarity to known proteins. However, the effector HopD2 has similarity to protein tyrosine phosphatases, including a conserved active domain. To investigate the *in planta* function of HopD2 I generated transgenic *Arabidopsis* plants expressing HopD2 under the control of an inducible promotor. Expression of HopD2 suppresses basal defense-associated callose deposition elicited by the *Pst* DC3000 *hrpA* mutant and flg22-induced resistance to *Pst* DC3000, suggesting that HopD2 blocks PAMP-induced innate immunity. Transgenic plants expressing HopD2 also allow the normally non-pathogenic *hrpA* mutant to multiply to high levels within the leaf tissue. The virulence function of HopD2 is dependent on an intact phosphatase catalytic site, as transgenic plants expressing a catalytically inactive derivative do not show these effects. Interestingly, expression of the catalytically inactive HopD2 has a dominant-negative effect on the function of the wild-type HopD2. Remarkably, genome-wide expression profiling analysis suggests that HopD2 acts on host targets without affecting host gene expression.

Introduction

Type III protein secretion systems (TTSSs) and associated secreted virulence effector proteins play a major role in the infection of eukaryotic hosts by bacterial pathogens belonging to many diverse genera including *Yersinia, Salmonella, Escherichia, Pseudomonas, Xanthomonas,* and *Ralstonia* (Cornelis and Van Gijsegem, 2000). The virulence functions of TTSS effectors from mammalian and human pathogens such as *Yersinia enterocolitica* and *Salmonella enterica* have been studied extensively and have been shown to participate in manipulation of host cytoskeleton and vesicle trafficking systems to allow bacterial entry into host cells and suppression of host defenses such as phagocytosis (Abrahams and Hansel, 2006; Navarro et al., 2005). In contrast to mammalian and human pathogens, relatively little is known about the virulence functions of TTSS effectors encoded by plant pathogenic bacteria. The recent availability of genome sequences for a number of plant pathogenic bacteria such as *P. syringae* pv. tomato (Buell et al., 2003), *X. campestris* pv. campestris (da Silva et al., 2002), *X. campestris* pv. vesicatoria (Thieme et al., 2005), and *R. solanacearum* (Salanoubat et al., 2002) has allowed researchers to mine these genomes for potential TTSS effector genes using a variety of bioinformatics and functional approaches (Chang et al., 2005; Fouts et al., 2002; Guttman et al., 2002; Noel et al., 2001; Petnicki-Ocwieja et al., 2002; Roden et al., 2004; Schecter et al., 2004; Zwiesler-Vollick et al., 2002). The results of these studies showed that plant pathogenic bacteria, in general, encode and secrete many more TTSS effector proteins than their mammalian- and human-pathogenic counterparts. For example, the human pathogen *Yersinia enterocolitica* secretes 6 TTSS effectors (Navarro et al., 2005) compared to 30-40 effectors secreted by *P. syringae* pv. tomato strain

DC3000 (*Pst* DC3000; Chang et al., 2005; Schechter et al., 2004). The importance of TTSS effectors in promoting virulence is evidenced by the observation that bacterial mutants defective in assembly of the TTSS and secretion of effectors, such as *hrp* (*hypersensitive response and pathogenicity*) mutants of *P. syringae*, are unable to cause disease on normally susceptible hosts (Lindgren et al., 1986). Given the important role of TTSS effector proteins in the ability of plant-pathogenic bacteria to cause disease, understanding the function of these proteins within the host cell should significantly advance the study of plant-microbe interactions.

Studying the virulence functions of TTSS effector proteins from plant pathogenic bacteria has proven to be difficult for a number of reasons. First, bacterial mutagenesis approaches to study the function of single effector knock-out mutants have been largely unsuccessful. Single effector mutants generally show little or no reduction in virulence, presumably due to functional redundancy among the many TTSS effectors encoded by a given plant pathogenic bacterium. Second, the gene and protein sequences of TTSS effectors share little or no homology to proteins of known function, rendering comparative approaches uninformative and hindering the ability to generate testable hypotheses for the function of individual effectors. However, limited sequence similarity, such as the presence of conserved enzyme active sites in some TTSS effectors, and new approaches such as transgenic expression of effectors directly in host cells have led to insights into the function of some effectors in the host. The *Pst* DC3000 effectors AvrRpt2 and AvrPphB have both been shown to be cysteine proteases that mediate the degradation of specific host proteins. AvrRpt2 causes the degradation of the *Arabidopsis* RIN4 protein, whereas AvrPphB degrades the *Arabidopsis* PBS1 kinase (Kim et al.,

2005; Shao et al., 2003). It is not yet clear how the degradation of RIN4 and PBS1 contributes to the virulence of *Pst* DC3000. Other studies suggest that a major virulence function of TTSS effectors from plant pathogens is the suppression of host defense responses. Several *Pst* DC3000 effectors have been demonstrated to suppress programmed cell death (PCD) associated with the hypersensitive response (HR; Abramovitch et al., 2003; Jamir et al. 2004). Most notably, AvrPtoB was found to suppress PCD and to possess E3 ubiquitin ligase activity, and this activity was required for PCD supression (Abramovitch et al., 2006; Janjusevic et al., 2006). In addition to HR suppression, other *Pst* DC3000 effectors such as HopM1, AvrPto, AvrRpm1, and AvrRpt2 have been shown to suppress defense readouts associated with PAMP-induced innate immunity (DebRoy et al., 2004; Hauck et al., 2003; Kim et al., 2005).

The *Pst* DC3000 TTSS effector HopD2 (also known as HopPtoD2 and HopAO1) contains a conserved motif (HCxxGxxRS/T) characteristic of protein tyrosine phosphateses (PTPs) and exhibits such enzymatic activities *in vitro* (Bretz et al., 2003; Espinoza et al., 2003). This potential enzymatic activity provides a starting point for studying the virulence function of this effector protein. HopD2 has previously been shown to contribute to virulence of *Pst* DC3000 as deletion mutants lacking HopD2 were reduced in virulence on *Arabidopsis* (10-100 fold reduction in multiplication; Bretz et al., 2003). Additionally, when expressed ectopically in *P. syringae* pv. phaseolicola (Espinosa et al., 2003) or *Pst* strain Psy61 (Bretz et al., 2003), HopD2 caused a delay in the onset of HR in the non-host plants *Nicotiana tabacum* and *N. benthamiana* respectively. When transiently expressed in *N. tabacum*, HopD2 was also able to suppress HR-associated PCD initiated by expression of a constitutively active form of the

mitogen-activated protein kinase kinase (MAPKK) NtMEK2 (Espinosa et al., 2003). However, in a screen for effectors that could suppress HR-associated PCD elicited by the effector HopPsyA, HopD2 failed to suppress PCD in either *N. tabacum* or *Arabidopsis* (Jamir et al., 2004). The contradictory nature of these results leaves open the question of the true virulence function of HopD2 in susceptible host plants.

To study the virulence function of HopD2 in the susceptible host *Arabidopsis*, I created transgenic plants expressing HopD2 or a catalytically inactive derivative under the control of an inducible promotor. In this chapter, I report that HopD2 does not suppress the HR when expressed transgenically in *Arabidopsis*. However, transgenic expression of HopD2 does enhance the multiplication of the normally non-pathogenic *Pst* DC3000 *hrpA* mutant and suppresses defense responses associated with PAMP-induced innate immunity. The phosphatase activity of HopD2 is required for these functions, as transgenic plants expressing the catalytically inactive derivative do not show these phenotypes. To address the function of HopD2 in suppressing host defenses, I investigated the activation of defense-associated MAPKs, gene expression changes, and lipid profiles in the transgenic plants.

Experimental Procedures

Bacterial strains and media

Bacterial cultures of *Escherichia coli* (*E. coli*) were grown at 37°C in low-salt (5g/L NaCl) Luria Bertani (LB) medium (Sambrook et al., 1989). *P. syringae* cultures were grown in low salt LB at 30°C. Antibiotics were used at the following concentrations unless otherwise specified - rifampicin 60 mg/L, kanamycin 50 mg/L, ampicillin 100 mg/L, spectinomycin, 50 mg/L.

Plant growth and bacterial enumeration

Arabidopsis thaliana plants were grown in controlled growth chambers at a constant temperature of 20°C with a 12 hr photoperiod under a light intensity of 100 $\mu E/m^2/s$. Four- to six-week-old plants were used for all experiments. Bacteria for plant inoculations were grown to the mid-logarithmic phase, centrifuged at 2500 x g, and re-suspended in sterile H_2O to the specified inoculum density. For vacuum-infiltration, Silwet L-77 (Osi Specialties, Friendship, WV) was added to bacterial suspensions at a concentration of 0.004%. After inoculation, plants were left uncovered until leaves were no longer water soaked, then covered with humidity domes until completion of experiments. Bacterial population in the plant apoplast was determined as previously described (Katagiri et al., 2002). The mean values of the bacterial populations are plotted

with the standard deviation displayed as error. To assess flg22-induced resistance to *Pst* DC3000, plants were sprayed with 30 μM dexamethasone 12 hrs prior to syringe-infiltration of 5 μM flg22 or water (control). After 24 hrs, plants were vacuum-infiltrated with *Pst* DC3000 (1 x 10^6 cfu/ml) and bacterial multiplication was assessed 2 days after inoculation. All bacterial multiplication assays reported here were performed at least 3 times with similar results.

Generation of transgenic plants

Genomic DNA was extracted from *Pst* DC3000 as described previously (Chen and Kuo, 1993). PCR was used to amplify the *hopD2* coding sequence using HIFI Taq polymerase (Invitrogen, Carlsbad, CA) and the following primers:
sense primer 5'-TACTCGAGCGAGATAGTTCATACAGCTATG-3'
antisense primer 5'-CAACTAGTGCGAGAAACACTAAAGGGC-3'.
The *hopD2* gene was cloned into the pBD vector (a gift from Jeff Dangl, University of North Carolina, Chapel Hill), allowing for inducible expression of the transgene after application of the rat glucocorticoid hormone dexamethasone (DEX). The HopD2 insert was sequenced and pBD/*hopD2* was transformed into *E. coli* DH5α and transferred into *Agrobacterium tumefaciens* strain C58C1 by tri-parental mating using the *E. coli* helper strain pRK600. Four pots each of *A. thaliana* Col *gl1* plants (5 plants per pot) were transformed with *A. tumefaciens* carrying pBD/*hopD2* using the floral dip method (Clough and Bent, 1998). Seeds collected from each pot were kept separate to ensure that independently transformed lines could be isolated. T1 pBD/*hopD2* seeds were sowed in

soil and allowed to germinate. Two-week-old T1 seedlings were sprayed with a 0.2% solution of the herbicide BASTA (glufosinate-ammonium, trade name Finale, AgroEvo Environmental Health, Montvale, NJ) to select transformants.

Site-directed mutagenesis of HopD2

A phosphatase catalytically-inactive derivative of *hopD2* (*hopD2*^{C378S}) was generated by site-directed mutagenesis using the QuickChange XL mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturers protocol. The mutagenesis primers used to change the conserved catalytic Cys_{378} to Ser were:
sense primer 5'- GAGTCGCTAGTTGTGCACAGTAACGGCGGTCGGGGCCG-3'
antisense primer 5'-CGGCCCCGACCGCCGTTACTGTGCACAACTAGCGACTC-3'.

Production of HopD2 antibody

The *hopD2* gene was amplified by PCR from *Pst* DC3000 genomic DNA using the following primers:
sense primer 5'-ATCATATGCCTTTCGTCAGCCAATTACTC-3'
antisense primer 5'-AGCTCGAGAAACACTAAAGGGCTG-3'.
The gene was cloned into the pET28(a) vector (Invitrogen, Carlsbad, CA) and transformed into *E. coli* BL21. Protein was induced by addition of 1 mM IPTG to a mid-log culture and incubated for 4 hrs at 37°C. HopD2 protein was extracted from *E. coli* cells using a standard protocol (Qiagen, Valencia, CA) and purified using Ni-NTA agarose. Purified HopD2 protein was analyzed by SDS-PAGE and the band corresponding to HopD2 was excised for further purification. The gel slice was destained

for 2 hrs in 30% MeOH and 10% acetic acid followed by rinsing in H_2O for 1 hr. The gel slice was then cut into pieces and further macerated by centrifugation in a 1 ml pipette tip loaded with copper wire coil. HopD2 protein was eluted from the macerated gel overnight at room temperature by rocking in 50mM Tris + 1% SDS. The final purified HopD2 protein was analyzed by SDS-PAGE and used to raise antibodies in rabbit at Cocalico Biologicals, Inc., Reamstown, PA. Pre- and post-immune sera was obtained and checked for the ability to recognize the antigen using immunoblot analysis.

DEX induction of transgene expression

Dexamethasone (Sigma Aldrich, St. Louis, MO) was dissolved in 100% ethanol at a concentration of 30 mM and stored at -20°C. This stock solution was diluted in water to the concentrations specified for individual experiments.

Microarray analysis

Pots of *Arabidopsis* Col *gl1*, HopD2, or HopD2^{C378S} were sprayed with 30 μM DEX to induce the *HopD2* transgene. After 24 hrs, pots were vacuum-infiltrated with a bacterial suspension of 1 x 10^8 cfu/ml of the *Pst* DC3000 *hrpA* mutant or a mock inoculum of sterile H_2O with 0.004% Silwet L77 (OSI Specialties, Friendship, WV). Plants were left uncovered until leaves were no longer water soaked and then covered with a humidity dome. After 7 hrs, all fully expanded leaves from 10-12 plants for each treatment were harvested, frozen in liquid N_2, and stored at -80°C for subsequent RNA isolation. For bacterial mutant analysis, *Arabidopsis* Col-0 plants were vacuum-infiltrated with bacterial suspensions of 1 x 10^8 cfu/ml *Pst* DC3000, *Pst* DC3000 *ΔhopD2* mutant,

or a mock inoculum of sterile H_2O with 0.004% Silwet L77. Infiltrated plants were left uncovered until leaves were no longer water soaked and then covered with a humidity dome. Tissue was harvested for RNA isolation 7 hrs after infiltration in the manner described above. Total RNA was isolated from *Arabidopsis* leaves using the Promega (Madison, WI) RNAgents total RNA isolation system. The RNA concentration was determined by absorbance at 260 nm and its quality was evaluated by separation on 2% denaturing agarose gels containing formaldehyde. The total RNA was further purified using Qiagen (Valencia, CA) RNeasy minicolumns. Biotinylated target complementary RNA (cRNA) was prepared from 16μg of total RNA using the procedure described by Affymetrix (Santa Clara, CA). Labeled target cRNA was subsequently purified, fragmented, and hybridized to *Arabidopsis* ATH1 GeneChip arrays according to protocols provided by the manufacturer (Affymetrix) in a Hybridization Oven model 640 (Affymetrix). The GeneChips were washed and stained with streptavidin-phycoerythrin using a GeneChip Fluidics Station model 400 and then scanned with a Gene Array Scanner (Hewlett-Packard, Palo Alto, CA). The gene expression data was normalized and analyzed using the Affymetrix Microarray Program Suite (MAS 5.0 statistical algorithms and the Data Mining Tool (version 2.0)). Output from all GeneChip hybridizations was scaled globally such that its average intensity (TGT value) was equal to an arbitrary target intensity of 500 allowing comparisons between GeneChips. Data was then compared between sample chips from the same biological replicate producing a Signal Log_2 Ratio (SLR) calculated from the GeneChip fluorescence signal intensity data. The software was used to determine whether there was a genuine change in mRNA accumulation (Change Call, D for Decrease, I for Increase) and Change Call p-value.

SLRs, Change Calls and p-values were determined for each bacterially inoculated sample compared with its corresponding mock control or bacterial mutant treated sample. Significance Analysis of Microarrays (SAM; Tusher et al. 2001) was used to identify significant genes based on their differential expression between sets of samples. The SAM analysis feature contained in the TIGR Microarray Experiment Viewer v4.0 (Saeed et al., 2003; http://www.tm4.org/mev.html) was used to conduct the analysis. The normalized signal $\log_2$ ratios for each replicate of each comparison were used for the analysis. The two datasets (transgenic plants and bacterial mutant) were independently analyzed using the multiclass design, with the default settings. The delta value was set at a level such that the median False Discovery Rate was less than 5%. Access database management software was used to further filter and query the data. The criteria used to identify reproducibly differentially expressed probe sets were a signal $\log_2$ ratio of at least -1.0, a gene expression change call of D (decrease) and p-value >0.995 or signal $\log_2$ ratio of at least 1.0, change call of I (increase) and p-value of <0.005 for all 3 biological replicates for at least one type of comparison.

Lipid profiling analysis

Arabidopsis Col *gl1*, HopD2, or HopD2^{C378S} plants were sprayed with 30 μM DEX to induce the transgene. After 24 hrs, 5 fully expanded leaves were harvested for extraction of total lipids. Harvested leaves were quickly immersed in isopropanol + 0.01% butylated hydroxytoluene (BHT; Sigma Aldrich, St. Louis, MO) preheated to 75°C in a water bath and incubated for 15 min. Samples were removed from water bath and 1.5 ml of chloroform and 0.6 ml water were added with vortexing. Samples were

incubated with shaking at room temperature for 1 hr. Lipid extracts for each sample were transferred to a separate tube and 4 ml chloroform:methanol (2:1) + 0.01% BHT was added to the leaf samples. Samples were incubated for 30 min. at room temperature with shaking. Lipid extracts were again removed and combined with the first extract. Extraction with 2:1 chloroform:methanol was repeated 4 times and extracts for each sample were combined. Extracts were rinsed with 1 ml of 1M KCl followed by 2 ml of H_2O. Solvents were evaporated by streaming nitrogen into the tubes and lipids were resuspended in 0.5 ml chloroform:methanol (2:1) + 0.01% BHT and stored at -80°C. Extracted leaves were dried overnight in an oven and weighed to obtain dry weights. Dry weights for each sample were 8-10 mg. Lipid samples were analyzed using high pressure liquid chromatography (HPLC) coupled to a Waters MicroMass dual quadrupole mass spectrometer using electrospray ionization (ESI) in the negative ion mode. To detect phosphatidylinositol lipids, a parent ion method was used to scan the mass spectrum for lipids that fragmented to yield parent ions of 241 m/z (representing the phosphatidylinositol headgroup with no reversible phosphorylation) or 321 m/z (representing the phosphatidylinositol headgroup with a single reversible phosphorylation). Scans were conducted for 3 min. per sample. Data on PI content reported here are from single MS/MS analyses of single lipid extracts for each sample.

Callose assay

Arabidopsis Col *gl1*, HopD2, or HopD2^{C378S} plants were sprayed with 30 μM DEX and after 24 hrs were vacuum-infiltrated with a suspension of the type III secretion-defective *Pst* DC3000 *hrpA* mutant (Wei et al., 2000) at a concentration of 1 x 10^8 cfu/ml

(OD_{600} = 0.2). Plants were left uncovered after infiltration and leaves were harvested after 7 hrs. Harvested leaves were cleared of pigment by vacuum-infiltrating alcoholic lactophenol (1:1:1:1:2 phenol:glycerol:lactic acid:water:ethanol) followed by 30 min. of incubation at 65°C. The leaves were then transferred to fresh alcoholic lactophenol solution and incubated overnight at room temp. Cleared leaves were rinsed briefly in 50% ethanol, then water, and stained with aniline blue solution (0.01% aniline blue [Sigma] in 150 mM K_2HPO_4, pH 9.5). Leaves were examined with a Zeiss Axiophot D-7082 fluorescence microscope with an excitation filter of 365 ± 25 nm, a 400 nm dichroic mirror, and a 450 nm longpass emission filter. The number of callose deposits (visualized as white spots against a blue background) was determined using Quantity One software (BioRad, Hercules, CA). More than 10 adjacent fields of view along the length of the leaf were analyzed and averaged. Average numbers of callose deposits are plotted with standard deviation displayed as error. Callose assays reported here were performed 3 times with similar results.

In-gel kinase assay

Arabidopsis Col *gl1*, HopD2, or $HopD2^{C378S}$ plants were sprayed with 30 μM DEX and after 24 hours were inoculated by vacuum-infiltration with a bacterial suspension of 1 x 10^8 cfu/ml of the *Pst* DC3000 *hrpA* mutant or a mock inoculum of H_2O. Leaf tissue samples were collected at 0 (immediately prior to vacuum-infiltration), 1, 2, 3, and 6 hrs after inoculation. Four leaf discs (1.5 cm^2) per sample were collected using a #5 cork borer and immediately frozen in liquid N_2. For analysis of kinase activation after inoculation of bacterial mutants, *Arabidopsis* Col *gl1* plants were inoculated with

bacterial suspensions of 1 x 10^8 cfu/ml of *Pst* DC3000, a *Pst* DC3000 *hopD2* deletion mutant (*ΔhopD2*; Bretz et al., 2003), or *Pst* DC3000 carrying the avirulence gene *avrRpt2*. Leaf tissue samples were collected at 0 (immediately prior to vacuum-infiltration), 3, 6, and 9 hours after inoculation as described above. Frozen leaf tissue was ground using plastic micro pestles and 200 µl of extraction buffer (100 mM Hepes, pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM DTT, 10 mM Na_3VO_4, 10 mM NaF, 50 mM β-glycerolphosphate, 1 mM phenylmethylsulfonyl fluoride, 5 µg/mL antipain, 5 µg/mL aprotinin, 5 µg/mL leupeptin, 10% glycerol) was added to each sample followed by further homogenization until no large leaf pieces remained. Homogenized samples were centrifuged at 12,000 x g at 4°C for 30 min and supernatants were transferred to new tubes and stored at -80°C. Protein concentration for each sample was determined using a Pierce BCA Protein Assay Kit (Pierce, Rockford, IL) according to the manufacturers instructions. 10 µg of protein for each sample was separated on 10% SDS polyacrylamide gels containing 0.25 mg/ml of myelin basic protein (MBP) and in-gel kinase assays were performed as described previously (Zhang and Klessig, 1997). All in-gel kinase assays reported here were performed two times on independent samples with similar results.

Immunoblotting and HopD2 protein fractionation

Following DEX treatment, one cm^2 of leaf tissue was collected using a #2 cork borer, homogenized in 100 µl of protein extraction buffer (0.125M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol) and denatured at 100°C for 10 min. Equal volumes of each sample were separated on 12% SDS-polyacrylamide gels and proteins were transferred onto Immobilon-P blotting membrane (Millipore, Billerica, MA) using a

Semi-Phor semi-dry transfer apparatus (Hoefer Scientific Instruments, San Francisco, CA). Immunoblotting was carried out using HopD2 antiserum and anti-rabbit alkaline phosphatase or horseradish peroxidase conjugated secondary antibodies. HopD2 protein bands were visualized by a standard color reaction using Sigma Fast BCIP/NBT reagent (Sigma Aldrich, St. Louis, MO) or by enhanced chemiluminescence using a Pierce SuperSignal chemiluminescent detection kit according to the manufacturers protocol (Pierce, Rockford, IL). For localization of HopD2 to membrane or soluble fractions of extracts of HopD2 transgenic *Arabidopsis* plants, plants were sprayed with 30 μM DEX to induce the transgene. After 24 hrs, 2.5 cm^2 of leaf tissue was collected using a #5 cork borer and homogenized in 200 μl extraction buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% glycerol, 10 μM β-mercaptoethanol, 1x Sigma plant protease inhibitor cocktail). Following homogenization, an additional 200 μl of extraction buffer was added to each sample. Samples were centrifuged at 10,000 x g for 30 min at 4°C and supernatants were transferred to new tubes. Samples were then centrifuged at 100,000 x g for 1 hour at 4°C. The supernatant (soluble fraction) was removed and the pellet (membrane fraction) was resuspended in 400 μl extraction buffer. Protein fractions were concentrated approximately 8-fold using Centricon microcentrifuge units (Millipore, Bellerica, MA) with a 10,000 MW cutoff. After concentration, 20 μl of each sample was mixed with 5x SDS-PAGE loading buffer (0.2M Tris-HCl pH 6.8, 40% glycerol, 10% SDS, 0.0005% bromophenol blue, 15% β-mercaptoethanol), loaded and separated on 12% SDS polyacrylamide gels, and blotted as described above. HopD2 was detected as described above.

Results

Arabidopsis thaliana transgenic plants expressing HopD2 exhibit chlorosis and necrosis upon induction.

To study the function of HopD2 in promoting virulence on susceptible host plants, I created transgenic *Arabidopsis* Col *gl1* plants expressing the *hopD2* gene or the catalytically inactive derivative *hopD2*^{C378S} under the control of a DEX-inducible promotor. Transgenic lines were selected on the basis of their resistance to the herbicide BASTA. Seven independent HopD2 transgenic lines and 11 independent HopD2^{C378S} lines were analyzed by western blotting to detect the presence of the HopD2 protein after induction with DEX. Each line was scored for the development of phenotypes after DEX treatment. Two HopD2-expressing lines and two HopD2^{C378S}-expressing lines were subsequently assayed for multiplication of the *Pst* DC3000 *hrpA* mutant. HopD2 line 141 and HopD2^{C378S} line 193 were chosen for further characterization and the experiments reported here were performed using these transgenic lines. Un-induced HopD2 and HopD2^{C378S} plants were phenotypically normal and indistinguishable from the parental Col *gl1* line. However, by 3 days after induction of the HopD2 protein by spraying with 30 μM DEX, HopD2 141 plants began to develop leaf chlorosis (Figure 4-1A). By 5 to 6 days after DEX treatment, leaves of HopD2 141 plants began to exhibit a necrotic phenotype and eventually collapsed, resulting in death of the transgenic plants (Figure 4-1B). In contrast, HopD2^{C378S} 193 plants did not develop these severe phenotypes after DEX induction and were generally indistinguishable from Col *gl1* plants (Figure 4-1A

and B). Occasionally, HopD2^{C378S} developed mild chlorosis after several days of DEX treatment.

A

Col *gl1* HopD2 HopD2^{C378S}

B

Col *gl1* HopD2 HopD2^{C378S}

Figure 4-1: Leaves of Col *gl1* or HopD2 transgenic plants 3 days **(A)** or 7 days **(B)** after treatment with 30 μM DEX. Plants were sprayed with DEX every 48 hours. Leaves of HopD2 transgenic plants developed chlorosis and eventually collapsed. Note that leaves of HopD2^{C378S} plants did not develop this phenotype.

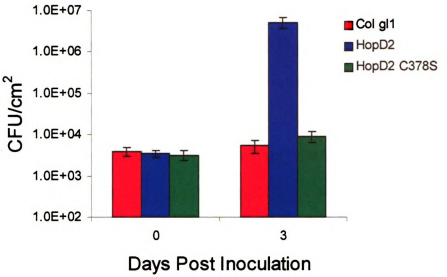
HopD2 transgenic plants allow enhanced multiplication of the *Pst* DC3000 *hrpA* mutant.

Collectively, TTSS effectors secreted by *Pst* DC3000 into host cells function to promote multiplication of the bacteria within the apoplastic space. Mutants that are defective in assembly of the TTSS such as the *hrpA* mutant cannot secrete effectors and are rendered completely non-pathogenic. Previous studies have demonstrated that transgenic expression of single *Pst* DC3000 TTSS effectors in *Arabidopsis* can be sufficient to promote significant multiplication of normally non-pathogenic *hrp* mutants (Hauck et al., 2003; Kim et al., 2005). To determine if transgenic expression of HopD2 in *Arabidopsis* could promote multiplication of normally non-pathogenic TTSS-deficient bacteria, HopD2 and HopD2^{C378S} plants were inoculated with the *Pst* DC3000 *hrpA* mutant. HopD2 transgenic plants allowed *hrpA* to multiply to significantly higher population levels than the parental Col *gl1* plants (Figure 4-2A). Importantly, this enhanced multiplication was dependent on an intact HopD2 phosphatase catalytic site as multiplication of *hrpA* in HopD2 ^{C378S} was indistinguishable from Col *gl1*. Although *hrpA* was able to achieve population levels approaching that of *Pst* DC3000 after 3 days, the TTSS-deficient mutant was not able to cause typical disease symptoms such as water soaking, chlorosis, or necrosis. DEX treated HopD2 plants inoculated with *hrpA* were visually similar to un-inoculated, DEX treated plants. Expression of HopD2 had no effect on the multiplication of *Pst* DC3000 (Figure 4-2B).

Interestingly, while HopD2^{C378S} plants did not allow the *hrpA* mutant to multiply, transgenic expression of HopD2 ^{C378S} had a dominant-negative effect on *Pst* DC3000 multiplication, causing a 5-10-fold reduction in population levels (Figure 4-2B). To

determine if this effect is a specific, dominant-negative effect on the virulence function of the native HopD2 secreted by *Pst* DC3000 during the course of an infection, the multiplication of *Pst* DC3000 *ΔhopD2*, a deletion mutant lacking *HopD2*, was investigated in Col *gl1*, HopD2 and HopD2 ^{C378S} plants. Expression of wild-type HopD2 *in planta* complemented the *ΔhopD2* mutant, allowing the mutant bacteria to achieve a population level similar to wild-type *Pst* DC3000. Multiplication of *ΔhopD2* was similar in Col *gl1* and HopD2 ^{C378S} (Figure 4-2B) suggesting that: 1) Expression of HopD2 ^{C378S} cannot complement the virulence defect of the *ΔhopD2* mutant and, 2) HopD2 ^{C378S} likely interferes specifically with the function of wild-type HopD2 delivered by *Pst* DC3000 as the virulence of the *ΔhopD2* mutant is not further reduced in HopD2 ^{C378S} plants.

A



B

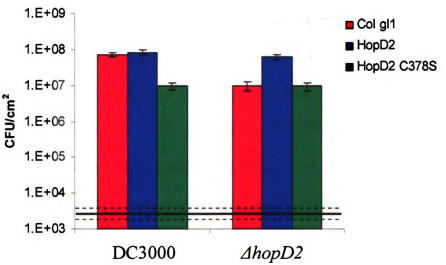


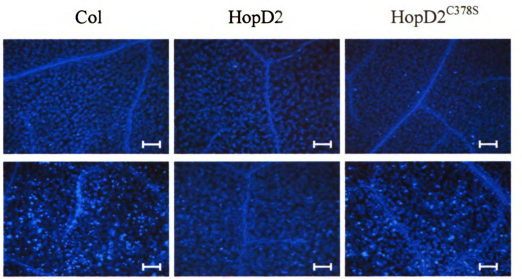
Figure 4-2: Bacterial multiplication in HopD2 transgenic plants. **(A)** Multiplication of the *Pst* DC3000 *hrpA* mutant in Col *gl1* (red bars), HopD2 (blue bars), or HopD2 ^{C378S} (green bars) plants. Plants were vacuum-infiltrated with a bacterial suspension of 1 x 10^6 cfu/ml. Error bars represent standard deviation for both charts. **(B)** Multiplication of *Pst* DC3000 or the *ΔhopD2* mutant in Col *gl1* (red bars), HopD2 (blue bars), or HopD2^{C378S} (green bars) plants. Plants were vacuum-infiltrated with bacterial suspensions of 1 x 10^6 cfu/ml and multiplication was assessed 3 days after inoculation. Bacterial populations immediately after inoculation (Day 0) were averaged and are represented by the solid horizontal line with standard deviation represented by the dashed horizontal lines.

Transgenic expression of HopD2 suppresses defense responses associated with PAMP-induced innate immunity.

TTSS effectors such as HopD2 can potentially promote multiplication and/or symptom development through several possible mechanisms. Effectors may contribute to virulence by suppressing host defense responses such as those associated with PAMP-induced innate immunity or the HR, or by manipulating host cells to promote the creation of an apoplastic environment that is more suitable for bacterial multiplication, potentially through the release of water and/or nutrients. Transgenic plants expressing HopD2 allow multiplication of the *hrpA* mutant, which is normally unable to multiply in *Arabidopsis* presumably due, at least in part, to it's inability to suppress PAMP-induced innate immunity. Therefore, I investigated the ability of HopD2 transgenic plants to mount defense responses associated with PAMP-induced innate immunity. One such defense response is the deposition of callose-rich papillae at the cell well (Gomez-Gomez et al., 1999; Soylu et al., 2005). Perception of PAMPs present on non-pathogens such as the *hrpA* mutant leads to the deposition of papillae that can be visualized by aniline blue staining for callose. After inoculation with the *hrpA* mutant, Col *gl1* leaves exhibited characteristic callose deposits (white spots on blue background) when stained with aniline blue (Figure 4-3A). In contrast, HopD2 plants exhibited a significant reduction in callose deposition (Figure 4-3A) to less than 50% of the number of callose deposits observed in Col *gl1* leaves (Figure 4-3B). An intact phosphatase catalytic site was required for HopD2 suppression of callose deposition as the number of callose deposits in HopD2^{C378} leaves was not significantly different than in Col *gl1* (Figure 4-3B).

To further test the effect of HopD2 on PAMP-induced innate immunity, the ability of treatment with the flg22 peptide to confer partial resistance to *Pst* DC3000 was investigated in Col *gl1*, HopD2, and HopD2^{C378S} plants. Infiltration of *Arabidopsis* leaves with the flg22 peptide prior to or along with *Pst* DC3000 has been shown to reduce bacterial multiplication (Zipfel et al., 2004). Consistent with published results, treatment of Col *gl1* leaves with 5 μM flg22 24 hrs prior to infiltration with *Pst* DC3000 resulted in an ~10-fold reduction in bacterial multiplication compared to H_2O-treated control leaves (Figure 4-4). Multiplication of *Pst* DC3000 in HopD2 transgenic plants did not differ significantly between leaves treated with flg22 and H_2O-treated (control) leaves, suggesting that HopD2 can block defenses activated by flg22. *Pst* DC3000 population levels in H_2O-treated HopD2^{C378S} leaves were ~5 fold less than in H_2O-treated Col *gl1* leaves, consistent with our prior observation of a dominant-negative effect of HopD2^{C378S} on *Pst* DC3000 multiplication. Multiplication of *Pst* DC3000 was further reduced in flg22-treated HopD2^{C378S} leaves, suggesting that HopD2 phosphatase activity is required for suppression of flg22-induced resistance to *Pst* DC3000.

A



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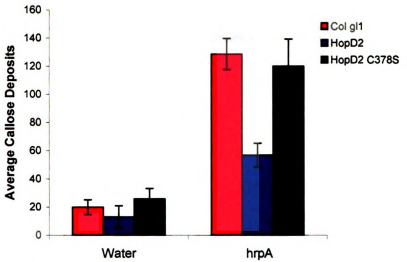


Figure 4-3: Papilla-associated callose deposition in HopD2 transgenic plants. Col *gl1*, HopD2, and HopD2^{C378S} plants were inoculated with *Pst* DC3000 *hrpA* mutant bacteria (1×10^8 cfu/ml) or sterile H_2O. Leaf tissue was collected after 8 hrs, cleared, and stained with aniline blue to detect callose. **(A)** Representative microscope images, callose deposits appear as white spots on the blue background. **(B)** Quantitation of callose deposition. Callose spots were counted for at least 10 microscope fields of view per treatment. Average numbers of deposits per field are plotted with standard deviation displayed as error.

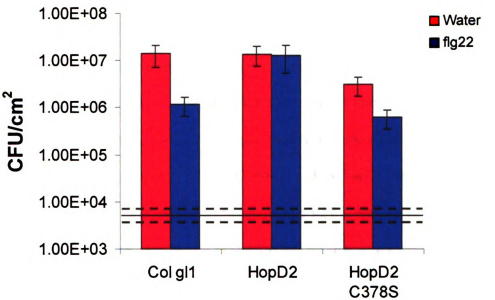


Figure 4-4: Transgenic expression of HopD2 blocks flg22-induced resistance to *Pst* DC3000. Multiplication of *Pst* DC3000 in Col *gl1*, HopD2, or HopD2 ^{C378S} plants after treatment with 5 μM flg22 (blue bars) or water (red bars) as a control. Leaves were syringe-infiltrated with flg22 or water 24 hrs prior to vacuum-infiltration with *Pst* DC3000 (1 x 10^8 cfu/ml). Multiplication was assessed 2 days after inoculation. Error bars represent standard deviation. Bacterial populations immediately after inoculation (Day 0) were averaged and are represented by the solid horizontal line with standard deviation represented by the dashed horizontal lines.

Development of the HR is not effected in HopD2 transgenic plants.

In previous studies, transient expression of HopD2 was sufficient to block or delay the onset of HR-associated PCD elicited by avirulent bacteria in the non-host plants *N. benthamiana* and *N. tabacum* (Bretz et al., 2003; Espinosa et al., 2003). However, in a screen for *Pst* DC3000 TTSS effectors that could suppress PCD elicited by the effector HopPsyA in *N. tabacum*, HopD2 failed to suppress or delay HR-associated PCD (Jamir et al., 2004). These experiments were all carried out by transiently expressing HopD2 in non-host *Nicotiana* plants. To determine if HopD2 could suppress or delay the onset of HR-associated PCD in stable transgenic *Arabidopsis* plants, we investigated the ability of the transgenic plants expressing HopD2 to mount the HR in response to *Pst* DC3000 carrying the avirulence gene *AvrRpt2*. AvrRpt2 is secreted by *Pst* DC3000 into *Arabidopsis* cells where it is recognized by the R protein RPS2, leading to the HR (Kunkel et al., 1993; Yu et al., 1993). Under our laboratory conditions, recognition of AvrRpt2 by RPS2 led to the onset of the HR in Col *gl1* plants approximately 14-16 hours after bacterial inoculation (Figure 4-5). Development of the HR in HopD2 and HopD2^{C378S} transgenic plants was indistinguishable from Col *gl1* in both the severity and timing of PCD-associated tissue collapse. Similar results were obtained when plants were inoculated with *Pst* DC3000 carrying the avirulence genes *AvrB* or *AvrPphB* (data not shown). These results suggest that the virulence function of HopD2 in the susceptible host *Arabidopsis* is not related to suppression of HR-associated PCD.

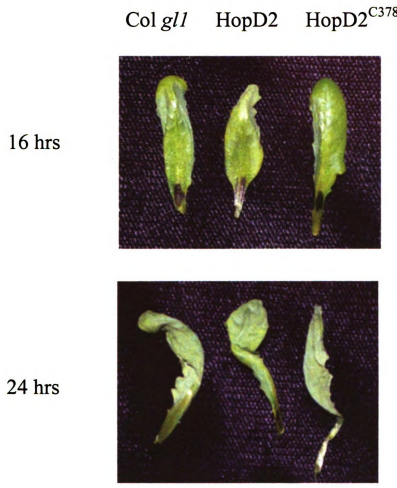


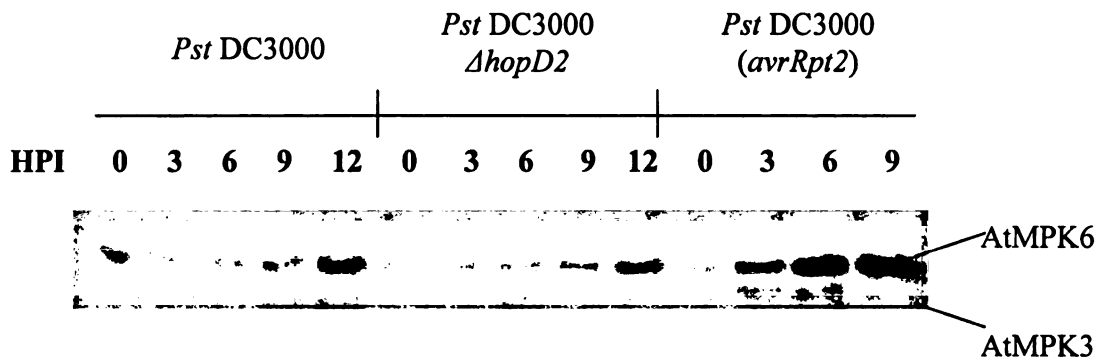
Figure 4-5: Onset of HR in Col *gl1* and HopD2 transgenic plants. Col *gl1*, HopD2, and HopD2^{C378S} plants were sprayed with 30 μM DEX 24 hrs prior to vacuum-infiltration with bacterial suspensions of 1 x 10^8 cfu/ml *Pst* DC3000 carrying the avirulence gene *avrRpt2*. Onset of HR was observed by monitoring tissue collapse associated with cell death. Representative leaves were detached for imaging at 16 hrs (upper panel) and 24 hrs (lower panel) after inoculation. Note that neither the timing, nor severity of the HR was affected by transgenic expression of HopD2.

Activation of the *Arabidopsis* MAPKs *At*MPK3 and *At*MPK6 is enhanced in HopD2 transgenic plants.

Our observations that transgenic expression of HopD2 allows multiplication of the *hrpA* mutant, reduces papilla-associated callose deposition, and blocks flg22-induced resistance to *Pst* DC3000 suggest that the virulence function of HopD2 in host plants is to suppress PAMP-induced innate immunity by dephosphorylating a host target or targets. There are several potential mechanisms through which HopD2 could act to block defenses associated with PAMP-induced innate immunity including blocking early signaling events initiated by pattern recognition receptors (PRRs) such as FLS2, altering gene expression changes associated with innate immunity, or blocking later events such as vesicle trafficking leading to deposition of papillae. Activation of mitogen-activated protein kinases (MAPKs) is known to be an early step in the signal transduction cascade initiated by the FLS2 receptor (Asai et al., 2002). The MAPKs AtMPK3 and AtMPK6 are activated in response to perception of flg22 by FLS2. Because MAPKs require tyrosine phosphorylation to become activated, we hypothesized that HopD2 may block PAMP-induced signaling initiated by PRRs by dephosphorylating and thus, inactivating AtMPK3, AtMPK6, or both. To test this hypothesis, we investigated the activation of AtMPK3 and AtMPK6 in HopD2 transgenic plants and in wild-type Col plants after inoculation with *Pst* DC3000 or the Δ*hopD2* mutant. We observed no significant difference in activation of AtMPK3 or AtMPK6 in Col *gl1* plants inoculated with *Pst* DC3000 or the *ΔhopD2* mutant. Both *Pst* DC3000 and the *ΔhopD2* mutant began to elicit activation of AtMPK6 above background levels by 9 hrs after vacuum-infiltration with more significant activation by 12 hrs after infiltration (Figure 4-6A). Activation of

AtMPK3 was not observed in response to either *Pst* DC3000 or the *ΔhopD2* mutant at the time-points observed in our experiments. As a control, Col *gl1* plants were inoculated with *Pst* DC3000 carrying the avirulence gene *avrRpt2*. This strain elicits the HR and caused strong activation of both AtMPK3 and AtMPK6 as early as 3 hours after infiltration. To our surprise, HopD2 transgenic plants exhibited significant activation of AtMPK6 and slight activation of AtMPK3 compared to Col *gl1* or HopD2^{C378S} prior to bacterial inoculation (0 hrs; Figure 4-6B). In addition to their role in signaling initiated by the FLS2 receptor, AtMPK3 and AtMPK6 are activated by multiple biotic and abiotic stresses including wounding. Constitutive activation of these kinases in plants expressing HopD2 may result from general stress as these plants develop chlorosis and necrosis after DEX treatment. AtMPK3 and AtMPK6 were activated even more strongly in HopD2 plants 1 hr after vacuum-infiltration with the *Pst* DC3000 *hrpA* mutant. HopD2^{C378S} plants also exhibited slightly higher levels of kinase activation 1 hr after inoculation compared to Col *gl1*. Taken together, our results with transgenic plants and the *ΔhopD2* bacterial mutant suggest that HopD2 does not exert its virulence activity by inactivating the MAPKs AtMPK3 or AtMPK6. These results derived from bacterial infection of stable transgenic plants are consistent with a recently published study in which HopD2 failed to block flg22-elicited activation of AtMPK3 or AtMPK6 in *Arabidopsis* protoplasts (He et al., 2006), suggesting that HopD2 acts downstream or independent of kinase activation to block PAMP-induced innate immunity.

A



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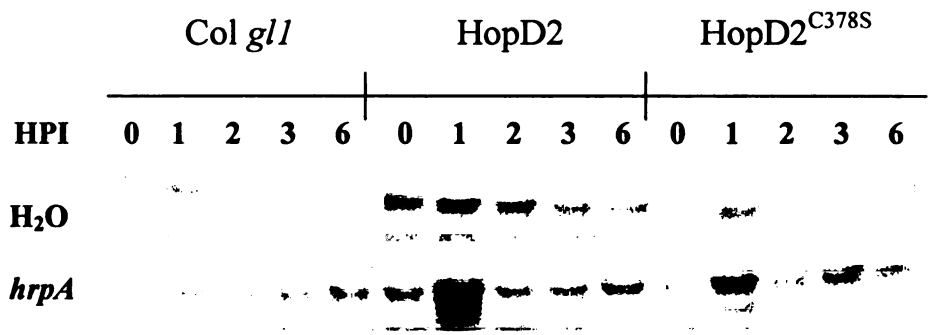


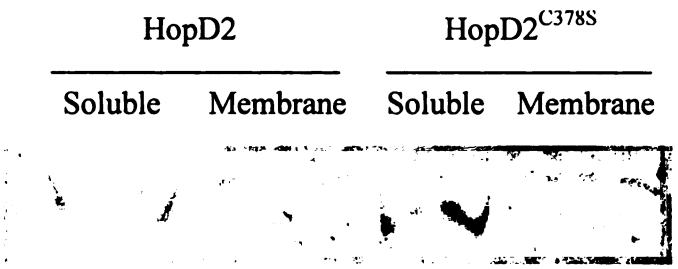
Figure 4-6: **(A)** Activation of the *Arabidopsis* MAPKs *At*MPK3 and *At*MPK6 in Col *gl1* leaves after vacuum-infiltration with 1 x 10^8 cfu/ml suspensions of *Pst* DC3000, *Pst* DC3000 *ΔhopD2*, or *Pst* DC3000 (*avrRpt2*). Leaf tissue was collected at the indicated time-points and protein extracts were used for in-gel kinase assays. **(B)** Activation of AtMPK3 and AtMPK6 in Col *gl1*, HopD2, or $HopD2^{C378S}$ leaves after inoculation with 1 x 10^8 cfu/ml *hrpA* mutant bacteria or a mock inoculum of sterile H_2O. AtMPK3 and AtMPK6 were identified by their electrophoretic mobility. In all panels, upper bands represent *At*MPK6 and lower bands represent *At*MPK3.

HopD2 is a non-transmembrane protein tyrosine phosphatase and is present in both the soluble and membrane fractions of *Arabidopsis thaliana* protein extracts.

Protein tyrosine phosphatases can be categorized into distinct subgroups based on several aspects of their sequences, subcellular localizations, and substrate specificity (Tonks, 2006). These proteins are classified as either transmembrane, receptor-like phosphatases, or intracellular phosphatases. HopD2 lacks transmembrane domains, and thus, is expected to be an intracellular phophatase. However, more specific information about the localization of HopD2 in *Arabidopsis* cells could potentially provide clues to help determine the substrate(s) of HopD2. Many TTSS effectors of *Pst* DC3000, including AvrPto and HopM1, have been discovered to be peripherally membrane associated through modifications such as myrisoylation and other unknown mechanisms (Nomura et al., 2006; Shan et al., 2000). The effector AvrPto is associated with membranes and membrane association is required for its ability to elicit the HR in resistant tomato plants carrying the Pto kinase (Shan et al., 2000) and seems to be required for its virulence function in *Arabidopsis* (Nomura and He, unpublished). To determine if HopD2 is localized to membranes in *Arabidopsis* cells, protein extracts from DEX-induced HopD2 and HopD2^{C378} plants were separated into soluble and membrane fractions by ultracentrifugation. Western blots with HopD2 antibodies revealed that HopD2 is detectable primarily in the soluble fraction of *Arabidopsis* protein extracts (Figure 4-7A). However, some HopD2 was detected in the membrane fraction and migrated at a slightly lower mobility, suggesting a potential post-translational modification. Non-transmembrane protein tyrosine phosphatases are further categorized into two classes based on substrate specificity. Classical protein tyrosine phosphatases

(PTPs) possess catalytic activity toward phosphotyrosine residues only, whereas dual specificity phosphatases (DSPs) can act on phosphoserine or phosphothreonine residues in addition to phosphotyrosine. Some DSPs have even been shown to act on non-protein substrates such as phosphorylated lipids (Robinson and Dixon, 2006). The amino acid sequence of HopD2 bears little similarity to known tyrosine phosphatases outside of the conserved catalytic motif. However, the conserved catalytic domain bears greater similarity to the canonical motif of PTPs than to that of DSPs (Figure 4-7B).

A



B

PTP --	P I V V H C S A G v G R T G
DSP --	r v l V H C q A G I S R S a
HopD2 --	S L V V H CN G G RG R T T

Figure 4-7: (A) Western blot analysis of soluble and membrane fractions of protein extracts from HopD2 and HopD2^{C378S} leaves. Plants were treated with 30 μM DEX 24 hrs prior to extraction of total proteins. Total proteins were fractionated by ultracentrifugation and fractions were analyzed by western blotting and detection with HopD2-specific polyclonal antibodies. **(B)** Amino acid sequence alignment of the consensus catalytic motifs of PTPs and DSPs (Fauman and Saper, 1996) with the catalytic region of HopD2. Capital letters in the PTP and DSP motifs represent highly conserved residues present in 90% or more of family members.

Microarray analysis of HopD2 function using transgenic plants and bacterial mutants.

As a protein tyrosine phosphatase, HopD2 has the potential to alter or disrupt signaling pathways in the host. Given that transgenic expression of HopD2 in *Arabidopsis* suppresses numerous defense readouts associated with PAMP-induced innate immunity, we hypothesized that HopD2 may alter host signaling pathways and changes in gene expression leading to the activation of basal defenses. To investigate the effect of HopD2 on gene expression changes in *Arabidopsis*, we conducted microarray analyses to profile gene expression changes associated with transgenic expression of HopD2 in *Arabidopsis*, and with the presence or absence of HopD2 in bacteria using the *ΔhopD2* bacterial deletion mutant. To determine if transgenic expression of HopD2 altered gene expression changes associated with perception of the non-pathogenic *hrpA* mutant (PAMP perception), Col *gl1*, HopD2, and HopD2^{C378S} plants were inoculated with the *hrpA* mutant (1 x 10^8 cfu/ml) or a mock inoculum of sterile H_2O 24 hrs after spraying with 30 μM DEX. Leaf tissue was collected 7 hrs after inoculation for RNA isolation, labeling, and analysis of transcripts using Affymetrix ATH1 genechips. Comparison of mock-inoculated samples also allowed the identification of HopD2-dependent changes in gene expression in the absence of bacterial challenge. A summary of the experiments and comparisons performed using HopD2 transgenic plants is presented in Table 4-1. To determine if transgenic expression of HopD2 is associated with gene expression changes in unchallenged plants, expression profiles of mock-inoculated HopD2 plants were compared to those of mock-inoculated Col *gl1* plants. Remarkably, only 11 genes were reproducibly differentially regulated in plants expressing HopD2 compared to wild-type

plants (Table 4-1, row 1). Furthermore, these changes in gene expression were not dependent on phosphatase activity, as these genes were regulated similarly in HopD2^{C378S} plants (Table 4-1, row 2). These results suggest that HopD2 phosphatase activity does not effect gene expression in unchallenged plants. To determine if HopD2 expression suppresses or alters gene expression changes induced by PAMP perception, the expression profiles of HopD2 plants inoculated with *hrpA* were compared to those of *hrpA*-inoculated Col *gl1*. This comparison revealed only 2 genes that were reproducibly differentially regulated (Table 4-1, row 3). Similar to the previous comparison, the alteration of expression observed for these genes was not dependent on HopD2 phosphatase activity (Table 4-1, row 4). Genes differentially regulated in HopD2 and HopD2^{C378S} plants compared to Col *gl1* (Table 4-1, rows 1 and 3) are displayed in Table 4-2. To verify the quality and reproducibility of our dataset, the genes reproducibly regulated in Col *gl1* by inoculation of the *hrpA* mutant were compared to the independently identified set of genes regulated by the same treatment described in Chapter 2. These datasets were found to be highly similar.

We also compared the expression profiles of Col *gl1* plants inoculated with either *Pst* DC3000, the *ΔhopD2* mutant, or a mock inoculum of H_2O. Similar to the results with our transgenic plants, we found no HopD2-specific gene expression changes in comparisons of plants inoculated with the *ΔhopD2* bacterial mutant to those inoculated with wild-type *Pst* DC3000. Taken together, these results suggest that the virulence function of HopD2 in *Arabidopsis* is not associated with alteration of gene expression or blocking early signaling events mediated by PAMP perception and that HopD2 likely blocks basal defenses at a relatively late stage, downstream of gene expression changes.

Comparison	Treatment	Repressed Genes[b]	Induced Genes[b]	Total	Expression Pattern Identified
HopD2 vs. Col *gl1*	mock, 7 hrs.	3	8	11	HopD2 regulation
HopD2 vs. HopD2^{C378S}	mock, 7 hrs.	0	0	0	Phophatase-dependent regulation
HopD2 vs. Col *gl1*	1x10^{8} cfu/ml *hrpA*, 7 hrs.	2	0	2	HopD2 alteration of PAMP regulation
HopD2 vs. HopD2^{C378S}	1x10^{8} cfu/ml *hrpA*, 7 hrs.	0	0	0	Phosphatase-dependent regulation

Table 4-1: Microarray analysis of HopD2 transgenic plants. Summary of treatments and comparisons used to identify gene expression changes resulting from transgenic expression of HopD2 and changes dependent on the phosphatase activity of HopD2.
[b] Criteria for gene selection is described in the "Experimental Procedures" section of the text.

A

Array Element	AGI	Description	Average Fold Change HopD2 vs. Col *gl1*	Average Fold Change HopD2^{C378S} vs. Col *gl1*
266385_at	At2g14610	PR-1 pathogenesis-related protein 1	3.9	4.8
265943_at	At2g19570	AtCDA1 cytidine deaminase 1	2.1	2.1
264648_at	At1g09080	putative luminal binding protein	3.5	3.3
264153_at	At1g65390	Putative TIR class disease resistance protein	-4.9	-2.9
254975_at	At4g10500	oxidoreductase, 2OG-Fe(II) oxygenase family protein	4.3	2.5
254042_at	At4g25810	AtXTR6 xyloglucan endotransglycosylase-related protein	5.7	13.0
251625_at	At3g57260	PR-2 pathogenesis-related protein 2, beta 1,3-glucanase	4.0	3.6
249032_at	At5g44910	putative disease resistance protein	-2.3	-2.3
248921_at	At5g45950	GDSL-motif lipase/hydrolase-like protein	-2.3	-5.2
247925_at	At5g57560	AtTCH4 xyloglucan endotransglycosylase	3.3	5.0
246302_at	At3g51860	AtCAX3 vacuolar Ca2+/H+-exchanging protein	4.8	4.4

B

Array Element	AGI	Description	Average Fold Change HopD2 vs. Col *gl1*	Average Fold Change HopD2^{C378S} vs. Col *gl1*
262399_at	At1g49500	expressed protein	-3.4	-2.2
260101_at	At1g73260	trypsin and protease inhibitor family protein	-4.9	-2.1

Table 4-2: Genes differentially regulated by transgenic expression of HopD2 and HopD2^{C378S}. **(A)** Differentially regulated genes in HopD2 vs. Col *gl1* and HopD2^{C378S} vs. Col *gl1* comparisons after mock inoculation with sterile H_2O (no bacterial challenge). **(B)** Differentially regulated genes in HopD2 vs. Col *gl1* and HopD2^{C378S} vs. Col *gl1* comparisons after inoculation with the *hrpA* mutant (1 x 10^8 cfu/ml).

Lipid profiling analysis of HopD2 transgenic plants.

Recent studies of protein tyrosine phosphatases have revealed that some DSPs, particularly those of the myotubularin family in mammalian cells, can act on phosphorylated lipids such as phosphoinositides (Robinson and Dixon, 2006). Phosphatidylinositol lipids can be reversibly phosphorylated and exist in a number of distinctly phosphorylated forms where the phosphotidylinositol headgroup can be reversibly singly, doubly, and triply phosphorylated. Phosphoinositides (PIs) have been shown to play an important role in regulating vesicle trafficking through interactions with protein components of the membrane trafficking machinery (Di Paolo et al., 2004; Wenk and Camilli, 2004). To determine if HopD2 dephosphorylates a PI lipid substrate in *Arabidopsis*, the content of phosphotidylinositol species in lipid extracts from Col *gl1*, HopD2, and HopD2^{C378S} leaves was analyzed by mass spectrometry. Previous studies have analyzed phosphotidylinositol species in plants that fragment to give a parent ion of mass-to-charge ratio (m/z) 241 using tandem mass spectrometry (Welti and Wang, 2004). This parent ion represents the phosphotidylinositol head group with no additional phosphate groups (ie. not reversibly phosphorylated). Using parent ion mode ESI-MS/MS, we analyzed lipid extracts from Col *gl1*, HopD2, and HopD2^{C378S} for lipids that fragmented to yield a parent ion of 241 m/z. In our lipid samples, the primary phosphotidylinositol species detected were PI 34:3 (831 m/z) and PI 34:2 (833 m/z; Figure 4-8). We did not observe any significant differences in the abundance of PI species between HopD2 transgenic plants and Col *gl1* (Figure 4-8). This result suggests that HopD2 does not dephosphorylate a mono-phosphorylated PI lipid species. We also examined the spectrum of lipids that fragmented to yield a parent ion of 321 m/z to

determine if HopD2 acts on a bi-phosphorylated PI lipid. Again, we found no significant differences in the samples derived from HopD2 plants compared to Col *gl1* plants (data not shown), suggesting that HopD2 does not dephosphorylate a bi-phosphorylated PI lipid species. Further analysis of our lipid extracts to detect PI species that fragment to yield a parent ion of 401 m/z will be required to determine if HopD2 acts on a tri-phosphorylated PI lipid. However, these initial results of analyzing the PI lipid content of HopD2 transgenic plants combined with the observation that the conserved active motif of HopD2 is more similar to that of PTPs rather than DSPs suggests that HopD2 is more likely to act on a protein substrate, and, more specifically, a tyrosine-phosphorylated protein substrate, than a phosphorylated lipid species.

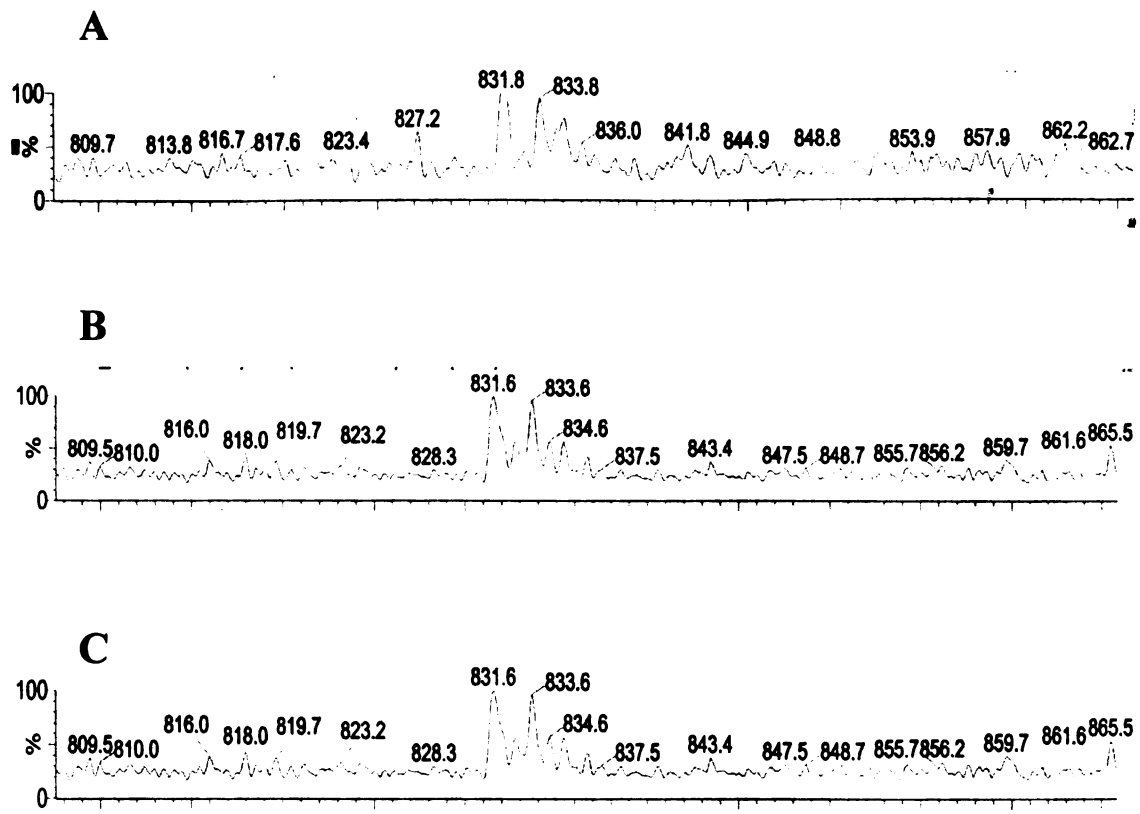


Figure 4-8: Phosphoinositide (PI) lipid content in extracts of **(A)** Col *gl1*, **(B)** HopD2, and **(C)** HopD2^{C378S} leaves 24 hrs after treatment with 30 μM DEX. Lipid extracts were analyzed by tandem mass spectrometry using a parent ion method to detect lipids fragmenting to yield a parent ion of 241 m/z. Peaks at 831 m/z represent PI 34:3 and peaks at 833 m/z represent PI 34:2. These data are from single MS/MS analyses of single lipid extracts for each sample.

Discussion

Effector proteins delivered into host cells by plant pathogenic bacteria through TTSSs are often essential virulence determinants. Emerging evidence suggests that a major role of TTSS effectors in promoting bacterial virulence is the suppression of host defense responses. However, the specific molecular mechanisms through which individual TTSS effectors function to suppress host defenses are poorly understood. The *Pst* DC3000 effector HopD2 is required for full virulence on the host plant *Arabidopsis* and is a protein tyrosine phosphatase (Bretz et al., 2003; Espinosa et al., 2003). Previous studies of HopD2 function carried out in non-host *Nicotiana* species suggested that HopD2 may promote bacterial virulence through suppression of the HR (Bretz et al., 2003; Espinosa et al., 2003). In this chapter I report that HopD2, when expressed transgenically in the host plant *Arabidopsis*, does not suppress the HR elicited by *Pst* DC3000 carrying the avirulence genes *avrRpt2*, *avrB*, or *avrPphB*, suggesting that the virulence function in the host plant is not related to suppression of the HR. Instead, transgenic expression of HopD2 suppressed several defense responses associated with PAMP-triggered innate immunity. Plants expressing HopD2 exhibited a reduced ability to deposit callose associated with papillae in response to inoculation with the non-pathogenic *hrpA* mutant and were completely impaired in resistance to *Pst* DC3000 induced by treatment with the flg22 peptide. Additionally, transgenic expression of HopD2 was sufficient to promote significant multiplication of *hrpA* mutant bacteria. Importantly, these effects of HopD2 expression were entirely dependent on an intact phosphatase catalytic site as transgenic plants expressing HopD2^{C378S} exhibited responses similar to the parental Col *gl1* plants.

HopD2, as a protein tyrosine phosphatase, may disrupt host signaling pathways leading to the activation of basal defenses. MAPKs were obvious candidates for virulence targets of HopD2 given their requirement for tyrosine phosphorylation for activation and their roles in signal transduction. Specifically, the MAPKs AtMPK3 and AtMPK6 have been shown to participate in signaling initiated by perception of bacterial flagellin by the FLS2 receptor kinase (Asai et al., 2002). However, contrary to this expectation, we found that transgenic expression of HopD2 caused enhanced activation of AtMPK3 and AtMPK6. Even more strikingly, expression of HopD2 had no effect on gene expression levels in *Arabidopsis*. No phosphatase-dependent alterations of gene expression were observed in transgenic *Arabidopsis* plants in microarray experiments, nor did HopD2 effect changes in gene expression in response to perception of *hrpA* bacteria. These results are in strong contrast with previous studies of transgenic plants expressing the *Pst* DC3000 effector AvrPto. Transgenic expression of AvrPto was found to have significant effects on gene expression and to cause changes in expression that were strikingly similar to those induced by inoculation with *Pst* DC3000 (Hauck et al., 2003). The results of our microarray experiments with HopD2 transgenic plants suggest that alteration of gene expression is not a universal characteristic of transgenically expressed TTSS effector proteins. Additionally, these results suggest that HopD2 acts at a significantly later stage to suppress basal defenses, downstream of initial signaling events and gene expression changes.

Protein tyrosine phosphatases are a broad group of enzymes including transmembrane, receptor-like phosphatases and intracellular phosphatases that act on diverse substrates including tyrosine-phosphorylated as well as serine- and threonine-

phosphorylated proteins and even phosphorylated lipids. HopD2 does not contain any predicted transmembrane domains and was localized primarily to the soluble fraction of protein extracts from HopD2 transgenic plants. However, some HopD2 protein was also detected in the membrane fraction, suggesting that HopD2 may be peripherally membrane associated. Several *Pst* DC3000 effectors, including HopM1, AvrPto, and AvrRpm1, have been shown to localize to host membranes (Nimchuk et al., 2000; Nomura et al., 2006; Shan et al., 2000). Both AvrPto and AvrRpm1 carry potential myristoylation signals that promote their membrane localization. HopD2 does not have any predicted myristoylation signals. It will be interesting to determine how HopD2 is targeted to host membranes and whether the membrane-localized pool of HopD2 is, in fact, the active form. Alternatively, the presence of HopD2 in membranes may be an artifact of overexpression in the plant cells.

Because our results suggest that HopD2 acts at a late stage to block PAMP-triggered innate immunity, we suspect that HopD2 may potentially interfere with host vesicle trafficking pathways leading to the deposition of papillae or secretion of antimicrobial compounds. Reversibly phosphorylated PI lipids are hypothesized to play a role in regulating vesicle trafficking events. We therefore examined the PI lipid content of our HopD2 transgenic plants using mass spectrometry. Our results suggest that HopD2 does not act on mono- or bi-phosphorylated PI lipids. However, we have not investigated the PI content of wild-type and HopD2 transgenic plants after induction of basal defenses by the *hrpA* mutant or flg22. It is possible that the substrate of HopD2 is only phosphorylated in response to detection of bacterial elicitors; therefore, further analysis of the PI lipids will be required to rule out these molecules as targets of HopD2

phosphatase activity. However, it is noteworthy that the conserved PTP catalytic domain of HopD2 bears greater similarity to that of tyrosine-specific phosphatases than to DSPs. Taken as a whole, these results point toward a tyrosine-phosphorylated protein(s) as the likely virulence target of HopD2.

Clearly, identifying the host target(s) of HopD2 phosphatase activity will be the next step toward a mechanistic understanding of its virulence function. Additionally, identifying the virulence target of HopD2 may provide novel information about the mechanisms of PAMP-triggered innate immunity. Currently, relatively little is known about the cell biological events leading to resistance to bacteria or about the compounds that ultimately antagonize bacterial multiplication. During the past 5 years, significant progress has been made toward understanding the early events of PAMP recognition including the identification of the flagellin receptor, signal transduction components such as MAPKs and transcription factors, and gene expression changes associated with innate immunity (Asai et al., 2002; Gomez-Gomez et al., 2000; Thilmony et al., 2006; Zipfel et al., 2004). However, the later events leading to enhanced resistance remain enigmatic. Deposition of callose-rich papillae has long been correlated with restriction of bacterial multiplication (Brown et al., 1995). However, the mechanisms by which plant cells target defense-related compounds to specific locations at the site of interaction with invading bacteria are unknown as are the specific compounds that act to restrict bacterial proliferation. Because HopD2 acts at a late stage to block PAMP-induced defenses, identifying the target(s) of HopD2 may also improve our understanding of these enigmatic components of innate immunity in *Arabidopsis*. Interestingly, the human pathogen *Yersinia pseudotuberculosis* also secretes a TTSS effector, YopH, that is a PTP.

Investigators were able to identify protein substrates of YopH by co-immunoprecipitation using a catalytically inactive derivative that was found to trap substrates in unproductive complexes in the absence of phosphatase catalytic activity (Black and Bliska, 1997). Our observation that transgenic expression of the catalytically inactive HopD2^{C378S} has a dominant-negative effect on the function of wild-type HopD2 delivered by *Pst* DC3000 suggests that HopD2^{C378S} may also form unproductive complexes with target proteins. Therefore, it may be possible to co-immunoprecipitate substrates of HopD2 using the catalytically inactive form as a substrate trap. Identifying the host target(s) of HopD2 should provide novel mechanistic insights into both bacterial virulence strategies and host innate immune responses.

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Chapter 5

Conclusions and Future Perspectives

The long-term goals of the study of plant-pathogen interactions are to improve our understanding of plant defenses against pathogens and the mechanisms used by pathogens to overcome these defenses and infect plants and to use this knowledge to improve agricultural production. There is significant potential for improvement in agricultural production through enhanced resistance to pathogens, as crop losses due to disease have been estimated to reduce worldwide agricultural productivity by 12% (Food and Agriculture Organization, 1993). In addition to increasing overall productivity and agricultural output, development of more resistant crop varieties also has the potential to reduce input costs associated with applications of pesticides and foliar sprays to control pathogen spread and proliferation. Reducing the reliance on chemical sprays and pesticides to control pathogens will also lessen the negative environmental impacts of using such chemicals. Increasing agricultural productivity and efficiency is likely to become very important in the near future, as increased demands will be placed on agriculture for the food needs of a steadily increasing human population. In addition to food needs, agriculture may also be relied on to provide other resources such as biofuels. An increase in demand for agricultural output in the form of fuel crops will likely challenge our agricultural systems to become more efficient in order to supply the demands for both food and fuel.

To develop and institute novel strategies for controlling crop losses due to pathogens will require a thorough understanding of the molecular nature of plant defenses, the mechanisms by which successful pathogens overcome these defenses, and the effects of varying environmental conditions on the effectiveness of defenses. It has become clear that plants defend themselves from pathogens by employing multiple layers

of defense responses. It is likely that durable pathogen resistance, often described as non-host resistance, is a combination of several successful layers of defense responses including surface defenses and preformed barriers, apoplastic innate immune responses, the hypersensitive response, and systemic acquired resistance. It will also be important to understand why these defense responses are effective against many potential pathogens, but fail to prevent infection by successful pathogens. The aims of the studies described here were to improve our understanding plant innate immunity triggered by perception of pathogen-associated molecular patterns (PAMPs) and the mechanisms used by a successful bacterial pathogen to overcome these defenses.

When these studies were initiated, relatively little was known about PAMP-induced innate immunity in plants. The discovery that *Arabidopsis thaliana* could perceive bacterial flagellin was a significant breakthrough that sparked an increased interest in innate immunity in plants (Felix et al., 1999). This discovery led to the identification of the flagellin receptor and several downstream signaling components and to the identification of several other PAMPs perceived by plants (Asai et al., 2002; Gomez-Gomez et al., 2000; Kunze et al., 2004; Zeidler et al., 2004). An understanding of PAMP perception and subsequent signaling events was beginning to take shape, however, downstream events such as gene expression changes and activation of defense responses were still poorly understood. We sought to investigate gene expression changes in *Arabidopsis thaliana* associated with perception of PAMPs presented on live bacteria by whole-genome microarray analysis. Additionally, we sought to analyze the specific effects of the two well-characterized *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000) virulence factors coronatine (COR) and the type III secretion system (TTSS) on

gene expression changes, including the effect of these virulence factors on expression changes associated with PAMP-induced innate immunity. Our expression profiling analyses described in chapter 2 identified 736 PAMP-regulated genes including 347 genes induced by PAMP perception. These expression profiles provide a molecular signature of PAMP-induced innate immunity in *Arabidopsis*. Additionally, our analyses identified candidate genes whose products may be involved in the downstream events leading to defense responses such as deposition of papillae. Two particularly interesting examples are the *EXO70H1* and *EXO70H2* genes that were strongly induced by PAMP perception (5-15 fold). EXO70 proteins are components of a complex known as the exocyst, which is composed of 8 subunits (Wang and Hsu, 2006). In yeast and mammalian cells, the exocyst complex has been shown to be involved in targeted exocytosis (Wang and Hsu, 2006). Although plants encode all 8 subunits of the exocyst complex (Elias et al., 2003), the role of this protein complex in plants has not been extensively characterized. Interestingly, a significant expansion of the EXO70 family of proteins has occurred in plants. The genome of the yeast *Saccharomyces cerevisiae* contains only a single *EXO70* gene, while the *Arabidopsis* genome contains 22 *EXO70* isoforms (Synek et al., 2006). The PAMP-induced *EXO70H1* and *EXO70H2* genes are closely related and form a distinct clade (Synek et al., 2006). We are currently working to identify T-DNA insertional mutants for each gene and to cross the mutants to determine if these EXO70 proteins have a specific role in responses to pathogens such as facilitating targeted vesicle trafficking leading to deposition of papillae. Our identification of transcriptional changes associated with PAMP-induced innate immunity and with

specific effects of COR and the TTSS should facilitate further inquiry into the molecular mechanisms of plant defense and COR and TTSS effector function in bacterial virulence.

Chapter 3 describes our discovery of bacterium-induced stomatal closure as an integral part of the plant innate immune response. Prior to this study, stomata had been generally regarded as passive ports of entry for bacteria. Although closure of stomata had previously been observed in response to fungal elicitors such as chitosan (Lee et al., 1999) and modulation of guard cell K^+ currents was observed during the hypersensitive response (HR) elicited by the *Cladosporium fulvum* avr protein Avr9 (Blatt et al., 1999), this study provided the first evidence that stomatal closure is a component of the plant innate immune response and effectively restricts entry of bacteria into leaf tissue. We found that stomata have the ability to perceive PAMPs present on the surface of bacteria such as flagellin and LPS. PAMP-induced closure of stomata was dependent on the presence of abscisic acid (ABA) and downstream components of the ABA signaling pathway. Additionally, we found that the virulent pathogen *Pst* DC3000 can overcome PAMP-induced stomatal closure. The phytotoxin COR was found to be the virulence factor that allows *Pst* DC3000 to overcome stomatal closure and enter leaf tissue to initiate infection. These findings open up many new areas for future research. Although ABA and components of the ABA signal transduction pathway are required for bacterium-induced stomatal closure, it is not yet clear if perception of PAMPs by pattern recognition receptors such as FLS2 leads to increased biosynthesis of ABA or relocalization of previously existing ABA pools. ABA and components of the ABA signaling pathway mediate plant responses to numerous environmental stresses. However, the signaling events upstream of ABA synthesis or mobilization are poorly

understood. Genetic screens are currently being pursued to identify signaling intermediates linking activation of pattern recognition receptors by PAMPs to the ABA signaling pathway (Zeng and He, unpublished).

The mode of action of COR in blocking stomatal closure or promoting opening of closed stomata is not yet clear. COR did not block synthesis of nitric oxide (NO) induced by PAMP perception, suggesting that COR acts downstream or independent of NO synthesis. However, the host target(s) of COR are yet to be discovered. Additionally, only five *P. syringae* pathovars are known to produce COR, yet bacterium-induced stomatal closure seems to be conserved among a variety of plant species, suggesting that other pathovars and species of phytopathogenic bacteria have evolved distinct virulence factors to overcome stomatal defense. Phytopathogenic bacteria are known to produce numerous toxins and it will be interesting to determine if any of these toxins are also involved in overcoming stomatal closure.

Guard cells are required to respond to numerous environmental stimuli including light, CO_2 concentration, humidity, and the presence of bacteria. It is likely that, in some cases, the plant may perceive conflicting signals necessitating a prioritization of the stomatal response. How guard cells prioritize input signals is not known, but is of significant relevance to plant-bacterium interactions. Severe outbreaks of bacterial disease in plants are often associated with conditions of rain or high humidity. It is possible that these conditions favor opening of stomata and allow more bacteria to enter leaf tissues, promoting infection. A thorough understanding of the response of stomata to numerous environmental inputs may facilitate the development of new strategies to control outbreaks of bacterial disease in crop plants.

Chapter 4 summarizes my work to characterize the virulence function of the *Pst* DC3000 TTSS effector HopD2 in *Arabidopsis*. HopD2 contains a conserved protein tyrosine phosphatase (PTP) catalytic motif and previous studies have shown that HopD2 possesses tyrosine phosphatase activity *in vitro* (Bretz et al., 2003; Espinoza et al., 2003). Additionally, the results of these studies suggested that HopD2 may promote bacterial virulence by blocking or delaying activation of the hypersensitive response (HR). However, I found that transgenic expression of HopD2 in *Arabidopsis* did not affect the timing or severity of the HR elicited by several *P. syringae* avr proteins. This result suggests that the virulence function of HopD2 in *Arabidopsis* is not related to suppression of the HR. Instead, I found that transgenic expression of HopD2 resulted in the suppression of defense responses associated with PAMP-induced innate immunity. Transgenic expression of HopD2 suppressed flg22-induced resistance to *Pst* DC3000, reduced papilla-associated callose deposition in response to inoculation with the *Pst* DC3000 *hrpA* mutant, and promoted significant multiplication of the normally non-pathogenic *hrpA* mutant bacteria. I also created transgenic *Arabidopsis* lines expressing the catalytically inactive HopD2^{C378S} and found that the effects of HopD2 were dependent on an intact phosphatase catalytic site.

Interestingly, transgenic expression of HopD2^{C378S} had a dominant-negative effect on wild-type HopD2 delivered from *Pst* DC3000, suggesting that the catalytically inactive HopD2^{C378S} may form unproductive complexes with host virulence targets of HopD2. PTPs in yeast and mammalian cells are often found to be involved in regulation of signal transduction pathways through the dephosphorylation of targets such as mitogen-activated protein kinases (MAPKs). We tested the ability of HopD2 to block

activation of the *Arabidopsis* MAPKs AtMPK3 and AtMPK6, which are known to be involved in signaling initiated by the FLS2 flagellin receptor (Asai et al., 2002). Contrary to our expection, activation of AtMPK3 and AtMPK6 was enhanced in HopD2 transgenic plants. Additionally, global gene expression profiling by microarray analyses revealed that HopD2 does not effect gene expression in *Arabidopsis*. These results suggest that HopD2 does not block signal transduction or gene expression changes leading to the activation of innate immunity, but likely acts downstream of these events at a later stage to block basal defenses.

Some PTPs, specifically those of the myotubularin family, have been shown to act on phosphotidylinositol lipid species (Robinson and Dixon, 2006). Phosphoinositides (PIs) are involved in numerous cell signaling and regulatory processes including the regulation of targeted vesicle trafficking events (Di Paolo et al., 2004; Wenk and Camilli, 2004). To determine if HopD2 targets *Arabidopsis* PIs, I analyzed the PI lipid content of the HopD2 transgenic plants by tandem mass spectrometry. The results of these analyses show that HopD2 does not act on mono- or di-phosphorylated PIs, however, the effect of HopD2 on tri-phosphorylated PIs has not yet been determined.

The next step toward understanding the virulence function of HopD2 will be the identification of the *Arabidopsis* target(s) that are dephosphorylated by this effector. Several approaches have already been pursued to identify physical interactors of HopD2 including yeast-two-hybrid analyses using HopD2 or HopD2^{C378S} as bait (Jin and He, unpublished) and co-immunoprecipitation experiments using the polyclonal HopD2 antibody (Underwood and He, unpublished). Thus far, our attempts to identify HopD2-interacting *Arabidopsis* proteins have not been successful. However, the observation that

HopD2^{C378S} has a dominant-negative effect on HopD2 function suggests that this catalytically-inactive protein may form unproductive complexes with host targets. The *Yersinia enterocolitica* TTSS effector YopH is also a PTP and a catalytically-inactive variant of YopH was found to trap substrates in unproductive complexes, allowing the identification of virulence targets by Co-IP (Black and Bliska, 1997). Further attempts at Co-IP, potentially using affinity-tagged versions of HopD2^{C378S}, may facilitate the identification of the virulence target(s) of HopD2. Additionally, DEX induction of the HopD2 transgene in *Arabidopsis* seedlings results in lethality. This seedling-lethal phenotype could be used as the basis for a suppressor screen to identify genetic interactors of HopD2. However, this approach assumes that the seedling-lethal phenotype is directly related to the virulence function of HopD2.

Our observation that HopD2 does not affect gene expression changes resulting from activation of basal defenses as a result of perception of the *Pst* DC3000 *hrpA* mutant suggests that HopD2 acts at a late stage to block PAMP-induced innate immunity. Relatively little is known about the molecular events leading to the activation of basal defense or the specific components of innate immunity that antagonize bacterial proliferation. Because HopD2 is likely to act at a late stage to block PAMP-induced innate immunity, identifying the virulence target(s) of HopD2 and their role in basal defenses may shed light on the cellular components mediating innate immunity in plants.

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Appendix A

This appendix contains supplementary information for Chapter 2

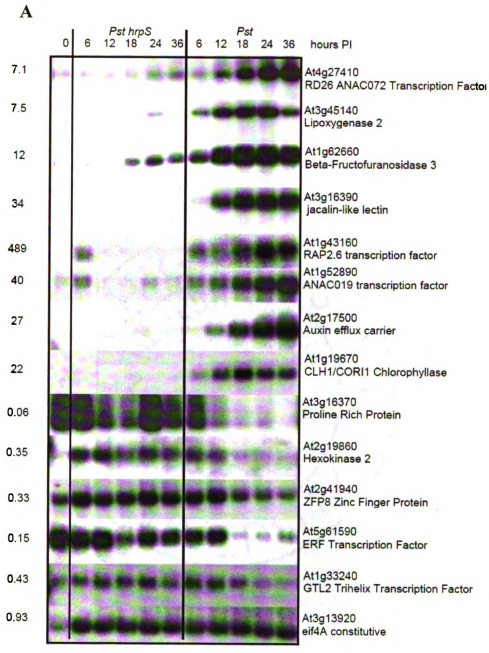
I would like to acknowledge Dr. Roger Thilmony for contribution of figures A-1, A-4, A-5, A-6, and A-7.

			Average Fold Change			
Array Element	AGI	Description	*hrpA*	COR⁻ *hrpS*	*E. coli*	DC3000
247740_at	At5g58940	receptor-like protein kinase precursor - like	1.91	2.30	1.66	-8.00
253819_at	At4g28350	lectin protein kinase family protein	2.64	1.95	2.64	-4.19
251054_at	At5g01540	receptor like protein kinase	2.46	2.19	2.35	-4.00
256378_at	At1g66830	leucine-rich repeat transmembrane protein kinase, putative	3.03	1.07	2.00	-3.82
266231_at	At2g02220	leucine-rich repeat transmembrane protein kinase, putative	3.73	2.05	3.25	-2.96
267165_at	At2g37710	lectin protein kinase, putative	1.70	2.89	1.70	-2.76
261836_at	At1g16090	AtWAKL7 WAK-like kinase	3.82	3.48	2.70	-2.76
246366_at	At1g51850	light repressible receptor protein kinase, putative	7.13	4.39	4.92	-2.64
257478_at	At1g16130	AtWAKL2 WAK-like kinase	3.25	1.91	2.96	-2.58
254241_at	At4g23190	AtCRK11 putative receptor-like kinase	4.09	2.52	2.89	-2.46
257479_at	At1g16150	AtWAKL4 WAK-like kinase	3.17	2.00	2.58	-2.24
246146_at	At5g20050	receptor-like protein kinase-like protein	2.58	1.74	2.30	-2.19
267289_at	At2g23770	putative protein kinase	2.35	1.70	1.78	-2.14
250909_at	At5g03700	S-receptor kinase-like protein	2.96	2.14	3.03	-2.09
255740_at	At1g25390	wall-associated kinase, putative	2.46	1.74	2.14	-1.95
264757_at	At1g61360	S-locus lectin protein kinase family protein	2.83	2.00	2.14	-1.87
255280_at	At4g04960	lectin protein kinase, putative	2.64	1.82	2.24	-1.78
261718_at	At1g18390	wall-associated kinase, putative	2.64	2.35	2.30	-1.78
260975_at	At1g53430	receptor-like serine/threonine kinase, putative	3.10	1.12	1.95	-1.70
246368_at	At1g51890	light repressible receptor protein kinase, putative	4.19	3.03	2.83	-1.66
257264_at	At3g22060	receptor kinase common family, putative	3.56	2.24	2.83	-1.52
256170_at	At1g51790	receptor protein kinase, putative	4.59	2.58	3.56	-1.35
248934_at	At5g46080	serine/threonine protein kinase-like protein	3.56	2.24	2.30	-1.32
254249_at	At4g23280	serine /threonine kinase - like protein	4.09	3.48	3.03	-1.23
256169_at	At1g51800	receptor protein kinase, putative	7.13	3.56	3.91	-1.20
251097_at	At5g01560	receptor like protein kinase	3.17	3.03	3.56	-1.12
260239_at	At1g74360	leucine-rich repeat transmembrane protein kinase, putative	2.46	2.89	2.09	-1.12
267436_at	At2g19190	AtFRK1 Flagellin-induced receptor-like kinase	9.40	4.19	5.53	-1.10
266203_at	At2g02230	putative phloem-specific lectin	3.32	2.89	3.10	-1.10
256181_at	At1g51820	leucine-rich repeat protein kinase, putative	9.40	3.25	5.53	-1.07
246373_at	At1g51860	receptor-like protein kinase, putative	4.29	3.48	4.70	-1.05
267624_at	At2g39660	putative protein kinase	2.09	2.30	2.35	1.00
255342_at	At4g04510	putative receptor-like protein kinase	3.32	3.65	2.83	1.02
252940_at	At4g39270	leucine-rich repeat transmembrane protein kinase, putative	2.89	2.00	2.05	1.02
264107_s_at	At2g13790	putative receptor-like protein kinase	1.23	2.30	1.32	1.07
257206_at	At3g16530	putative lectin	3.56	1.87	1.82	1.10
251096_at	At5g01550	receptor like protein kinase	4.39	1.87	2.96	1.12
251479_at	At3g59700	AtHLECRK member of Receptor kinase-like protein family	3.17	2.19	2.83	1.23
256177_at	At1g51620	protein kinase, putative	3.48	3.91	3.56	1.32
263232_at	At1g05700	putative light repressible receptor protein kinase	6.35	7.29	7.13	1.32
254271_at	At4g23150	serine/threonine kinase - like protein	2.00	3.40	1.91	1.35
255340_at	At4g04490	putative receptor-like protein kinase	2.52	2.89	1.95	1.41
247145_at	At5g65600	receptor protein kinase-like protein	8.19	4.92	2.64	1.45
246943_at	At5g25440	protein kinase family protein	1.78	2.41	1.59	1.48

254660_at	At4g18250	receptor serine/threonine kinase-like protein	3.56	3.91	2.14	1.48
246858_at	At5g25930	receptor-like protein kinase-like	3.73	1.74	2.41	1.62
256981_at	At3g13380	leucine-rich repeat family protein/protein kinase family protein	1.95	2.89	2.19	1.70
261501_at	At1g28390	protein kinase family protein	3.82	1.91	3.10	1.70
251063_at	At5g01850	protein kinase, putative	1.45	2.35	-1.35	1.74
247532_at	At5g61560	protein kinase family protein	5.04	1.82	3.40	1.96
254410_at	At4g21410	serine threonine kinase - like protein;	5.28	2.58	4.09	2.46
250640_at	At5g07150	leucine-rich repeat kinase family protein	2.14	6.65	1.95	2.64
252511_at	At3g46280	protein kinase-related	8.19	4.92	6.06	3.17
254605_at	At4g18950	protein kinase - like protein	2.19	1.48	2.09	4.19
248381_at	At5g51830	pfkB-type carbohydrate kinase family protein	2.76	2.70	1.87	6.50
248090_at	At5g55090	putative protein; similar to NPK1-related protein kinase	7.82	3.03	5.53	14.59

Table A-1: Known and predicted kinases induced by PAMP perception. Line within table denotes cutoff for PAMP-induced genes that are repressed or not significantly induced (less than 1.5-fold) by *Pst* DC3000. Genes below the line are PAMP- and *Pst* DC3000-induced. Grey shading indicates genes previously identified as induced by the bacterial PAMP flagellin.

Figure A-1


A
Pst hrpS
Pst
0 6 12 18 24 36 6 12 18 24 36 hours PI
7.1
At4g27410
RD26 ANAC072 Transcription Factor
7.5
At3g45140
Lipoxygenase 2
12
At1g62660
Beta-Fructofuranosidase 3
34
At3g16390
jacalin-like lectin
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RAP2.6 transcription factor
40
At1g52890
ANAC019 transcription factor
27
At2g17500
Auxin efflux carrier
22
At1g19670
CLH1/CORI1 Chlorophyllase
0.06
At3g16370
Proline Rich Protein
0.35
At2g19860
Hexokinase 2
0.33
At2g41940
ZFP8 Zinc Finger Protein
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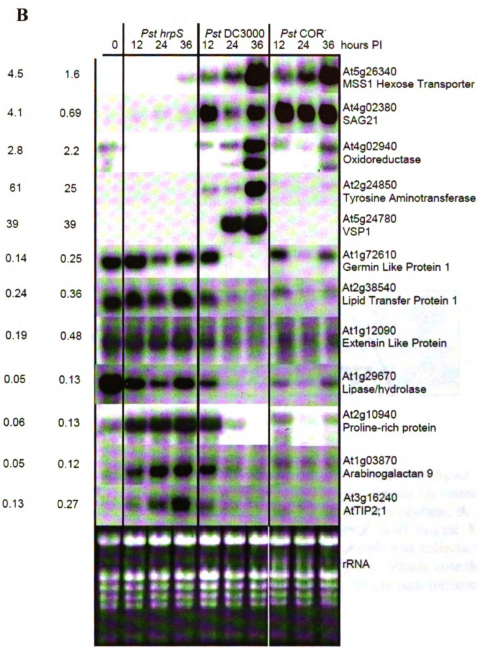


Figure A-1: RNA blot analysis of differentially expressed genes identified by microarray analysis. **(A)** Samples were harvested from uninoculated, *Pst hrpS* and *Pst* DC3000 inoculated tissues 6, 12, 18, 24 and 36 hours after infiltration with $1x10^6$ bacteria/mL. The results for 8 induced genes, 5 repressed genes, and a constitutively expressed control is shown. The average microarray ratio for a similar comparison is shown on the left. **(B)** Samples were harvested from uninoculated, *Pst hrpS*, *Pst* DC3000 and *Pst* DC3118 COR$^-$ inoculated tissues 12, 24 and 36 hours after infiltration with $1x10^6$ bacteria/mL. The average microarray ratio for the *Pst* DC3000 vs *Pst* COR$^-$*hrpS* and *Pst* DC3000 vs *Pst* COR$^-$ comparison is shown on the left. The AGI identifier and gene annotation is listed on the right.

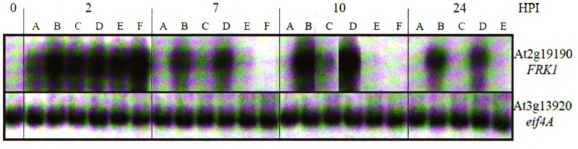


Figure A-2: RNA blot analysis of the PAMP-responsive gene *Flagellin-Induced Receptor Kinase 1* (*FRK1*). RNA samples were isolated from leaf tissues harvested at 0, 2, 7, 10, and 24 hours after inoculation with the following: **A.** Mock inoculum; **B.** *Pst hrpA*, 10^8 cfu/ml; **C.** *Pst* COR$^-$*hrpS,* 10^6 cfu/ml; **D.** *Pst* COR$^-$*hrpS,* $5x10^7$ cfu/ml; **E.** *Pst* DC3000, 10^6 cfu/ml; **F.** *Pst* DC3000, 10^8 cfu/ml. No 24 hour sample was collected for treatment F as tissue collapse had begun to occur by this time point. Please note that at 2 HPI, *FRK1* is induced in all treated tissues, presumably due to the vacuum-infiltration procedure.

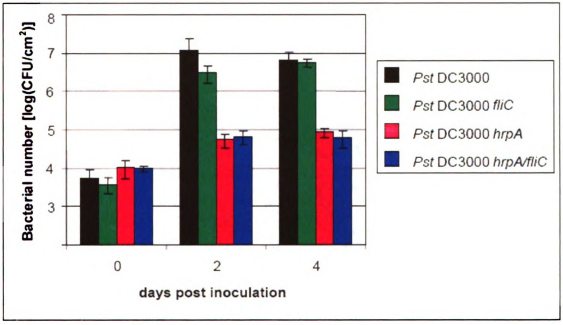


Figure A-3: *In planta* multiplication of bacterial flagellin mutants. *Arabidopsis* Col-0 leaves were vacuum infiltrated with $1x10^6$ bacteria/mL suspensions of *Pst* DC3000 (black), the *Pst* DC3000 *fliC* mutant (green), the *Pst* DC3000 *hrpA* mutant (red), or the *Pst* DC3000 *hrpA fliC* (blue) double mutant bacteria. Bacterial numbers per cm^2 leaf tissue was determined 0, 2, and 4 days after inoculation.

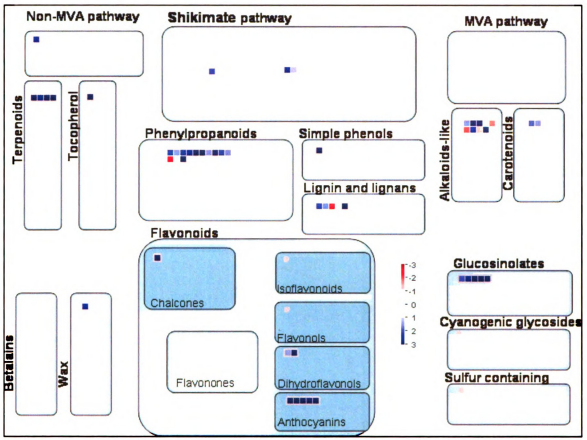
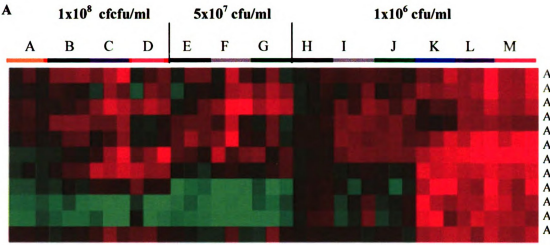


Figure A-4: COR toxin induces genes involved in secondary metabolism and TTSS effectors repress genes involved in photosynthesis. The average fold change (of the 3 biological replicates) is shown as illustrated in the fold change color bar in each panel. **(A)** The MapMan 'Secondary Metabolism' display created using the 944 COR toxin-regulated genes identified from the *Pst* DC3000 vs *Pst* COR$^-$ comparison (24 HPI, 1x10^6 bacteria/mL) is shown.

Figure A-5: *Pst* regulates the gene expression of enzymes in the tryptophan, glucosinolate and related biosynthetic pathways. **A.** The expression profile of the genes for 7 enzymes and the regulatory transcription factors *ATR1* and *ATR2* following *Pst* inoculation is shown. **B.** The expression profiles of 7 other genes which encode enzymes within the pathways displayed in C are shown. Treatments: A- *E. coli* v mock; B- *hrpA* v mock; C- DC3000 v mock; D- DC3000 v *hrpA*; E- *corhrpS* v mock; F- *cor* v mock; G- *cor* v *corhrpS*; H- *corhrpS* v mock; I- *cor* v mock; J- *cor* v *corhrpS*; K- DC3000 v *cor*; L- DC3000 v mock; M- DC3000 v *hrpA*. **C.** The enzymes encoded by the genes displayed in A and B are placed on their corresponding biosynthetic pathways. See Figure 2-1 legend for a description of the gene expression display.

Figure A-5


A
1x10^8 cfcfu/ml
5x10^7 cfu/ml
1x10^6 cfu/ml
A B C D E F G H I J K L M
At5g05730 ASA1 Anthranilate Synthase Alpha-1
At5gAt5g05730 ASA1 Anthranilate Synthase Alpha-1
At5g46760 ATR2 bHLH transcription factor
At3g57850 anthranilate phosphoribosyltransferase-like
At1g25220 ASB1/ASB2 anthranilate synthase beta
At4g39950 CYP79B2 cytochrome P450
At2g22330 CYP79B3 cytochrome P450
At1g07780 PAI1/PAI2 phosphoribosylanthranilate isomerase
At5g60890 ATR1 AtMyb34 transcription factor
At1g16400 CYP79F1/CYP79F2 cytochrome P450
At4g03060 AOP2 2-oxoglutarate dependent dioxygenase
At3g44320 NIT3 Nitrilase 3

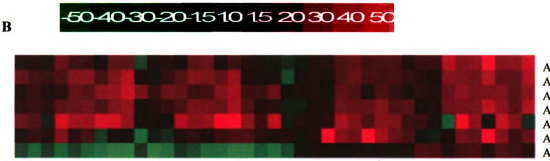

B
-50 -40 -30 -20 -15 -10 15 20 30 40 50
At5g17990 PAT anthranilate phosphoribosyltransferase
At3g54640 TSA1/TRP3 tryptophan synthase alpha
At5g54810 TSB1/TSB2 tryptophan synthase beta
At2g04400 IGPS indole-3-glycerol phosphate synthase
At3g26830 PAD3 CYP71B15 cytochrome P450
At5g38530 tryptophan synthase beta-like
At4g13770 CYP83A-1 cytochrome P450

Figure A-5 (cont'd).

C

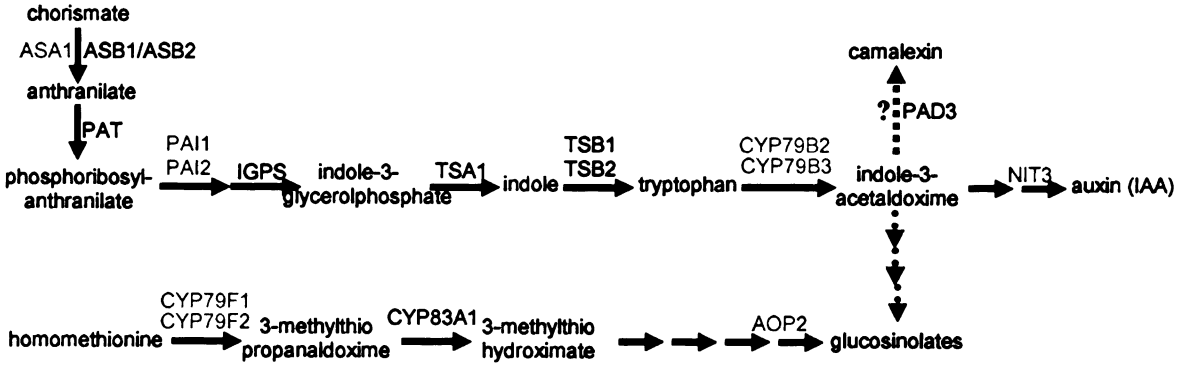


Figure A-6: Bacterial inoculation alters auxin-related gene expression. The expression profile of 44 auxin-related genes is shown. See Figure 2-1 legend for a description of the gene expression display. Treatments: A- *E. coli* v mock; B- *hrpA* v mock; C- DC3000 v mock; D- DC3000 v *hrpA*; E- *corhrpS* v mock; F- *cor* v mock; G- *cor* v *corhrpS*; H- *corhrpS* v mock; I- *cor* v mock; J- *cor* v *corhrpS*; K- DC3000 v *cor*; L- DC3000 v mock; M- DC3000 v *hrpA*.

Figure A-6

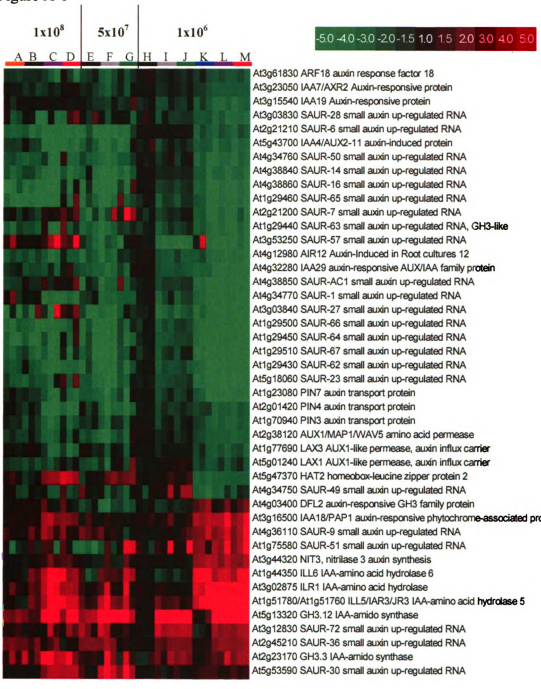

1x10^8
5x10^7
1x10^6
-5.0 -4.0 -3.0 -2.0 -1.5 1.0 1.5 2.0 3.0 4.0 5.0
A B C D E F G H I J K L M
At3g61830 ARF18 auxin response factor 18
At3g23050 IAA7/AXR2 Auxin-responsive protein
At3g15540 IAA19 Auxin-responsive protein
At3g03830 SAUR-28 small auxin up-regulated RNA
At2g21210 SAUR-6 small auxin up-regulated RNA
At5g43700 IAA4/AUX2-11 auxin-induced protein
At4g34760 SAUR-50 small auxin up-regulated RNA
At4g38840 SAUR-14 small auxin up-regulated RNA
At4g38860 SAUR-16 small auxin up-regulated RNA
At1g29460 SAUR-65 small auxin up-regulated RNA
At2g21200 SAUR-7 small auxin up-regulated RNA
At1g29440 SAUR-63 small auxin up-regulated RNA, GH3-like
At3g53250 SAUR-57 small auxin up-regulated RNA
At4g12980 AIR12 Auxin-Induced in Root cultures 12
At4g32280 IAA29 auxin-responsive AUX/IAA family protein
At4g38850 SAUR-AC1 small auxin up-regulated RNA
At4g34770 SAUR-1 small auxin up-regulated RNA
At3g03840 SAUR-27 small auxin up-regulated RNA
At1g29500 SAUR-66 small auxin up-regulated RNA
At1g29450 SAUR-64 small auxin up-regulated RNA
At1g29510 SAUR-67 small auxin up-regulated RNA
At1g29430 SAUR-62 small auxin up-regulated RNA
At5g18060 SAUR-23 small auxin up-regulated RNA
At1g23080 PIN7 auxin transport protein
At2g01420 PIN4 auxin transport protein
At1g70940 PIN3 auxin transport protein
At2g38120 AUX1/MAP1/WAV5 amino acid permease
At1g77690 LAX3 AUX1-like permease, auxin influx carrier
At5g01240 LAX1 AUX1-like permease, auxin influx carrier
At5g47370 HAT2 homeobox-leucine zipper protein 2
At4g34750 SAUR-49 small auxin up-regulated RNA
At4g03400 DFL2 auxin-responsive GH3 family protein
At3g16500 IAA18/PAP1 auxin-responsive phytochrome-associated protein 1
At4g36110 SAUR-9 small auxin up-regulated RNA
At1g75580 SAUR-51 small auxin up-regulated RNA
At3g44320 NIT3, nitrilase 3 auxin synthesis
At1g44350 ILL6 IAA-amino acid hydrolase 6
At3g02875 ILR1 IAA-amino acid hydrolase
At1g51780/At1g51760 ILL5/IAR3/JIR3 IAA-amino acid hydrolase 5
At5g13320 GH3.12 IAA-amido synthase
At3g12830 SAUR-72 small auxin up-regulated RNA
At2g45210 SAUR-36 small auxin up-regulated RNA
At2g23170 GH3.3 IAA-amido synthase
At5g53590 SAUR-30 small auxin up-regulated RNA

Figure A-7: *Pst* alters host cytokinin-related gene expression. Seven type A response regulator genes, one type B response regulator (*ARR14*) and *IPT3,* a cytokinin synthase are repressed following *Pst* inoculation. Two cytokinin oxidase genes (*CKX4* and *CKX5*) are induced. See Figure 2-1 legend for a description of the gene expression display. Treatments: A- *E. coli* v mock; B- *hrpA* v mock; C- DC3000 v mock; D- DC3000 v *hrpA*; E- *corhrpS* v mock; F- *cor* v mock; G- *cor* v *corhrpS*; H- *corhrpS* v mock; I- *cor* v mock; J- *cor* v *corhrpS*; K- DC3000 v *cor*; L- DC3000 v mock; M- DC3000 v *hrpA*.

Figure A-7

1x10^8 cfu/ml
5x10^7 cfu/ml
1x10^6 cfu/ml
A
B
C
D
E
F
G
H
I
J
K
L
M
At1g74890 ARR15
At2g40670 ARR16
At5g62920 ARR6
At1g19050 ARR7
At3g48100 ARR5
At1g10470 ARR4
At3g63110 IPT3
At3g57040 ARR9
At2g01760 ARR14
At4g29740 CKX4
At1g75450 CKX5
-50 -40 -30 -20 -15 -10 15 20 30 40 50