INTERPLAY BETWEEN BACTERIAL VIRULENCE AND PLANT INNATE IMMUNITY IN *PSEUDOMONAS-ARABIDOPSIS* INTERACTIONS

by

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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Department of Plant Pathology
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ABSTRACT

Plants activate innate immune responses or innate immunity upon pathogen infection. There are two types of plant innate immunity: PAMP-triggered innate immunity (PTI) and effector-triggered innate immunity (ETI). The molecular basis for ETI has been well documented. However, the study on PTI and its interplay with pathogen virulence is in its infancy. My research focuses on the interplay between PTI and bacterial virulence in *Pseudomonas-Arabidopsis* interactions.

NHO1, a gene required for nonhost resistance to *Pseudomonas syringae*, encodes for the 3-glycerol kinase in *Arabidopsis* genome. *NHO1* functions, at least in part, by depriving glycerol from nonhost bacteria cells. *NHO1* is induced by a well-known bacteria PAMP flg22. The induction of *NHO1* correlates well with the resistance against *Pseudomonas syringae* pv. *tabaci* because a mutant strain of *P. s.* pv. *tabaci* deficient in *NHO1* induction gains partial virulence on *Arabidopsis* plants. *P. s.* pv. *tomato* strain DC3000 induces transient *NHO1* expression that is suppressed in a type III secrection system-dependent manner. Using protoplast assay, nine DC3000 effectors that are able to suppress *NHO1* were identified. One of them, HopAI1, induces leaf chlorosis and helps nonpathogenic bacterial growth when expressed in *Arabidopsis* plants, suggesting that HopAI1 has virulence activity *in planta*.

To study AvrB virulence activity in *Arabidopsis* plants, one mutant compromised in AvrB-specific *RAR2.6* induction has been characterized in detail. *rrb3* is more susceptible to a nonhost bacteria *P. s.* pv. *tabaci* strain 6505, a virulent bacteria *P. s.* pv. *tomato* strain DC3000 and an avirulent bacteria strain DC3000 (*avrB*). The mutant allele *rrb3* carries a point mutation at the end of RAR1 CHORD II domain. *RRB3* (*RAR1*), together with *NDR1*, is involved in the type II nonhost resistance to *P. s.* pv. *tabaci* but not in the type I nonhost resistance to *P. s.* pv. *phaseolicola*. *RAR1* participates in basal resistance against DC3000 by antagonizing COI1 activity. AvrB targets RAR1 to trigger AvrB-dependent leaf chlorosis and enhanced bacterial growth. The AvrB-dependent enhanced bacterial growth but not leaf chlorosis requires COI1, suggesting that AvrB targets JA signaling pathway to promote parasitism.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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DEDICATION

This work is dedicated to my past beloved uncle Qinhe Li.

CHAPTER 1

LITERATURE REVIEW

THE MOLECULAR BASIS OF PLANT HOST AND NONHOST RESISTANCE

Introduction

Plants are sessile and encounter various environmental challenges, abiotic or biotic, during their lifecycle. To protect themselves, plants have developed a complicated defense system. Upon the invasion of biotic offenders, plants use both preformed barriers and active defense responses to keep the invaders out. One of the well-characterized preformed barriers for foliar pathogen is the plant surface structure. Pathogen infection could be hindered by the wax and cuticle layer covering plant epidermal cells, or by the unfavorable size, location and shapes of stomata and lenticels. Besides the physical barriers, plants also produce antimicrobial chemicals such as phytoalexin to restrict pathogen infection. Once the preformed barriers are overcome, plants initiate active defense responses upon pathogen infection. There are two major types of plant induced defense responses, systemically induced resistance (SIR) and localized innate immunity (LII). SIR refers to resistance that is induced in uninfected above-ground part of plants by a chemical treatment or pathogen infection. The well-studied SIR includes systemic acquired resistance (SAR, Durrant and Dong, 2004), induced systemic resistance (ISR, van Loon et al., 1998) and wound inducible resistance (WIR, Kessler et al., 2002). SAR is triggered by a group of small signaling molecules, including salicylic acid (SA) and its analogs, or by pathogen infection (Durrant and Dong, 2004). ISR is elicited by nonpathogenic rhizobacteria colonizing roots (van Loon et al., 1998). WIR is induced upon tissue damage typically caused by feeding insects (Kessler et al., 2002). The signaling network of SIR involves salicylic acid (SA), jasmonic acid (JA) and ethylene with intertwining crosstalk (Pieterse and Van Loon, 2004). Recently, brassinosteroids and abscisic acid were also found to play a role in SIR (Nakashita et al., 2003; Ton and Mauch-Mani, 2004).

LII refers to local defense responses triggered by plant pattern recognition receptors (PRRs) upon recognition of pathogen associated molecular patterns (PAMPs) or by resistance (R) proteins upon recognition of avirulence (AVR) proteins (Ausubel, 2005). Often the plant LII is associated with a rapid, localized hypersensitive response (HR) elicited by gene-for-gene resistance, which accounts for most cultivar level resistance and in some cases, species level resistance (Zhao et al., 2005). The species level resistance conferred by an entire plant species

to a whole pathogen species or pathovar is also called nonhost resistance (Thordal-Christensen, 2003). In the past twenty years, the understanding of gene-for-gene resistance has been greatly expanded with the intensive study of AVR-R interactions. However, the molecular basis for nonhost resistance is poorly explored until recently. With the discovery of PAMPs and their corresponding receptors in plants, PAMP triggered immunity (PTI) has been established as an important molecular mechanism for nonhost resistance and is emerging as a research front in the field of molecular plant-microbe interactions. PTI, together with effector-triggered immunity (ETI), forms the important layers of plant innate immunity (reviewed in Thordal-Christensen, 2003; Cunha et al., 2006; Chisholm et al., 2006) and shares striking similarity with animal innate immunity (reviewed in Nurnberger and Brunner, 2002; Buttner and Bonas, 2003; Nurnberger et al., 2004;).

To counteract plant resistance, pathogens have developed sophisticated virulence strategies to avoid, suppress or break through host surveillance systems (reviewed in Hornef et al., 2002; Finlay and McFadden, 2006). One of the strategies used by pathogen is the modification of PAMPs to avoid host recognition (Hornef et al., 2002). Pathogens such as bacteria, fungi and oomycetes can also avoid recognition by losing or mutating an AVR gene whose product is recognized by the hosts. In some cases, lost of a functional AVR gene will incur a fitness penalty on susceptible host, thus imposing a selection pressure on pathogen population (Leach et al., 2001; McDonald and Linde, 2002). Other strategies include the secretion of toxins, cell wall degrading enzymes and virulence effectors. In this chapter, recent advances on PAMP and AVR perception, suppression of PAMP or AVR signaling by pathogen virulence machineries, the molecular basis of nonhost resistance and potential application of plant innate immunity in disease management will be discussed.

PAMPs and Their Recognition by the Hosts

PAMPs, also called MAMPs (microbe associated molecular patterns), refer to pathogen structures or components, usually indispensable for the microbial lifestyle, that are not found in potential hosts (Nurnberger et al., 2004). Some PAMPs are not critical for bacteria survival but mediate bacterial virulence activity and are required for bacteria pathogenesis. For example,

flagellum is dispensable for bacteria viability whereas critical for bacteria motility. A bacterium lacking functional flagellin synthesis or flagellum assembly genes is unable to exert full virulence on its hosts (Takeuchi et al., 2003; Tans-Kersten et al., 2001). Due to their indispensability for bacteria survival or virulence, PAMPs have been used as ideal targets by host surveillance systems.

The perception of PAMPs is mediated by a group of pattern recognition receptors (PRRs), including transmembrane Toll-like receptors (TLRs) and cytosolic Nod proteins in animals as well as receptor like kinases (RLKs) and NBS-LRR proteins in plants (Ausubel, 2005). TLRs are *Toll* family proteins characterized by an extracellular leucine-rich repeat (LRR) domain and an intracellular Toll/IL-1 receptor (TIR) domain (Medzhitov, 2001). Both LRR and TIR domains are conserved protein-protein interaction modules found in proteins involved in ligand recognition or signal transduction (Medzhitov, 2001). The first Toll family protein, *Drosophila Toll*, was identified in fruitfly determining dorsal-ventral embryonic polarity (Hashimoto et al., 1988). TLR family proteins were then found in mammals and other vertebrates. In plants, clear TLR homologues are lacking (Zipfel and Felix, 2005). However, a group of RLKs and RLPs (receptor like proteins) have been identified as important players in plant growth, development and plant-microbe interactions (Torii, 2004). In the *Arabidopsis* genome, there are more than 600 RLK family members (Morillo and Tax, 2006). About 200 of them, including the well-characterized RLKs such as CLV1 (CLAVATA1, Clark et al., 1997), BRI1 (brassinosteroid-insensitive 1, Li and Chory, 1997), FLS2 (flagellin sensing 2, Gomez-Gomez and Boller, 2002) and the recently identified EF-Tu receptor EFR1 (elongation factor Tu receptor 1, Zipfel et al., 2006), belong to the LRR-RLK subfamily (Torii, 2004).

Nod proteins are a family of intracellular PRRs characterized by their nucleotide-binding oligomerization domain (NOD) (Inohara and Nunez, 2003; Inohara et al., 2005). The well-characterized Nod proteins include apoptosis protease activating factor 1 (APAF1), mammalian NOD-LRR proteins and plant NBS-LRR proteins (Inohara and Nunez, 2003). Most Nod proteins are composed of a variable N-terminus effector-binding domain that is responsible for signaling, a conserved NOD domain that mediates self-oligomerization and a C-terminus ligand recognition domain that interacts with cytosolic ligands. The structure

homology among APAF1, NOD-LRR and plant NBS-LRR proteins indicates that they may use similar mechanism to activate downstream signaling (Inohara and Nunez, 2003).

PAMP Perception and Signaling in Animal Systems

In animal systems, TLR-mediated PAMP perception triggers NFκB activation, cytokine production and inflammatory responses via a series of phosphorylation events. A well-characterized example is the LPS receptor TLR4 (Medzhitov et al., 1997). TLR4 recognize LPS with the assistance of LBP (soluble LPS-binding protein), CD14 (membrane attached co-receptor) and MD-2 (soluble protein associated with TLR4). Upon ligand binding, the cytosolic TIR domain of TLR4 recruits myeloid differentiation factor 88 (MyD88) adaptor protein, interleukin-1 receptor-associated kinases (IRAKs), Toll-interacting protein (TOLLIP) and TNF-receptor associated factor 6 (TRAF6) to form a signaling complex, which subsequently phosphorylates IκB (inhibitor of NFκB) and activates the transcription factor NFκB (Miller et al., 2005). Similar receptor complexs and signaling cascades were also found in other TLR-mediated innate immune response except TLR3 (Sioud, 2006). Recently, cytosolic Nod proteins have been shown to mediate PAMP perception in addition to TLRs. How these cytosolic Nod proteins recognize PAMPs and activate downstream activities is not fully understood. However, APAF1, a well-studied Nod protein, was found to activate caspases and apoptosis through self-oligomerization (Zou et al., 1997). In the absence of death stimuli, APAF1 self-oligomerization is suppressed by its N-terminus WD40 domain (Hu et al, 1998; Srinivasula et al., 1998). In response to cell damage, cytochrome c is released from mitochondria and binds to the WD40 domain of APAF1. APAF1 is then oligomerized and pro-caspase-9 is recruited (Saleh et al., 1999; Benedict et al., 2000). Deletion of the WD40 domain leads to constitutive activation of procaspase-9 independent of cytochrome c (Hu et al, 1998; Srinivasula et al., 1998), indicating WD40 is a negative regulator of APAF1 activation.

PAMP Perception and Signaling in Plants

In plants, bacteria PAMPs such as peptidoglycan, cold shock protein, LPS, flagellin, and EF-Tu elicit medium alkalinization (Felix and Boller, 2003), oxidative burst (Meyer et al., 2001), nitric oxide production (Zeidler et al., 2004), callose deposition (Gomez-Gomez et al.,

1999), PR gene expression and other basal defense responses (Newman et al., 2002; Keshavarzi et al., 2004). Other PAMPs like chitin, β-glucan, xylanse and cell wall proteins from fungi and oomycetes trigger phytoalexin and ethylene production (Zhang et al. 2002; Ramonell et al., 2002; Ron and Avni, 2004), electrolyte leakage and PR gene expression (Ron and Avni, 2004). Based on their localization, these PAMPs can be grouped into three classes, the surface exposed PAMPs, the cytosolic PAMPs and the secreted PAMPs.

The surface exposed PAMPs include flagellin, LPS, peptidoglycan from bacteria and chitin, β-glucan, cell wall proteins from oomycetes. Some of these surface exposed PAMPs are well studied with their PRRs in plants identified. For example, the β-glucan elicitor (GE), released from *Phytophthora sojae* cell wall component 1,3-β-glucan, has been characterized as a defense elicitor in soybean and other *Fabaceae* family plants (Fliegmann et al., 2004). The corresponding receptor protein, GE binding protein (GEBP), has been purified through ligand-affinity chromatography. The soybean GEBP protein is membrane localized and composed of a GE binding domain and a β-glucan endoglucosidase domain with endo-1,3-β-glucanase activity. GEBP protein represents a unique type of PRR with dual functions in PAMP perception. It releases GE from β-glucans present in fungal or oomycete cell walls with its endo-1,3-glucanase activity. The released GE then binds to the GEBP and induces phytoalexin production (Fliegmann et al., 2004; Fliegmann et al., 2005). However, it is not determined whether GE perception plays a role in plant disease resistance. Another surface exposed PAMP, chitin, was recently found to bind chitin elicitor binding protein (CEBiP) purified from rice cell cultures. CEBiP is a plasma membrane protein with two extracellular LysM motifs (Kaku et al., 2006). Interestingly, LysM motifs are also present in Nod factor receptor kinases that recognize lipochitooligosaccharides, chitin oligosaccharides modified by fatty acid, sulfate or sugars (Madsen et al., 2003). Knock-down of CEBiP leads to loss or reduction of chitin induced responses and gene expression, indicating CEBiP is the bona fide PRR for chitin perception (Kaku et al., 2006). A loss-of-function mutation of chitin responsive gene has been linked to increased susceptibility against *Erysiphe cichoracearum,* the causal agent of powdery mildew disease on *Arabidopsis* (Ramonell et al., 2005), suggesting a correlation between chitin perception and disease resistance.

Most of our current knowledge on PAMP signaling and regulation in plants is provided by Boller's group working on flagellin perception. Flagellin is conserved in eubacteria. It has been identified as a general elicitor in boiled *Pseudomonas syringae* pv. *tabaci* crude extracts which induce strong medium alkalization in tomato cell cultures (Felix et al., 1999). A conserved N-terminal 22 amino acid peptide of flagellin (flg22) has full elicitor activity and triggers strong growth inhibition in *Arabidopsis thaliana* seedlings (Gomez-Gomez et al., 1999). The *fls2* gene controlling the flg22-induced growth inhibition was identified in a genetic screen. FLS2 is an LRR-RLK sharing high sequence similarity with the resistance protein Xa21 (Gomez-Gomez and Boller, 2000). Flg22 directly binds to FLS2 (Chinchilla et al., 2006). Upon flagellin binding, FLS2 rapidly activates a downstream MAPK (mitogen-activated protein kinase) cascade, WRKY family transcription factors (Asai et al., 2002), ion channels, the NADPH oxidase complex (Gomez-Gomez and Boller, 2002), defense gene expression (Zipfel et al., 2004) and callose deposition (Gomez-Gomez et al., 1999; Kim et al., 2005). The FLS2 mediated signaling cascade is negatively regulated by a kinase-associated protein phosohatase (KAPP) and FLS2 internalization (Gomez-Gomez and Boller, 2002; Gomez-Gomez et al., 2001; Robatzek et al., 2006), maintaining the transient status of flagellin signaling.

The second class of PAMPs, cytosolic PAMPs, has been characterized recently. To date, there are two bacteria cytosolic proteins found to elicit innate immune responses in plants: the cold shock protein (CSP) and the elongation factor EF-Tu. Cold shock protein was identified in an experiment initially designed to characterize peptidoglycan, a surface exposed PAMP. Peptidoglycan from Gram-positive bacteria activates innate immunity in animal system (Michel et al., 2001). Commercially available peptidoglycan from *Micrococcus lysodeikticus* elicits strong medium alkalinization in cultured cells of *Solanales* species such as tobacco, potato and *L. peruvianum*. Interestingly, a proteinaceous fraction in the peptidoglycan preparation was 100 fold more active than the nonproteinaceous fraction. Further analysis of this fraction led to the purification of CSP as a new elicitor that is present in all bacteria. Similar to flagellin, the conserved N-terminal 15-22 amino acid residues spanning a RNA-binding motif RNP-1 possess the full elicitor activity (Felix and Boller, 2004). The PRR

recognizing CSP has not been identified. However, PRR recognizing another cytosolic PAMP, EF-Tu, has been recently characterized, providing a good example of cytosolic PAMP perception. EF-Tu triggers medium alkalinization in *Arabidopsis* and other *Brassicaceae* family plants. Peptides containing the N-terminus 18-26 amino acids, elf18 to elf26, retain the full elicitor activity. *Arabidopsis* plants treated with elf18 exhibit enhanced oxidative burst, ethylene production and resistance to bacterial pathogens (Kunze et al., 2004). EF-Tu and flagellin induce a common set of responses and are perceived by closely related LRR-RLKs (Zipfel et al., 2006). Similar to FLS2, the EF-Tu receptor EFR has an extracellular LRR domain, a transmembrane domain and an intracellular serine/threonine kinase domain. EF-Tu perception activates defense responses that limit *A. tumefacien* infection in *Arabidopsis* leaves. Interestingly, tobacco plants that are highly sensitive to *Agrobacterium* transformation do not respond to EF-Tu. Transient expression of *Arabidopsis* EFR gene in tobacco leaves restores EF-Tu responses, indicating that lacking a functional EFR is responsible for the high transformation efficiency in tobacco (Zipfel et al., 2006). It is noteworthy that CSP is recognized by *Solanales* but not by *Brassicaceae* (Felix and Boller, 2004) whereas EF-Tu is only recognized by *Brassicaceae* (Zipfel et al., 2006). PAMP perception may be a molecular mechanism for nonhost resistance that contributes to host determination. How the bacteria cells release cytosolic PAMPs is not fully understood, but it is conceivable this occurs upon bacteria lysis. It is well known that plants secrete various proteases and enzymes into intercellular spaces, which may directly attack and break the invading bacteria cells.

The only known PRR recognizing a secreted PAMP is the ethylene-inducing xylanase (EIX) receptor. Xylanase is a 22-kD fungal protein purified from *Trichoderma viride.* Xylanase induces ethylene biosynthesis, electrolyte leakage, PR protein expression and a HR like response (Ron and Avni, 2004). The xylanase and the elicitor activity of EIX are independent of each other (Furman-Matarasso et al., 1999; Rotblat et al, 2002). EIX binds to two tomato RLPs with a cytosolic endocytosis signal motif, LeEix1 and LeEix2. However, only LeEix2 transduces the HR induction signal. The HR inducing capability of EIX through LeEix2 is dependent on its endocytosis signal. Interestingly, it has been shown that EIX was transported into cytoplasm after binding the plasma membrane, indicating a role of LeEix2 in

both EIX endocytosis and HR induction (Ron and Avni, 2004). Another well known secreted PAMP is harpin, a group of water soluble, HR eliciting proteins that are acidic, heat stable, and glysine rich encoded by the hypersensitive response and pathogenesis (hrp) island (Wei et al., 1992; Galan and Collmer, 1999). As other PAMPs, harpin is conserved across bacteria species and required for bacterial virulence on compatible hosts (Wei et al., 1992; He et al., 1993; Lee et al., 2001). The first harpin, hrpN, was identified in *Erwinia amynovora* (Wei et al., 1992). Similar proteins have then been found in *Pseudomonas syringae* (hrpZ; He et al., 1993; Preston et al., 1995) and *Rolstonia solanacearum* (popA; Arlat et al., 1994). Harpin is secreted from TTSS pilus tip into the plant apoplast (Li et al., 2002) and binds to lipid bilayers of plant plasma membrane to form ion-conducting pores (Lee et al., 2001). Whether this pore-forming activity is mediated by membrane localized receptor protein(s) remains unknown. Identification of harpin binding protein, if there is one, will provide a great example of a HR eliciting protein that recognizing a PAMP. Resistance conferred by such a protein is likely to provide durable and broad-spectrum resistance (Staskawicz et al., 2001; Chisholm et al., 2006).

Polymorphism of PAMP Recognition

The recognition of PAMPs is highly structure-dependent. For example, human TLR4 receptor recognizes hexa-acylated but not penta-acylated LPS from *P. aeruginosa* (Hajjar et al., 2002). Variation in one or more amino acid residues in the conserved flg22 peptide reduces or abolishes its recognition by FLS2 (Zipfel et al., 2004; Sun et al., 2005). Interestingly, the flagellins from *P. s.* pv. *tabaci* and *P. s.* pv. *glycinea* sharing identical amino acid sequence are differentially recognized due to different patterns of glycosylation (Takeuchi et al., 2003; Taguchi et al., 2006). In fact, shielding or modification of exposed PAMPs is an important strategy used by bacteria to evade host recognition and immune response (Hornef et al., 2002). Recently, it was shown that the oligomerization status and the localization of flagellin are also important for its recognition (Simth et al., 2003; Miao et al., 2006; Franchi et al., 2006). Human TLR5 recognizes *Salmonella typhimurium* flagellin at its C-terminus D1 domain. Deletion of 28 amino acid residues in this domain abrogate the ability of flagellin to trigger TLR5 mediated NF-kB activation (Smith et al., 2003). Interestingly, the D1 domain recognized by TLR5 is buried inside the flagellar filaments. Given that polymers of flagellin do not bind to TLR5 and

flagellin filaments trigger NF-kB activation only after deplomerization, monomeric flagellin, instead of flagellar filaments must be recognized by TLR5 (Smith et al., 2003). A recent report showed that the biologically active monomeric flagellin is not a product of flagellar shearing. In fact, it is secreted upon contact with host cells. Host produced lysophospholipids act as a signal molecule in flagellin secretion (Subramanian and Qadri, 2006). Monomeric flagellin can also be delivered into host cytosol and recognized by IPAF1 (or CARD12, Caspase recruitment domain family member 12) in a TLR5-independent manner, making the mechanism of PAMP perception more complicated (Miao et al., 2006; Franchi et al., 2006). Similar to *Salmonella* flagellin D1 domain, *Pseudomonas* flg22 is also embedded in the inner core of flagellin filament (Zipfel and Felix, 2004). How this hidden peptide binds to FLS2 remains mysterious. It is possible that flagellin monomer instead of flagellar filament acts as the defense-eliciting PAMP. If this is true, it will be interesting to ask how this flagellin monomer is produced and whether the production of monomeric flagellin needs a host signal.

On the other hand, polymorphism in individual PRR alleles also affects PAMP recognition. Human TLR4 polymorphisms led to variation in ligand recognition or downstream signaling events, which in turn changed the disease outcomes upon bacteria infection (Arbour et al., 2000; Miller et al., 2005). In plants, point mutations in the FLS2 kinase domain reduced flagellin binding whereas point mutations in the LRR domain totally abolished the ligand binding activity of FLS2 (Gomez-Gomez et al., 2001; Gomez-Gomez and Boller, 2000; Bauer et al., 2001). The sensitivity of PAMP perception indicates that it is a tightly regulated and evolutionary active biological activity in the arms race between pathogens and their corresponding hosts.

In general, PAMPs are conserved molecules required for bacterial survival or virulence. The recognition of PAMPs by plant PRRs triggers defense responses at the species level. It is interesting that plant recognize even cytosolic proteins. However, an important bacterial virulence determinant, the conserved TTSS pillus protein, which is in close contact with plant cells, is not recognized. In fact, the *Yersinia* TTSS base forming protein, YscF, does trigger an antibody response when injected into mice (Matson et al., 2005), indicating that the TTSS apparatus could be recognized by the hosts. Recent phylogenic study of hrpA genes from

22 *Pseudomonas syringae* bacteria strains reveals that it is the diversifying selection that keeps hrpA from being recognized (Guttman et al., 2006). Identification of PRRs that targeting HrpA and driving the diversifying selection will provide a useful model for studying the evolutionary relationship between PAMPs and PRRs (Guttman et al., 2006). Whether other PAMPs are undergoing similar selection process is an open question. It appears that PAMP recognition by PRRs might also be dynamic and undergoing selection albeit the process may be slower than that of AVR recognition by R proteins.

AVR Recognition and R Protein Activation in Plants

It has been 13 years since the cloning of the first plant R gene Pto (Martin et al., 1993). To date, more than 60 R genes and more than 40 AVR genes have been identified. Most of these R proteins have a conserved nucleotide binding domain (NBS) and a C-terminal leucine rich repeat (LRR). Based on the structural characteristics of their N-terminus domain, NBS-LRR type of R proteins can be classified into TIR- and CC-NBS-LRR proteins (reviewed in Baker et al., 1997; Staskawicz et al., 2001; Martin et al., 2003). Other R proteins include transmembrane receptor like proteins, such as Xa21 from rice and Cf family proteins from tomato (Romeis, 2001; Rivas and Thomas, 2005), variants of NBS-LRR proteins, such as Pita from rice, RRS1 and SLH1 from *Arabidopsis* (Bryan et al., 2000; Lahaye, 2004; Noutoshi et al., 2005), cytosolic kinase like proteins such as Pto from tomato and Rpg1 from maize, or R proteins that do not fall into any of the above classes (reviewed in Martin et al., 2003; McDowell and Woffenden, 2003). Recently, an inducible R gene, Xa27, has been characterized in rice-*Xanthamonas oryzae* interaction (Gu et al., 2005). Xa27 shares identical sequence in the coding region with its susceptible allele. However, only the resistant allele is directly induced by *Xanthomonas oryzae* pv. *oryzae* strains containing avrXa27, a AvrBs3/PthA family effector protein with conserved nucleotide binding motifs and transcription activation domain. The differential expression of Xa27 resistant and susceptible alleles is caused by the sequence variation in their promoter region that is responsible for the disease outcome (Gu et al., 2005). The identification and characterization of Xa27 and its avirulence effector avrXa27 provide a new model of AVR-R interaction in which avirulence effector directly binds to the promoter region of R genes and induces R gene expression.

Recognition of AVR Effectors

Except for Xa27, most R genes rely on their protein products to carry out AVR recognition. There are two widely discussed models explaining how R proteins recognize AVR proteins: the ligand-receptor model and the guard-guardee model. In the ligand-receptor model, R proteins act as receptors for AVR effectors. The binding of the AVR protein triggers R protein activation and disease resistance. The most likely candidate R proteins that fit in this model should have been the typical transmembrane receptor like R proteins because they structurally mimic known receptor proteins. However, direct binding between these R proteins and their AVR effectors has never been demonstrated (Luderer et al., 2001). At present, there are only three examples of direct AVR-R interaction: The AvrPita-Pita interaction in *Magnaporthe grisea*-rice pathosystem (Bryan et al., 2000), the PopP2-RRS1 interaction in *Rostonia solanacearum-Arabidopsis* pathosystem (Deslandes et al., 2003) and the AvrL567-L5, 6, 7 interactions in *Melampsora lini*-flax pathosystem (Dodds et al., 2006). Both Pita and RRS1 are NBS-LRR proteins. Pita has a typical NBS domain, a N-terminal CC domain and a C-terminal LRR domain (Bryan et al., 2000). RRS1 is a TIR-NBS-LRR type of R protein with unique features. RRS1 has two tandem TIRs at its N terminus and a WRKY domain at its C terminus. Upon binding PopP2, RRS1 translocates to the nucleus and regulates gene expression (Deslandes et al., 2003). L5, 6, 7 are flax rust resistance TIR-NBS-LRR proteins recognizing the secreted, 127 amino acid Avr567 protein variants encoded in the highly polymorphic *Avr567* locus (Dodds et al., 2004). There were 12 Avr567 variants identified and named from A to L. Each individual variant has been tested for its interaction with L5, L6 and L7 respectively in the yeast two-hybrid system. The specificity and strength of the interactions correlate well with the HR eliciting ability of Avr567 variants and with the virulence level of rust strains carrying those variants. For example, Avr567-D interacts with L6 but not L5. Consistently, AvrL567-D specifically induces a necrotic response in L6 and is not able to infect L6 plants. Another Avr567 variant, Avr567-B slightly interacts with L6 and only triggers a weak chlorotic response and weak resistance in L6 plants (Dodds et al., 2006).

Compared to the ligand-receptor model, the guard-guardee model seems to explain the activity of a greater number of R proteins. The central idea of guard-guardee model, or the

guard hypothesis, is that AVR effectors mediate virulence activity by targeting and modifying host component(s). R proteins detect the modification and trigger disease resistance. Based on this hypothesis, the host targets should interact with the AVR effectors *in planta* and enhance pathogen virulence in the absence of the corresponding *R* gene. To date, there are four plant proteins identified as targets of AVR effectors. They are *Arabidopsis* RIN4 (RPM1 interacting protein 4) targeted by *Pseudomonas syringae* effectors AvrB, AvrRpm1 and AvrRpt2 (Mackey et al., 2002; Mackey et al., 2003; Axtell et al., 2003), *Arabidopsis* PBS1 (required for AvrPphB/RPS5-mediated resistance) by *Pseudomonas syringae* effector AvrPphB (Shao et al., 2003), tomato Pto (resistance to *Pseudomonas syringae* pv. *tomato*) by *Pseudomonas syringae* effector AvrPto (Tang et al., 1996; Pedley and Martin, 2003; Mucyn et al., 2006) and tomato Rcr3 (required for Cf-2 mediated resistance) by *Cladosporium fulvum* effector Avr2 (Kruger et al., 2002; Rooney et al., 2005. However, none of these plant targets have been shown to assist virulence activity of AVR effectors, jeopardizing the virulence criterium for host targets in guard-guardee model. To explain this, the guard hypothesis has been modified, and a guard-multiple guardee model has been proposed (Lim and Kunkel, 2004; Belkhadir et al., 2004). Supporting the modified guard hypothesis, a second host target for AvrB has recently been identified (Shang et al., 2006). RAR1 (required for Mla12 resistance), a well-studied signaling component required for R gene activity, is found to negatively regulate cell wall defense and mediate AvrB triggered leaf chlorosis and bacterial growth enhancement. A point mutation at the end of RAR1 CHORII domain abolished its interaction with SGT1b (suppressor of the G2 allele of Skp1), a cochaperone of HSP90 (heat shock protein), and the AvrB virulence activity in *Arabidopsis* (Shang et al., 2006), suggesting the involvement of SGT1 in AvrB virulence activity. Like RIN4, RAR1 could be an ancient basal defense regulator that is exploited by bacterial effecors to carry out virulence activity. Further exploration of how AvrB modifies RAR1 and how R proteins guard the AvrB-RAR1 complex will shed light on the molecular mechanism of AVR recognition. Besides these two models, it is noteworthy that some effectors are processed by host proteins before coming into contact with host targets. A good example is AvrRpt2. AvrRpt2 is secreted as a 28kD protein into plant cell. Inside the plant cell, AvrRpt2 is able to cleave its own N terminus 71 amino acid peptide (Mudgett et al., 1999). The self-process and the HR eliciting ability of AvrRpt2 require

AtROC1, an *Arabidopsis* cyclophilin peptidyl-prolyl cis/trans isomerase (Coaker et al., 2005; Coaker et al., 2006). Cyclophilin is a well-know chaperone functioning in protein folding. Refolding and processing of type III effectors in the host might be a general mechanism for their activation (Joosten et al., 1999; Coaker et al., 2006).

R Protein Activation

Although many downstream components of R signaling have been identified through intensive genetic screening (reviewed in Glazebrook, 2001; Nimchuk et al., 2003), the exact mechanism of R protein activation remains a mystery. It is known that plant NBS-LRR proteins share significant sequence homology with mammalian Nod proteins. The question is: do plant NBS-LRR proteins act in a similar way as Nod proteins? One relavant question in this regard is, do plant NBS-LRR proteins rely on NOD oligomerization to carry out their mission? This important question had not been answered until recently (Mestre and Baulcombe, 2006). Mestre and Baulcombe detected N protein oligomerization in the presence of its corresponding AVR protein, TMV helicase P50. Oligomerization of N protein is required for resistance upstream of EDS1 and NPG1, signaling components required for N-mediated disease resistance. The authors were able to detect the elicitor dependent N protein oligomerization in a transient assay system that does not elicit cell death, ensuring enough protein for co-immunoprecipitation experiments. This breakthrough discovery will encourage similar exploration for other NBS-LRR proteins and provide new insight into the mechanism of R protein activation. Interestingly, like APAF-1, a well-characterized mammalian Nod protein, some NBS-LRR proteins are also negatively regulated. Point mutations in the conserved NBS domain (Shirano et al., 2002; Bendahmane et al., 2002; Howles et al., 2005), the LRR (Bendahmane et al., 2002; Howles et al., 2005) domain or in the region between NBS and LRR (Zhang et al., 2003) led to constitutive activation of defense responses independent of pathogen infection. The molecular mechanism of this negative regulation is not clear. It is possible that the R protein LRR domain plays a role in negative regulation resembling the APAF-1 model. Supporting this hypothesis, a truncated TIR-NBS R protein PRR1A (Michael Weaver et al., 2006) is constitutively active. A similar effect was also found in a truncated CC-NBS R protein RPS2 (Tao et al., 2000). On the contrary, deletion of LRR domain in other R proteins generated

inactive, instead of constitutive active R proteins (Moffett et al., 2002; Leister et al., 2005; Mestre and Baulcombe, personal communication), indicating that the negative regulation might be specific for certain R proteins.

Suppression of PAMP and Avr Signaling by Pathogen Virulence Systems

To avoid plant surveillance, pathogens have developed various strategies to evade or suppress plant innate immunity. One of the best-characterized virulence systems used by bacteria is the type III secretion system (TTSS). Bacteria are able to deliver effectors into host cells through TTSS to interfere with host signal transduction and metabolism. For example, *Pseudomonas* bacteria inject more than 50 type III effectors into host cells via the TTSS. Many of these effectors are able to suppress plant innate immunity through various mechanisms (Grant et al., 2006). Identifying specific pathways targeted by type III effectors will shed light on new resistance strategies to control disease in plants.

Suppression of PAMP Signaling

PTI (PAMP triggered immunity) is an important layer of plant disease resistance against pathogen infection. However, in most compatible interactions, PTI is not effective although PAMPs are perceived normally. For example, flagellin from the virulent *Pseudomonas syringae tomato* strain DC3000 share identical flg22 with the nonhost strains *P. s.* pv. *tabaci* and *P. s.* pv. *phaseolicola* that induce *NHO1* expression upon infection. However, this active flagellin does not elicit an effective resistance against DC3000 in *Arabidopsis*. DC3000 lacking the flagellin synthesis gene grows similarly compared to wild type DC3000 strain (Li, et al., 2005), indicating that the flagellin-induced immunity is overcome by DC3000. Further evidence shows that flg22-induced expression of *NHO1* is suppressed by DC3000 in a TTSS dependent manner. Nine out of nineteen effectors tested are able to suppress *NHO1* expression in a protoplast transient assay, suggesting that the induced expression of *NHO1* is a general target for type III effectors (Li et al., 2005). The *NHO1* gene has multiple W boxes in its promoter region, which are the potential binding sites for WRKY transcription factors. Since flagellin activates WRKY transcription factors through FLS2 and the MAPK cascade, it is possible that DC3000 targets this signaling pathway to suppress *NHO1* induction. Indeed,

HopAI1, one of the effectors suppressing *NHO1*, is able to block AtMAPK3 and AtMAPK6 activation at or downstream of AtMEKK1 (Zhang J. and Zhou J.M., unpublished). Recently, OspF, a HopAI1 homolog in *Shigella*, was also found to regulate MAPK signaling, which is responsible for the postivasion virulence in T84 intestinal cells (Zurawski et al., 2006). A MAPK signaling cascade is an evolutionary conserved signaling module mediating multiple biological activities of eukaryotic cells. In plants, MAPK cascades are involved in plant development, cell cycle regulation, hormone sensing and abiotic and biotic stress tolerance (Tena et al., 2001). The role of MAPKs in PAMP signaling, HR cell death and disease cell death regulation has also been documented (Asai et al., 2002; de Polo et al., 2004; Pedley and Martin, 2005). In *Arabidopsis*, AtMEKK1 positively regulates disease resistance against *Pseudomonas* bacteria. Constitutive active AtMEKK1 results in enhanced disease resistance against virulent *Pseudomonas syringae* bacteria strains (Asai et al., 2002). To identify DC3000 effectors that target MAPK cascades in *Arabidopsis*, He and colleagues tested ten effectors with demonstrated defense suppressing activity. Two of them, AvrPto and AvrPtoB, are able to block PAMP activated MAPK signaling at or upstream of AtMEKK1. Significantly, DC3000 lacking of these two effectors showed reduced virulence when growing in *Arabidopsis* leaves, indicating the suppression of PTI is critical for bacteria pathogenicity (He et al., 2006). Intriguingly, it has recently been pointed out that the AvrPtoB mediated basal defense suppression is dependent on the absence of a functional FLS2, the receptor kinase for bacterial flagellin (de Torres et al., 2006). Supporting this observation, FLS2, even when expressed at low levels, partially relieves MAPK suppression by AvrPtoB (He et al., 2006), revealing a feed back interference of PRRs against PTI suppression.

Although suppression of a MAPK cascade is an important strategy for bacteria to overcome host defense, other effectors such as AvrRpm1 and AvrRpt2, are not able to interfere with MAPK signaling (He et al., 2006). However, AvrRpm1 and AvrRpt2 suppress flagellin induced callose deposition as well as defense gene expression (Kim et al., 2005). The PTI suppressing activity of AvrRpm1 and AvrRpt2 is likely carried through by RIN4. RIN4 is a host target phosphorylated by AvrRpm1yet cleaved by AvrRpt2 (Mackey et al., 2002; Mackey et al., 2003; Axtell et al., 2003). RIN4 is not required for the virulence activity of AvrRpm1 and

AvrRpt2 measured by bacterial growth but is hypothesized to mediate PTI suppressing activity of these two effectors (Lim and Kunkel, 2004; Belkhadir et al., 2004; Kim et al., 2005). How AvrRpm1 and AvrRpt2 exert their PTI suppressing activity through RIN4 is not further clarified in the model proposed in Kim et al. (2005). AvrRpm1 and AvrRpt2 do not seem to interfere with MAPK signaling (He et al., 2006), thus there may not be a link between RIN4 and the specific MAPK pathway tested by He et al. (2006). Nevertheless, there are more than 60 MAPKKK, 10 MAPKK and 20 MAPK candidate genes in the *Arabidopsis* genome (Nakagami et al., 2005). The possibility that RIN4 regulates some of these MAPKs to suppress PTI cannot be excluded.

Type III dependent basal defense suppression also occurs in *Xanthomonas campestris* pv. *Vesicatoria (Xcv)* infected pepper plants (Keshavarzi et al., 2004). An *Xcv* hrpA mutant induced strong callose deposition and cell wall strengthening. The defense inducing elicitor was identified as lipopolysaccharides (LPS). LPS also induced rapid NOS (NO synthase) expression and a strong nitric oxide (NO) burst in *Arabidopsis* (Zeidler et al., 2004). NO production is responsible for down stream defense gene expression, indicating a role of NO in basal defense. An *Arabidopsis* AtNOS mutant that was unable to produce NO was more susceptible to a virulent *Pseudomonas* strain DC3000, confirming the positive regulation of basal defense by NO signaling. It remains to be determined if any DC3000 effectors suppress NO production or signaling.

Recently, both flagellin and LPS were found to induce stomatal closure that blocks host-entry by nonhost bacteria (Melotto et al., 2006). Stomatal closure induced by flagellin but not LPS is fully dependent on FLS2, indicating that active PTI is involved. The PAMP-induced stomatal closure requires the SA and ABA signaling pathway. Significantly, DC3000 induces transient stomatal closure early after infection and is able to force stomata to reopen in a coronatine dependent manner (Melotto et al., 2006), revealing a masked wrestling between PTI and bacterial virulence. All together these data suggest that the battle between bacterial virulence and plant innate immunity commences even before bacteria enter into host tissues.

Suppression of AVR Signaling---Layered Gene-for-Gene Resistance?

To colonize the host, pathogens need to overcome multiple layers of defense responses (Thordal-Christensen, 2003). Gene-for-gene resistance is believed to be the last barrier of plant defense. To overcome gene-for-gene resistance, pathogens have evolved virulence effectors to actively suppress HR and disease resistance. There are a number of bacteria and oomycete effectors that are able to suppress AVR triggered cell death. For example, *P. s.* pv. *phaseolicola* effectors AvrPphC, AvrPphF or VirPphA are able to suppress HR induced by other AVR proteins (Jackson et al., 1999; Tsiamis et al., 2000). Other effectors that are able to suppress AVR-triggered HR include AvrPphEpto, AvrPpiB1pto, AvrPtoB, HopPtoD2, HopPtoE, HopPtoF, AvrRpt2, HopPtoN (reviewed in Espinosa and Alfano, 2004; Nomura et al., 2005; Grant et al., 2006) and Avr3a (Bos et al., 2006). Among these effectors, some are general program cell death (PCD) suppressors that are able to suppress Bax-induced PCD in both yeast and plants (Jamir et al., 2004; Abramovitch et al., 2003). Some are able to suppress HR on nonhost plants (Abramovitch et al., 2003; Epinosa et al., 2003; Jamir et al., 2004; Lopez-Solanilla et al., 2004). Only two of them specifically suppress HR induced by certain AVR proteins (Ritter and Dangl, 1995; Bos et al., 2006). The exact mechanisms of most HR suppressing activities are not fully understood although some of them have their biochemical activity characterized. For example, AvrPtoB is an E3 ubiquitin ligase involved in protein degradation (Janjusevic et al., 2006; Abramovitch et al., 2006), HopPtoD2 is a putative protein tyrosine phosphatase, whereas HopPtoN is a papain-like cysteine protease (reviewed in Grant et al., 2006). Identification of the specific substrates for the above effectors will lead to a better understanding of how pathogens overcome gene-for-gene resistance.

To guard their gene-for-gene resistance, plants have developed multiple layers of R genes counteracting the cell death suppressing effectors (Chisholm et al., 2006). To date, the best-illustrated example for the layered gene-for-gene resistance is the RPM1 and RPS2 mediated resistance. It has been shown that AvrRpm1 and AvrB target RIN4 and induce RIN4 phosphorylation (Mackey et al., 2002). The perturbance of RIN4 is somehow recognized by RPM1 that elicits the first layer of gene-for-gene resistance (Mackey et al., 2002). To suppress the RPM1 mediated resistance, AvrRpt2 evolved in *Pseudomonas* bacteria to block AvrRpm1

or AvrB triggered HR through eliminating RIN4 (Chisholm et al., 2006). The elimination of RIN4 is subsequently recognized by RPS2 and a second layer of gene-for-gene resistance is initiated (Mackey et al., 2003; Axtell et al., 2003).

The Molecular Basis of Nonhost Resistance

Although R genes are effective resources for crop breeding, resistance mediated by a single R gene is rarely durable because it can be easily defeated by a single loss-of-function mutation in the corresponding AVR gene. To improve the crop protection against pathogen invasion, alternative strategies such as R gene pyramiding and multiline deployment have been proved successful (McDowell and Woffenden, 2003). Another important source of resistance, the nonhost resistance, however, has not been explored. Nonhost resistance is believed to be effective and durable against the vast majority of pathogens. Successful identification of genes involved in nonhost resistance in model plants provides a new strategy to study nonhost resistance in crop plants through either mutational or gene silencing analyses (Lu et al., 2001; Collins et al., 2003; Peart et al., 2002). According to Mysore and Ryu (2003), there are two types of nonhost resistance. Type II nonhost resistance is characterized by a visible HR while type I nonhost resistance does not trigger any visible response. Whether type I and type II nonhost resistance are activated through distinct or similar mechanisms is an open question. It seems like some defense genes are induced earlier and faster in type II nonhost resistance. However, the majority of defense genes share almost identical expression pattern in both types of nonhost resistance (Oh et al., 2006). Interestingly, genes involved in R-mediated resistance have been associated to type II but not type I nonhost resistance, indicating that type II nonhost resistance shares similar signaling pathways with gene-for-gene resistance. For example, silencing of NbSGT1, an important signaling component for N gene-mediated resistance, compromised nonhost resistance against *P. s.* pv. *maculicola* and *Xanthomonas axonopodis* pv. *vesicatoria* which cause HR on tobacco. Loss of NbSGT1 function does not affect nonhost resistance resistance against CaMV and *Xanthomonas campestris* pv. *campestris,* which do not elicit HR response on tabacco (Peart et al., 2002). Similarly, AtRAR1, a SGT1 interactor, is required for nonhost resistance against HR eliciting *P. s.* pv. *tabaci* but not against non-HR

eliciting *P. s.* pv. *phaseolicola* in *Arabidopsis* (Li X. and Zhou J.M., unpublished data). These data suggest that different mechanisms are exploited in different types of nonhost resistance.

There are three possible mechanisms explaining the molecular basis of nonhost resistance. First of all, gene-for-gene resistance can lead to nonhost resistance. Second, PAMP recognition and signaling contribute to nonhost resistance. Third, defense genes that directly restrict pathogen infection or growth play a role in nonhost resistance. It is known that gene-for-gene resistance is the major mechanism of intraspecies or cultivar level resistance. Examples of *R* gene functioning in nonhost resistance are few. It has been shown that nonhost resistance in wheat against *Blumeria graminis f. sp. secalis* and *B. graminis f. sp. agropyri*, the rye and wheatgrass mildew fungi, is mediated by *R* gene pyramids (Matsumura and Tosa, 1995). Similar mechanism also exists in cereal rust systems (Sanghi and Luig, 1974) and *Magnaporthe grisea*-gramineous plant system (Hiura, 1978; Kang et al., 1995; Swegard et al., 1995). To explain the formation of R pyramid-mediated nonhost resistance, Tosa has proposed a model describing the evolution of *formae speciales* and races (Tosa, 1992). In his model, the pathogen has a set of ancient *AVR* genes (*Ax1-4* and *Ay1*-4) to control characters other than avirulence. Some of the AVR effectors are recognized by plant species X and Y carrying the corresponding *R* genes. To colonize X and Y, pathogen chooses to lose the corresponding *AVR* genes while at the same time preserve the other effector genes that may incur fitness penalties. The species level resistance determined by *R-AVR* recognition has been maintained over the years by balancing and stabilizing selection (Tosa, 1992). This model has not been proved at the molecular level. However, single *R* gene mediated nonhost resistance has been reported in maize. The maize *Rxo1* (reaction to *Xanthomonas oryzicola* 1) gene is responsible for nonhost HR and resistance against the rice pathogen *X. o.* pv. *oryzicola* bearing *AvrRxo1*, the causal agent of bacteria streak disease. *Rxo1*-transgenic rice is more resistant to *X. o.* pv. *oryzicola* infection compared to nontransgenic rice, implicating a potential role of nonhost resistance in disease management. However, maize lines that do not carry *Rxo1* are still resistant to the rice pathogen, indicating the involvement of other genes or other mechanisms (Zhao et al., 2005). Besides *R* genes, several signaling components involved in gene-for-gene resistance such as SGT1 (Peart et al., 2002), RAR1 (Li X. and Zhou J.M., unpublished), EDS1 (Yun et al., 2003),

PAD4 and SAG101 (Lipka et al., 2005) are also involved in nonhost resistance. Significant overlaps in defense response gene profiling between nonhost resistance and gene-for-gene resistance have been detected in *Arabidopsis* plants treated with nonhost and avirulent strains of *Pseudomonas* bacteria (Tao et al., 2003; Navarro et al., 2004). Among the nonhost bacteria regulated genes, approximately 30% of them are also regulated by flg22, indicating a role of PAMP signaling in nonhost resistance (Navarro et al., 2004).

Supporting the idea that PAMP signaling participates in nonhost resistance, species or family level difference in PAMP recognition has been suggested to be associated with nonhost resistance (Felix and Boller; 2001; Fliegmann et al., 2004; Zipfel et al., 2006). Meanwhile, pathogen mutants lacking a functional PAMP have been shown to gain at least partial virulence on nonhost plants (Shimizu et al., 2003; Li et al., 2005). These two lines of evidence indicate that PAMP recognition is an important nonhost barrier at least in the tested pathosystems. The evolutionary process linking up PAMP recognition, suppression of PAMP signaling by pathogen virulence, gene-for-gene resistance and suppression of HR by pathogen effectors has been proposed (Chisholm et al., 2006). It seems that the game started from the pathogen side. Pathogens rely on their PAMPs to carry out life cycle and pathogenesis. PRRs are subsequently evolved to recognize pathogen PAMPs and trigger nonhost resistance. Because the pathogens could not afford losing PAMPs, they have evolved the ability to suppress PRR recognition thus breaching nonhost resistance and establish pathogenesis.

The third type of nonhost resistance genes plays a role in directly restricting pathogen infection or growth. To establish pathogencity, pathogens need to enter host tissue to obtain nutrients and counteract host defense. *Pseudomonas* bacteria choose to use natural opening or wounds on plant leaf surface whereas some fungal pathogens directly penetrate plant cell wall. In the latter case, there are several preformed (wax, cuticle layer, cell wall) and inducible barriers (papilla) to prevent pathogen penetration. Intensive mutant screening in *Arabidopsis* yielded three PEN genes involved in limiting *Blumeria graminis f. sp. hordei* (*Bgh*) penetration (reviewed in Ellis, 2006). PEN1 encodes a SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) domain containing protein involved in membrane fusion and secretion events (Collins et al., 2003; Assaad et al., 2004). PEN2 encodes a putative glycosyl

hydrolases with undefined biochemical activity (Lipka et al., 2005). PEN3 is an ATP binding cassette (ABC) transporter protein (Stein et al., 2006). All three proteins accumulate at the penetration sites during *Bgh* infection despite their different subcellular localization before infection (Ellis, 2006), indicating their role in direct prevention of *Bgh* penetration. Considering that formation of papillae beneath the penetration site is strongly induced upon *Bgh* infection, it is reasonable to hypothesize that PEN genes are involved in papillae mediated cell wall defense. In-deed, the rate of papillae formation is reduced in pen1 mutant (Assaad et al., 2005). However, the *Arabidopsis* mutant *pmr4* (powdery mildew resistance 4) lacking a callose synthase gene is more resistant instead of susceptible to *Bgh* infection. The resistance of *pmr4* against *Bgh* is caused by the SA dependent cell death. It is likely that plants are able to initiate a more effective defense response, the localized cell death once the cell wall defense failed. Supporting this idea, the EDS1-PAD4-SAG101 signaling complex (Feys et al., 2005) known to participate in gene-for-gene resistance is required for postpenetration defense against *Bgh* infection. Recently, a role of actin cytoskeleton in preinvasion nonhost resistance has also been demonstrated (Yun et al., 2004; Opalski et al., 2005; Shimada et al., 2006). Inhibition of the actin skeletal function in combination with the *eds1* mutation severely compromise nonhost resistance in *Arabidopsis* against wheat powdery mildew (Yun et al., 2004). Actin filament polariztion is induced in *Arabidopsis* toward the appressorial contact sites by nonhost *Colletotrichum* species and contributes to preinvasion resistance as well as papillae callose formation (Shimada et al., 2006). The synergistic effect of both pre- and postinvasion defenses eventually contributes to *Arabidopsis* nonhost resistance (Lipka et al., 2005).

Similar to resistance against fungal pathogens, plant resistance against bacteria pathogens is also controlled by layered defense responses (Cunha et al., 2006). One of the major challenges for *Pseudomonas* bacteria is to obtain nutrients. Virulent bacteria strains have various strategies to suppress host defense and access to nutrients. On the other hand, plants are able to deprive nutrients from nonhost bacteria. For example, glycerol is an important carbon source for *Pseudomonas* bacteria. Glycerol can be entrapped in plant cells in the form of G3P, catalyzed by 3-glycerol kinases. The *Arabidopsis nho1* mutant lacking the functional 3-glycerol kinase gene accumulates high level of glycerol that is permeable to the plant plasma

membrane. Nonhost bacteria strains are able to actively uptake the intercellular glycerol and grow in the *nho1* mutant (Li X. and Zhou J.M., unpublished), suggesting that nutrient deprivation is an important layer of nonhost resistance in *Arabidopsis* plants. Supporting the presence of an additive nonhost resistance in *Arabidopsis* against *Pseudomonas* bacteria, the *NHO1* mediated nutrient deprivation works synergistically with FLS2 mediated innate immunity against the nonhost bacteria *P. s.* pv. *tabaci* (Li X. and Zhou J.M., unpublished), Intriguingly, *NHO1* itself is induced by a flagellin PAMP, flg22, via a FLS2 dependent pathway (Li et al., 2005). Further experiments are needed to better understand plant nonhost resistance against *Pseudomonas* bacteria.

Conclusions and Perspectives

One of the major goals of studying plant-pathogen interaction is to develop strategies to protect crop plants from various diseases and reduce the loss in crop production. As the most important source of resistance in molecular breeding, *R* genes have been widely deployed in crop protection. In order to broaden the spectrum and improve the durability of R gene mediated resistance, multiple *R* genes recognizing different AVR effectors should be bred or transformed into one crop variety to form R gene pyramids, which is technically difficult and time consuming. Another way to achieve durable resistance is to use *R* genes recognizing an AVR effector that will impose a great fitness penalty on the pathogen. Such *R* genes may naturally exist in plants. For example, the Arabidopsis *RPW8* and the maize *Rpg1* confer broad-spectrum resistance against powdery mildew and stem rust respectively. There are two genes at the *RPW8* locus, *RPW8.1* and *RPW8.2*, both encoding proteins that contain a membrane anchor fused to a putative CC domain (Xiao et al., 2001). The resistance mediated by RPW8 proteins requires EDS1, EDS5, PAD4, NPR1 and SGT1b but not NDR1, RAR1 and PBS3 (Xiao et al., 2005). *Rpg1* encodes a receptor kinase with two tandem serine/threonine kinase domain which provides broad range and durable resistance against the barley stem rust fungi *Puccinia graminis f. sp. Tritici* (Brueggeman et al., 2002; Nirmala et al., 2006). *RPW8* and *Rpg1* are thought to recognize either a PAMP or an AVR effector indispensible for pathogen virulence. Further investigation of their ligand recognition and resistance activation will bring new ideas in breeding durable disease resistance. Besides

naturally existing *R* genes, chimeric *R* genes with PAMP recognizing and HR-eliciting abilities could be constructed in the future based on the in depth knowledge of PRR and R protein activation. It has been shown that chimeric RLK joining the BRI1 receptor domain and the Xa21 kinase domain is able to trigger HR cell death in rice cell culture in the presence of brassinosteroids ligand (He et al., 2000). In plants, there are a number of well-characterized RLKs including two PRRs and more than 10 R proteins. The recombination between the PAMP recognition and HR eliciting domain of these RLKs may generate new *R* genes that mediate highly effective and durable resistance. Direct transformation of PRRs that determine species level resistance into crop plants may also help. For example, tobacco plants are more susceptible to Agrobacterium infection due to the lack of the EF-Tu recognizing receptor EFR (Zipfel et al., 2006). Stable transgenic tobacco lines expressing EFR may restore the resistance against the *Agrobacterium*. With the discovery of more PAMP-PRR pairs, there will be more nonhost resistance genes available for disease management.

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CHAPTER 2

FLAGELLIN INDUCES INNATE IMMUNITY IN NONHOST INTERACTIONS THAT IS SUPPRESSED BY *PSEUDOMONAS SYRINGAE* EFFECTORS

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Abstract

Arabidopsis NONHOST1 (*NHO1*) is required for limiting the *in planta* growth of nonhost *Pseudomonas* bacteria but completely ineffective against the virulent bacterium *Pseudomonas syringae* pv. *tomato* DC3000. However, the molecular basis underlying this observation remains unknown. Here we show that *NHO1* is transcriptionally activated by flagellin. The nonhost bacterium *P. syringae* pv. *tabaci* lacking flagellin is unable to induce *NHO1,* multiplies much better than does the wild-type bacterium and causes disease symptoms on *Arabidopsis*. DC3000 also possesses flagellin that is potent in *NHO1*-induction, but this induction is rapidly suppressed by DC3000 in a type III secretion system-dependent manner. Direct expression of DC3000 effectors in protoplasts indicated that at least nine effectors, HopS1, HopAI1, HopAF1, HopT1-1, HopT1-2, HopAA1-1, HopF2, HopC1, and AvrPto, are capable of suppressing the flagellin-induced *NHO1* expression. One of the effectors, HopAI1, is conserved in both animal and plant bacteria. When expressed in transgenic *Arabidopsis* plants, HopAI1 promotes growth of the nonpathogenic *hrpL*$^-$ mutant bacteria. In addition, the purified phytotoxin coronatine, a known virulence factor of *P. syringae,* suppresses the flagellin-induced *NHO1* transcription. These results demonstrate that flagellin-induced defenses play an important role in nonhost resistance. A remarkable number of DC3000 virulence factors act in the plant cell by suppressing the species level defenses, and that contributes to the specialization of DC3000 on *Arabidopsis*.

Introduction

Nonhost resistance refers to resistance shown by an entire plant species to a specific parasite (Heath, 1987). This resistance is expressed by every plant towards the majority of potential phytopathogens and differs from the cultivar level resistance conditioned by gene-for-gene interactions (Martin et al., 2003; Belkhadir et al., 2004). Plant defenses can be induced by "general elicitors" of pathogen or plant origin, including oligosaccharides, lipids, polypeptides, and glycoproteins (Nurnberger et al., 2004). However, a role of these elicitors in plant disease resistance in a natural setting is often difficult to establish, because plants' responses to elicitors do not differentiate resistant and susceptible plants. Many of the elicitors are now known as Pathogen-Associated Molecular Patterns (PAMPs). The best-characterized PAMP known to activate innate immunity in plants is flagellin from *Pseudomonas* bacteria (Felix et al., 1999). A conserved N-terminal peptide of flagellin, flg22, is a highly potent elicitor of defense responses in tomato and *Arabidopsis* (Felix et al., 1999; Gomez-Gomez et al., 1999). In *Arabidopsis*, flg22 is perceived by FLS2, a receptor-like kinase that activates downstream events through a MAP kinase cascade (Gomez-Gomez and Boller, 2000; Asai et al., 2002). Pre-treatment of *Arabidopsis* with flg22 peptide not only globally induces defense gene expression, but also protects plants from subsequent infection of the virulent DC3000 (Zipfel et al., 2004). *Arabidopsis* plants lacking *FLS2* exhibit enhanced disease susceptibility to DC3000 under certain circumstances (Zipfel et al., 2004). While these work elegantly demonstrated the functional significance of flagellin-sensing in plant defense, whether flagellin-signaling plays a role in the species level resistance remains unknown.

The bacteria enter plants through natural openings such as stomata or wounds and proliferate in the intercellular spaces. A major bacterial pathogenesis mechanism is mediated by the so-called Type-III Secretion System (TTSS) through which gram-negative bacteria inject a repertoire of effectors into host cells (Alfano and Collmer, 2004). Type III effectors play an important role in bacterial pathogenesis. In *P. syringae*, a growing number of effector genes, such as *avrRpt2, avrRpm1, virPphA(hopAB1), avrPto,* and *hopAB2(avrPtoB),* are known to contribute to virulence (Chen et al., 2000; Ritter and Dangl, 1995; Jackson et al.,

1999; Tsiamis et al., 2000; Shan et al., 2000; Abramovitch et al., 2003). *avrRpt2,* for example, suppresses plant PR gene expression and interferes with the RPM1-specified resistance (Chen et al., 2000). *avrPtoB, hopX1(avrPphE$_{pto}$), hopAM1(avrPpiB$_{pto}$)*, *hopAO1*(*hopPtoD2), hopE1(hopPtoE), hopF2(hopPtoF), hopF1(avrPphF),* and *hopN1*(*hopPtoN)* all appear to suppress cell death in plants (Abramovitch and Martin, 2004; Alfano and Collmer, 2004). In addition to type III effectors, certain *P. syringae* strains, including DC3000, produce the phytotoxin coronatine, which also plays a role in bacterial virulence (Bender et al., 1999). A role of TTSS or coronatine in overcoming nonhost resistance has not been examined closely.

In previous studies we showed that the *Arabidopsis NHO1* gene is required for resistance to multiple strains of nonhost *P. syringae*, but completely ineffective against DC3000 (Lu et al., 2001). Interestingly, *NHO1* transcripts are induced by the nonhost strains, but suppressed by DC3000 (Kang et al., 2003). This suppression is apparently of functional significance, because plants overexpressing *NHO1* exhibit enhanced resistance to DC3000 (Kang et al., 2003).

Here, we show that the flg22 peptide strongly induces the transcription of *NHO1*. A *P. syringae* pv. *tabaci* (*Ptab*) strain, to which *Arabidopsis* is a nonhost plant, induces *NHO1* in a flagellin-dependent manner. A *Ptab* strain lacking the flagellin gene *fliC* elicits disease symptoms and multiplies in *Arabidopsis* plants, demonstrating that flagellin-signaling contributes to nonhost resistance. In contrast to nonhost bacteria that give a prolonged induction of *NHO1,* DC3000 only transiently induces *NHO1* transcription, also in a flagellin-dependent manner. While the wild-type DC3000 rapidly suppresses the *NHO1* induction, DC3000 mutant strains defective in TTSS are diminished in their ability to suppress *NHO1.* Expression of the DC3000 effectors HopS1, HopAI1, HopAF1, HopT1-1, HopT1-2, HopAA1-1, HopF2, HopC, and AvrPto in the plant cell blocks the *NHO1*-induction by flg22. In addition, purified coronatine suppresses the fl22- and *P. syringae* pv. *phaseolicola* (*Pph*)-induced *NHO1* expression. Furthermore, expression of HopAI1 in transgenic plants promotes nonpathogenic bacterial growth. Together these results demonstrate the importance of flagellin-induced innate immunity mechanism in nonhost resistance and a role of DC3000 virulence factors in suppressing the flagellin-induced innate immunity.

Materials and Methods

Construction of *NHO1*-LUC Reporter Line

An 1.8 kb *NHO1* promoter sequence was PCR-amplified from Col-0 genomic DNA with the following primers: 5'- CAGTCGACTTCCTTACAGTCCAGACAT G-3' and 5'-TTCCCGGGGGTAAAGGTGAAGAACGATGCT-3'. The PCR product was digested with *Sal*I and *Sma*I and cloned into a modified pBI121 vector with the LUC reporter gene (He et al., 2004). The *NHO1*-LUC construct was introduced into *Agrobacterium tumefaciens* strain GV3101 and transformed into Col-0 plants by floral-dipping (Clough and Bent, 1998). The T4 progeny of a selected homozygous transgenic line with a single insertion was used for all experiments.

Bacterial Strains and Bacterial Growth Assay

Bacterial strains used in this study include DC3000, *Pph* NPS3121 (Lu et al., 2001), *Ptab* 6505 wild-type and 6505 *fliC*$^-$ mutant (Shimizu et al., 2003), DC3000 *hrpL*$^-$ mutant (Zwiesler-Vollick et al., 2002), DC3000 *hrpA*$^-$ and *hrcC*$^-$ mutants (Roine et al., 1997; Yuan and He, 1996), and DC3000 *fliC*$^-$ mutant (previously referred to as *flaA*; Hu et al., 2001). Bacteria were grown overnight at room temperature in King's medium B with appropriate antibiotics, precipitated, washed twice with ddH_2O and diluted to the desired concentration with ddH_2O for plant inoculation. Bacteria used for growth assay was diluted to 10^5 cfu/ml and syringe-infiltrated into young and fully expanded *Arabidopsis* leaves. All experiments were repeated at least twice with similar results.

Luciferase Activity Assay

Bacterial cultures used for luciferase activity assay were diluted with 0.2 mM luciferin to 10^8 cfu/ml and syringe-infiltrated into *Arabidopsis* leaves. The leaves were removed from plants at the indicated time points and sprayed with 1mM luciferin containing 0.01% Triton X-100. Luminescence images were captured by using a low light imaging system, and relative luciferase activity was calculated with the WinView software (RoperScientific, Trenton, NJ; He et al., 2004).

Flagellin and Coronatine Treatment

Polypeptides containing 22 conserved N-terminal residues of flagellin from *P. aeruginosa, Ptab,* and *Agrobacterium tumefaciens* were synthesized by Bio-Synthesis, Inc (Lewisville, TX) as the following: flg22$^{P.\ aeruginosa}$: QRLSTGSRINSAKDDAAGLQIA; flg22$^{A.\ tumefaciens}$: ARVSSGLRVGDASDNAAYWSIA, and flg22$^{P.\ s.tabaci}$: TRLSSGLKINSAKDDAAGLQIA. Flg22 peptides were dissolved in ddH_2O and diluted to 1 μM with 0.2 mM luciferin before inoculation. Coronatine (kindly provided by Carol Bender) was dissolved in ddH_2O and diluted to 100 ng/ml with 0.2 mM luciferin before inoculation.

Construction of Effector Gene Expression Plasmids

A transient expression vector pUC19-35S-FLAG-RBS containing the CaMV 35S promoter, 3x FLAG, and a *Rubisco Small Subunit* terminator (Zou Y. and Zhou J.M., unpublished results; accession number DQ077692) was used for transient expression of effector genes in protoplasts. The effector genes were PCR-amplified with primers listed in Supporting Table 1. After restriction digestion, the *avrPto* PCR product was inserted between the *Xho*I and *Spe*I sites of pUC19-35S-3xFLAG-RBS, resulting in the 35S-AvrPto construct. Other effector genes were inserted between *Xho*I and *Csp45*I of pUC19-35S-FLAG-RBS, resulting in 35S-Effector-FLAG constructs.

Protoplast Transfection Assay

Protoplasts were isolated from 6-week-old *NHO1*-LUC plants according to Sheen (**http://genetics.mgh.harvard.edu/sheenweb/**). Protoplasts were transfected with either an effector construct or the empty vector, incubated in 0.4 M mannitol and 1 μM flg22$^{P.\ s.tabaci}$ for 12 h. LUC activity was measured after adding 50 μM luciferin to the transfected protoplasts.

Construction of Estradiol-Inducible *hopAI1* Expression Plants

The HopAI1-FLAG fragment was excised from the 35S-HopAI1-FLAG plasmid with *Xho*I and *Spe*I and inserted into pER8 (Zuo et al., 2000). The construct was transformed into *Arabidopsis* plants (Col-0) by *Agrobacterium*-mediated transformation. Transgenic plants

were selected on MS plates containing hygromycin. For *hopAI1* induction, plants were sprayed with 25 μM estradiol containing 0.02% silwet L-77.

Results

Flagellin Induces *NHO1* Transcription

To further investigate the regulation of *NHO1* expression in response to *Pseudomonas* bacteria, an *NHO1*-LUC reporter line was constructed. An 1.8 kb *NHO1* upstream sequence was fused to the firefly luciferase gene coding sequence and introduced into *Arabidopsis* plants (Col-0). Consistent with the expression of endogenous *NHO1* mRNA (Kang et al., 2003), the *NHO1*-LUC expression in a homozygous reporter line was strongly induced by *Pph*, but not DC3000 (Fig. 2-1a). Detailed analysis revealed a transient *NHO1*-LUC induction 3 h after DC3000-inoculation, but the *NHO1*-LUC expression returned to the base line by 12 h (Fig. 2-1b). In contrast, *Pph* induced a strong and sustained expression of *NHO1*-LUC. The strong induction by nonhost bacteria is not strain-specific, because another nonhost strain *Ptab* also induced *NHO1*-LUC to a high level (Fig. 2-2a).

We previously hypothesized that a PAMP derived from the nonhost *Pseudomonas* bacteria induces the expression of *NHO1* (Kang et al., 2003). Flagellin is a well-known PAMP that induces innate immune responses in plants and animals. We therefore tested if flagellin induces the *NHO1*-LUC reporter gene. flg22 peptides corresponding to *P. aeruginosa, A. tumefaciens,* and *P. s. tabaci* were tested for their ability to induce *NHO1*-LUC. Fig. 2 shows that the active peptide flg22$^{P.\ aeruginosa}$ was fully capable of inducing *NHO1*-LUC. Flg22$^{P.\ s.\ tabaci}$ was similarly active in *NHO1*-LUC induction (Fig. 2-6a). In contrast, flg22$^{A.\ tumefaciens}$, which is inactive in plant immune response induction (Felix et al., 1999), was unable to induce *NHO1*-LUC (Fig. 2-1c).

Flagellin Is Required for *NHO1* Induction and Resistance in Nonhost Interaction

If flagellin is required for the *NHO1*-induction by a nonhost bacterium, then bacteria lacking flagellin should be defective in *NHO1* induction. A *Ptab* mutant strain lacking the flagellin gene *fliC*⁻ (Shimizu et al., 2003) induced poorly the *NHO1*-LUC expression (Fig.

2-2a), indicating that flagellin is largely responsible for the observed induction of *NHO1* by this bacterium. To test if flagellin contributes to nonhost resistance in *Arabidopsis* plants, the *fliC*⁻ mutant was compared with the wild-type *Ptab* for disease symptoms and bacterial growth *in planta*. Fig. 2-2b shows that *fliC*⁻ caused visible disease symptoms on *Arabidopsis*. In contrast, the wild-type bacterium caused no visible symptoms. The mutant bacteria multiplied at least 10 fold four days after inoculation, whereas the wild-type *Ptab* failed to multiply in the four-day period (Fig. 2-2c). Together these data demonstrate that flagellin is a major PAMP responsible for the induction of *NHO1* and resistance to this nonhost *P. syringae* bacterium.

Transient Induction of *NHO1* by DC3000 Requires Flagellin

The possibility that transient *NHO1*-LUC induction by the wild-type DC3000 depends on flagellin was also tested. The *fliC*⁻ mutant of DC3000, previously referred to as *flaA* (Hu et al., 2001), failed to induce *NHO1*-LUC at any tested time point after inoculation (Fig. 2-3a). In addition, wild-type DC3000 bacteria killed by exposure to kanamycin prior to inoculation also induced a strong and sustained *NHO1*-LUC expression, whereas the *fliC*⁻ bacteria killed by kanamycin did not induce *NHO1*-LUC (data not shown). Bacterial growth assay indicated that the wild-type and *fliC*⁻ mutant of DC3000 grew similarly when infiltrated into *Arabidopsis* plants (Fig. 2-3b). The two strains also caused indistinguishable disease symptoms (Supporting Fig. 2-3). These results demonstrate that, like *Ptab,* DC3000 flagellin is fully capable of inducing *NHO1*. However, unlike *Ptab,* the response to DC3000 flagellin is abrogated and does not result in resistance in the plant.

TTSS Is Essential for DC3000 to Suppress *NHO1*

The lack of sustained *NHO1*-LUC induction by DC3000 flagellin is consistent with our hypothesis that this bacterium actively suppresses the *NHO1*-mediated nonhost resistance (Lu et al., 2001). Therefore, a role of DC3000 virulence/pathogenicity genes in the active suppression of *NHO1* was tested. Fig. 2-4a shows that DC3000 strains lacking the TTSS structure genes *hrpA* and *hrcC* induced much greater *NHO1*-LUC expression compared with the wild-type DC3000, indicating that TTSS is largely responsible for the suppression. The DC3000 mutant lacking the regulatory gene *hrpL* gave an even stronger induction than did

hrpA^{-} and *hrcC*$^{-}$ mutants (Fig. 2-4a). The strength and kinetics of the *hrpL*$^{-}$ mutant induced *NHO1*-LUC expression resemble those of *Pph* (Fig. 2-4b and Fig. 2-1b). *hrpL* encodes a sigma factor that regulates both TTSS and coronatine biosynthetic genes through the *hrp* box (Boch et al., 2002). These results demonstrate that TTSS is essential for DC3000 to suppress the *NHO1* expression.

Type III Effectors Suppress *NHO1* Expression

The hypothesis that type III effectors suppress *NHO1* expression was systematically tested by using a protoplast-based transient assay. Protoplasts were isolated from plants carrying the *NHO1*-LUC reporter and transfected with constructs carrying DC3000 effector genes under the control of the CaMV 35S promoter. A total of 19 effectors were tested (Supporting Table 2-1; http://www.*Pseudomonas-syringae*.org). Most of these effectors were selected because their function in virulence had not been reported previously. For control, protoplasts were transfected with an empty vector. The transfected protoplasts were subsequently induced with flg22$^{P.\ s.tabaci}$. As seen in Fig. 4c (contributed by Lin H.), flg22 $^{P.\ s.tabaci}$ induced *NHO1*-LUC in protoplasts transfected with empty vector compared to uninduced protoplasts, recapitulating the *NHO1*-LUC induction observed in intact leaves. Transfection of nine effector genes, *hopS1, hopAI1, hopAF1, hopT1-1, hopT1-2, hopAA1-1, hopF2, hopC1,* and *avrPto*, strongly reduced the flagellin-induced *NHO1* expression in repeated experiments. Among these, *hopAI1, hopT1-1, hopAA1-1, hopF2,* and *hopC1* completely abolished the *NHO1* induction. Other effector genes did not show a consistent effect on *NHO1* induction. These results indicate that almost 50% of the tested DC3000 effectors are functionally redundant and suppress the flagellin-induced *NHO1* expression.

Southern blot analysis was carried out to determine if any of these nine effector sequences exist in the two nonhost strains used (Supporting Fig. 2-4, contributed by Lin H.). Not all the nine effectors described in this work are unique to DC3000. HopAA1 is encoded by the conserved effector locus (CEL) that exists in all known *P. syringae* pathovars (Alfana et al., 2000). Southern blot analysis indicated that the *hopT1-1* and *hopAA1* sequences exist in *Ptab,*

whereas the *hopAF1, hopT1-2,* and *hopAA1* sequences are present in *Pph*. Thus it appears that the delivery of a few of these effectors by the bacterium is not sufficient for the suppression.

HopAI1 Promotes Parasitism in Plants

To determine if any of the tested effectors promote virulence, a FLAG-tagged hopAI1 was introduced into *Arabidopsis* plants as a stable transgene using an estrodial-inducible system (Zuo et al., 2000). This effector was chosen because it shares 35% identity with the *Salmonella enterica serovar typhimurium* VirA, a mouse killing factor (Gulig and Chiodo, 1990; Supporting Fig. 2-1). A search of the GenBank database indicated that similar proteins also exist in *S. choleraesuis, Shigella flexneri* and *Chromobacterium violaceum.* Fig. 2-5a shows that induced expression of hopAI1 in a transgenic line exhibited chlorosis, reminiscent of disease symptoms. The expression of hopAI1 also enhanced the growth of the hrpL- mutant bacteria by at least 30 fold (Fig. 2-5b, contributed by Zhang J.). Similar results were observed in 6 primary transgenic plants (Supporting Fig. 2-2, contributed by Zhang J.). These results indicate that the suppression of NHO1 by HopAI1 is relevant to the virulence function.

The role of hopAI1 in NHO1-suppression was further tested by using a DC3000 mutant strain carrying truncated hopAI1. Consistent with a redundant role of multiple effectors in NHO1-suppression, the hopAI1 mutation did not produce a measurable effect on NHO1-LUC suppression (Li X. and Zhou J.M., unpublished data).

Coronatine Partially Suppresses *NHO1* Expression

Previous work suggested that both TTSS and the phytotoxin coronatine modulate the expression of a similar set of plant genes (He et al., 2004; Alfano et al., 2000). This prompted us to test if coronatine also contributes to the observed suppression of *NHO1*. Figs. 2-6a and 2-6b show that co-infiltration of purified coronatine diminished the *NHO1*-LUC expression induced by flg22$^{P.\ s.\ tabaci}$ or *Pph*. However, a DC3000 mutant that is blocked in the synthesis of coronatine was only marginally compromised in *NHO1*-LUC suppression (Supporting Fig. 2-5). Together these results suggest that coronatine plays a minor role in *NHO1*-suppression. A role of coronatine and the requirement of COI1 in *NHO1* suppression (Lu et al., 2003) indicate that jasmonate signaling may play a role in *NHO1* suppression. Consistent with this possibility,

exogenous application of methyl jasmonate partially suppressed the *Pph*-induced *NHO1*-LUC expression (Supporting Fig. 2-6).

Discussion

The molecular basis of nonhost resistance is poorly understood. It is speculated that PAMP-induced innate immunity plays an important role in the species level resistance, but direct evidence is lacking (Nurnberger et al., 2004). The results presented here show that flg22, a known PAMP, mimics nonhost bacteria and induces the expression of *NHO1*. In contrast, the inactive peptide flg22$^{A.tumefaciens}$ is unable to induce *NHO1*. Thus the induced expression of the nonhost resistance gene *NHO1* is a typical PAMP-mediated innate immune response.

Recent results showed that *Pseudomonas* bacteria carry at least two additional PAMPs, a cold-shock protein and elongation factor-TU, both inducing defense responses in plants (Felix and Boller, 2003; Kunze et al., 2004). The results presented here indicate that flagellin is the primary PAMP in *Ptab* responsible for *NHO1* induction, because the *fliC*$^-$ mutant strain is largely inactive in *NHO1* induction. The induction of *NHO1* is likely of functionally importance, because *Arabidopsis* plants overexpressing *NHO1* display enhanced resistance to DC3000 (Kang et al., 2003). The *Ptab* strain lacking *fliC* gains partial virulence on wild-type *Arabidopsis* when directly infiltrated into leaves. This strain also displays enhanced virulence on tomato plants (Shimizu et al., 2003). It should be noted that the *fliC*$^-$ mutant is not fully pathogenic on *Arabidopsis*. One plausible explanation is that PAMPs other than flagellin also contribute to species level resistance (Zipfel et al., 2004). Nevertheless, these results demonstrate that flagellin plays a critical role in eliciting nonhost resistance.

Although nonhost resistance is effective to the vast majority of potential pathogens, it is breached by a small number of pathogens, presumably because the latter has evolved specialized virulence mechanisms that enable them to successfully overcome this resistance. Flagellin is highly conserved among Pseudomonads, including DC3000 that is virulent on *Arabidopsis*. *NHO1*-LUC reporter assay revealed a transient induction by DC3000, and this induction is flagellin-dependent. The induction is quickly suppressed within 6 h after inoculation, coincides with the *in planta* expression of type III genes in DC3000 (Xiao et al.,

2004). We previously hypothesized that DC3000 suppresses *NHO1* by using type III effectors (Kang et al., 2003). Indeed, the *hrpA*$^-$, *hrcC*$^-$ and *hrpL*$^-$ mutants of DC3000 all induce *NHO1*-LUC to a much greater level than does the wild-type strain. Most importantly, direct expression of nine DC3000 effector genes in the plant cell or exposure to purified coronatine strongly suppresses the flg22-induced expression of *NHO1*-LUC, providing direct evidence that type III effectors suppress the flagellin-induced immune responses. These observations are consistent with the knowledge that exogenous flagellin only protects *Arabidopsis* plants against DC3000 when applied one day before the bacterial inoculation, but ineffective when infiltrated simultaneously with the DC3000 bacterium (Zipfel et al., 2004). Together these results provide strong evidence that a major target for DC3000 is innate immunity that acts at the species level to limit nonhost *Pseudomonas* bacteria. Consistent with the role of DC3000 TTSS in overcoming species level resistance, recent work shows that the DC3000 TTSS actively suppresses and tolerates the production of antimicrobial root exudates that are inhibitory to nonhost bacteria, although which effector(s) does so remains to be determined (Bais et al., 2005). The ability of a bacterium to overcome the species level resistance may represent a major evolutionary step that enables a *P. syringae* bacterium to colonize on a new host species.

The results presented here indicate that a surprisingly large proportion of the DC3000 effectors possess the ability to suppress the flagellin-induced *NHO1* expression. Among the nine effectors that suppress *NHO1* expression, at least HopAI1 and AvrPto are capable of promoting nonpathogenic bacterial growth when expressed in plants (Kim et al., 2005). HopAI1 shares significant homology with virulence proteins of animal bacteria. This raises an intriguing possibility that flagellin-induced innate immunity in the host is similarly targeted by diverse pathogenic bacteria. Expression of AvrPto in the plant also suppresses callose deposition induced by the *hrcC* mutant bacteria (DebRoy et al., 2004). Because callose deposition can be induced by flagellin (Felix et al., 1999), AvrPto might suppress cell wall defense and *NHO1* expression through a common step required for flagellin signaling. A recent report shows that AvrRpt2 and AvrRpm1 can suppress flagellin-induced callose deposition when directly expressed in plants (Kim et al., 2005). These observations re-enforce the notion

that flagellin-induced defenses are targeted by diverse effectors, although they do not appear to share a conserved biochemical function.

A large number of *P. syringae* effectors have been shown to target various host defenses including callose deposition, defense gene expression and cell death induced by gene-for-gene interaction or nonhost interactions (Abramovitch and Martin, 2004). Often, the defense-suppression by an individual effector is revealed either when the latter is directly expressed at a high level in the plant cell or delivered along with other effectors in the bacterium. It remains to be determined whether these effectors, when individually delivered by *P. syringae*, are sufficient to suppress host defenses. It is possible that a successful defense-suppression by a bacterium requires synergistic action of a large set of the bacterium-delivered effectors. For instance, CEL, which exists in all *P. syringae,* is required by DC3000 for pathogenicity and suppression of callose deposition in *Arabidopsis* (DebRoy et al., 2004). However, the vast majority of *P. syringae* is nonpathogenic on *Arabidopsis*. Thus, the function of CEL-encoded effectors is likely to be assisted by other effectors unique to DC3000. Similarly, several effectors activate COI1-dependent gene expression when delivered by DC3000 but not when delivered by *Pph* (He et al., 2004). DC3000 type III effectors and coronatine act synergistically to modulate the JA signaling in *Arabidopsis* (He et al., 2004; Zhao et al., 2003). These may explain why some of the effector sequences carried by *Ptab* and *Pph* do not appear to suppress the *NHO1* expression. It may be that the suppression of the flagellin-induced expression of *NHO1*, which is known to involve the JA signaling pathway (Kang et al., 2003), requires a synergistic activity from a large set of these effectors and coronatine that target the JA signaling pathway.

The RPM1-interacting protein RIN4 was shown recently to negatively regulate the flagellin-induced callose deposition (Bias et al., 2005). RIN4 also interacts with AvrRpt2 and AvrRpm1 (Mackey et al., 2002; Mackey et al., 2003; Axtell and Staskawicz, 2003). AvrRpt2 is a cysteine protease that cleaves RIN4, leading to the degradation of RIN4 (Day et al., 2005), whereas AvrRpm1-RIN4 interaction results in the phosphorylation of RIN4 (Mackey et al., 2002). It is suggested that RIN4 and/or RIN4-associated proteins are manipulated by these two effectors to suppress the flagellin-induced cell wall defense (Bais et al., 2005). It remains to be

shown if and how the AvrRpt2-mediated degradation of RIN4 leads to the suppression of callose deposition. An important area of future research will be to determine if a common mechanism is used by various effectors to suppress flagellin-induced defenses.

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Figure Legend

Figure 2-1. *NHO1* is transcriptionally induced by nonhost bacteria and flagellin. (a). A luciferase image of *NHO1*-LUC transgenic leaves inoculated with water, nonhost strain *Pph,* or virulent strain DC3000 for 24 h. (b). Time course of *NHO1*-LUC expression in plants inoculated with water, *Pph,* or DC3000 bacteria. (c). *NHO1*-LUC activity of plants inoculated with 1 μM $flg22^{P.aeruginosa}$ or $flg22^{A.tumefaciens}$ at the indicated hours. Bacteria were inoculated at 10^7 cfu/ml suspended in 0.2 mM luciferin. *Arabidopsis* leaves were detached at the indicated time points after inoculation to examine the luciferase activity. These experiments were repeated at least six times with similar results.

Figure 2-2. Flagellin is required for *NHO1* induction and resistance to *Ptab*. (a). *NHO1*-LUC activity of plants inoculated with the wild-type and *fliC*⁻ mutant strains of *Ptab.* (b). Disease symptoms of *Arabidopsis* plants (Col-0) 7 days after inoculation with the wild-type (WT) and *fliC*⁻ mutant strains of *Ptab* (10^6 CFU/ml). (c). Bacterial growth of the wild-type (WT) and *fliC*⁻ mutant *Ptab* strains on *Arabidopsis* plants. For luciferase assay, a bacteria inoculum of 10^7 cfu/ml was used. For disease symptom development, a bacteria inoculum of 10^6 cfu/ml was used. For bacteria growth assay, a bacteria inoculum of 10^5 cfu/ml was used. These experiments were repeated three times with similar results.

Figure 2-3. DC3000 flagellin transiently induces *NHO1* and fails to confer disease resistance. (a). *NHO1*-LUC expression in plants inoculated with water, the wild-type (WT) or *fliC*⁻ mutant DC3000 strains at the indicated hours after inoculation. (b). Bacterial growth assay of Col-0 plants infiltrated with the wild-type (WT) or *fliC*⁻ mutant strains of DC3000. For luciferase assay, a bacteria inoculum of 10^7 cfu/ml was used. For bacteria growth assay, a bacteria inoculum of 10^5 cfu/ml was used. These experiments were repeated three times with similar results.

Figure 2-4. DC3000 requires type III effectors to suppress *NHO1* expression. (a). *NHO1*-LUC plants were inoculated with the wild-type (WT), *hrpA*⁻, *hrcC*⁻, or *hrpL*⁻ mutant DC3000 strains, and relative luciferase activity was measured 0, 12, and 24 h after inoculation. (b). Kinetics of *NHO1*-LUC expression in response to the wild-type (WT) and *hrpL*⁻ mutant

DC3000 strains. A bacteria inoculum of 10^7 cfu/ml was used for these luciferase assays. (c). Expression of DC3000 effectors blocks flg22-induced *NHO1*-LUC expression. Protoplasts were transfected either with the empty vector (V) or the indicated effector constructs, and relative LUC activity was measured 12 h after addition of flg22. Vector-transfected protoplasts treated with ddH_2O were used as a control for basal *NHO1*-LUC expression (V-). Each data point consists of three replicates. The error bar represents standard error. The experiments were repeated three times with similar results.

Note: Figure 2-4c is contributed by Lin H.

Figure 2-5. HopAI1 promotes virulence in plants. (a). *hopAI1* expression induces chlorosis. Transgenic *hopAI1-FLAG* (line 2) and wild-type (WT) plants were sprayed with 50 μM estradiol and photographed 5 days later. (b). *hopAI1* expression enhances bacterial growth in plants. Transgenic *hopAI1-FLAG* (line 2) and wild-type (WT) plants were sprayed with either buffer or 50 μM estradiol one day prior to inoculation with the *hrpL*$^-$ mutant. Bacteria population in the leaf was determined at the indicated times. Error bars indicate standard error. The experiments were repeated three times with similar results.

Note: Figure 2-5 is contributed by Zhang J.

Figure 2-6. Coronatine partially suppresses *NHO1* expression. (a). Coronatine inhibits the flagellin-induced *NHO1*-LUC expression. *NHO1*-LUC transgenic plants were inoculated with 1 μM $flg22^{P.\ s.tabaci}$ alone, 1 μM $flg22^{P.\ s.tabaci}$ plus 200 ng/ml coronatine, or 1 μM $flg22^{A.tumefaciens}$. *Arabidopsis* leaves were detached at 0, 3 and 9 hours after inoculation for luciferase assay. (b). Coronatine inhibits the *Pph*-induced *NHO1*-LUC expression. *NHO1*-LUC activity of plants inoculated with water, *Pph*, or *Pph* plus 200 ng/ml coronatine (cronatine+*Pph*). An inoculum of 10^7 cfu/ml was used for *Pph* in the luciferase assay. The experiments were repeated twice with similar results.

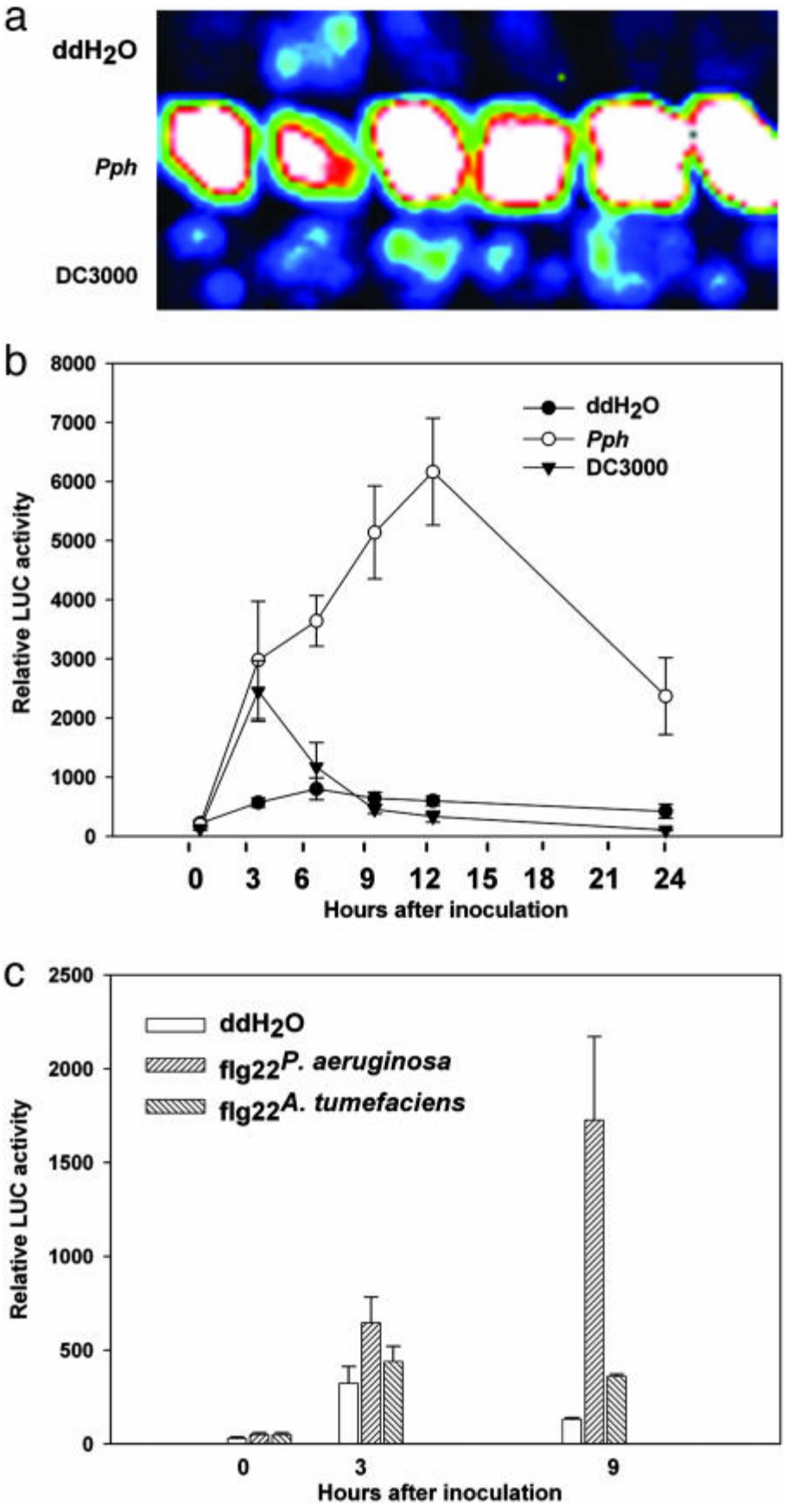

Figure 2-1 *NHO1* is transcriptionally induced by nonhost bacteria and flagellin

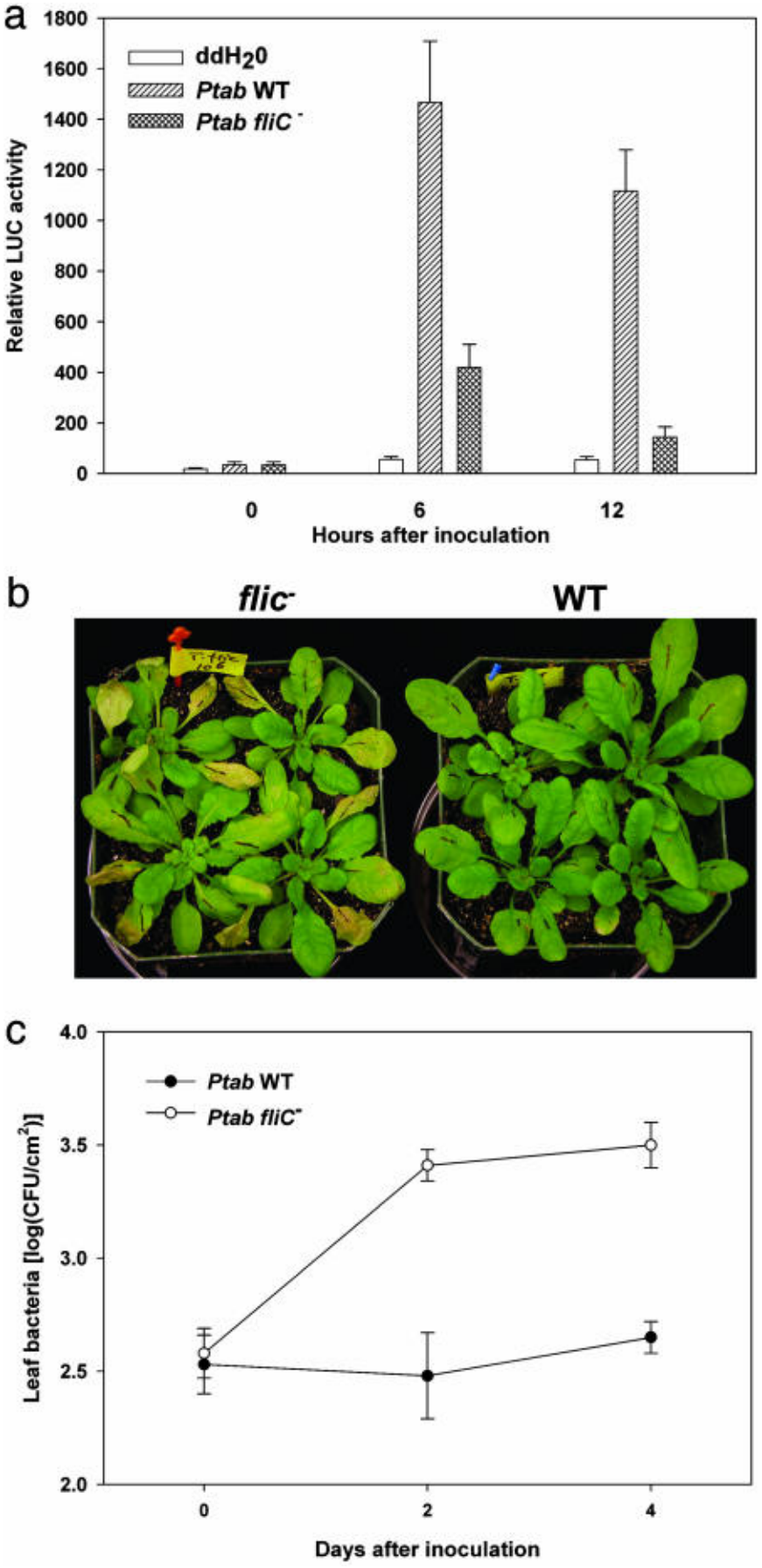

Figure 2-2 Flagellin is required for *NHO1* induction and resistance to *Ptab*

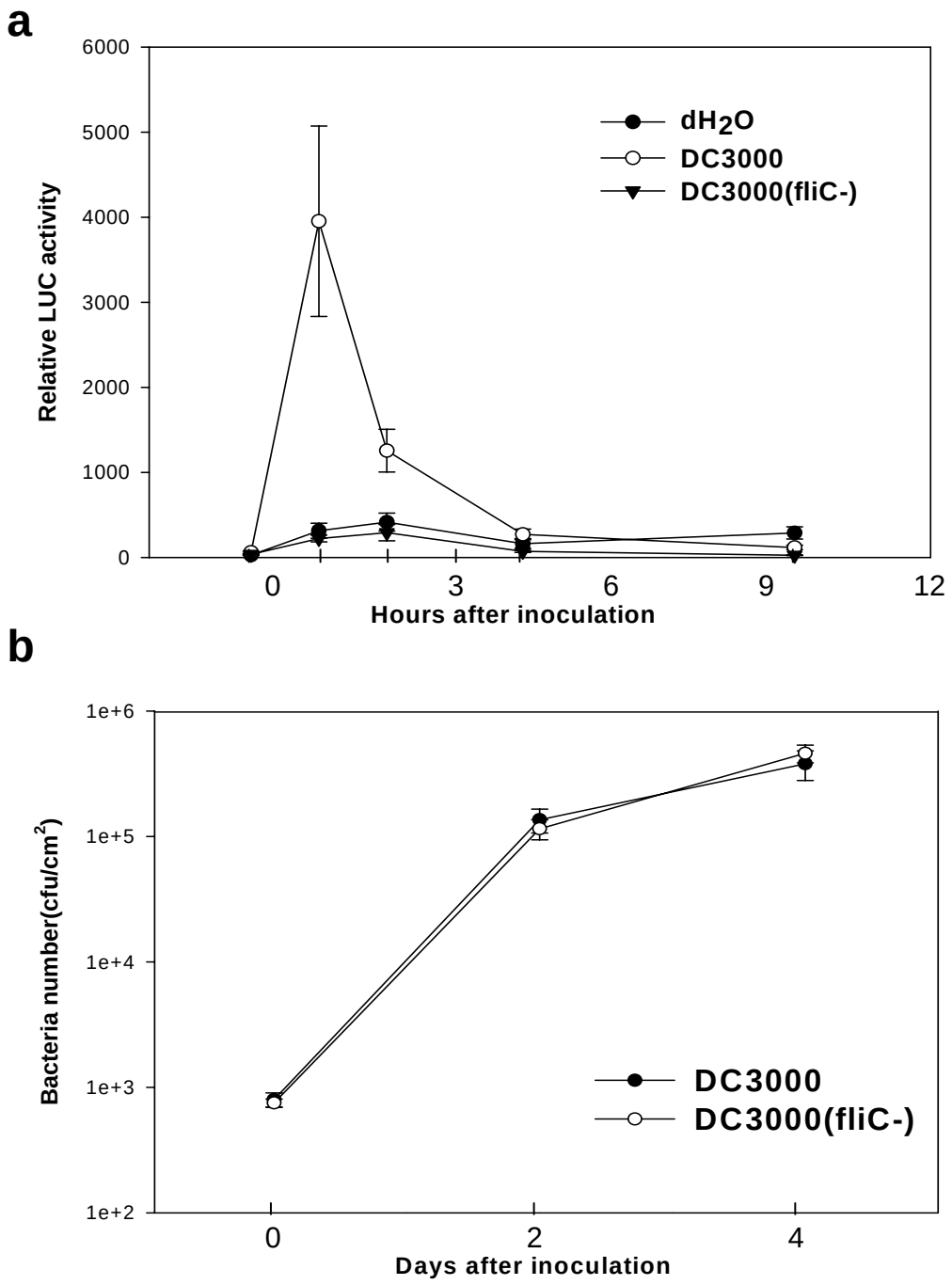

Figure 2-3 DC3000 flagellin transiently induces *NHO1* and fails to confer disease resistance

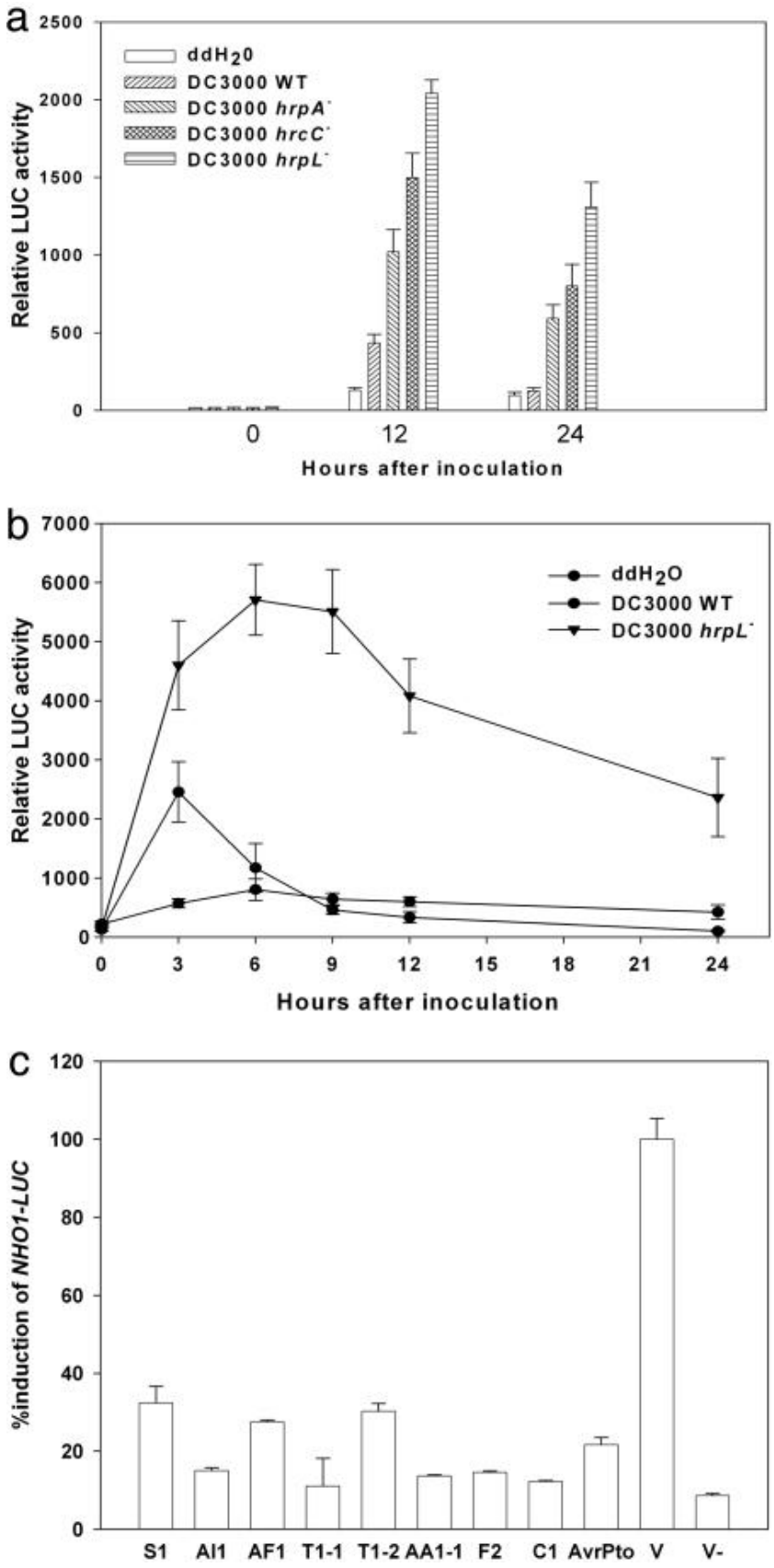

Figure 2-4 DC3000 requires type III effectors to suppress *NHO1* expression

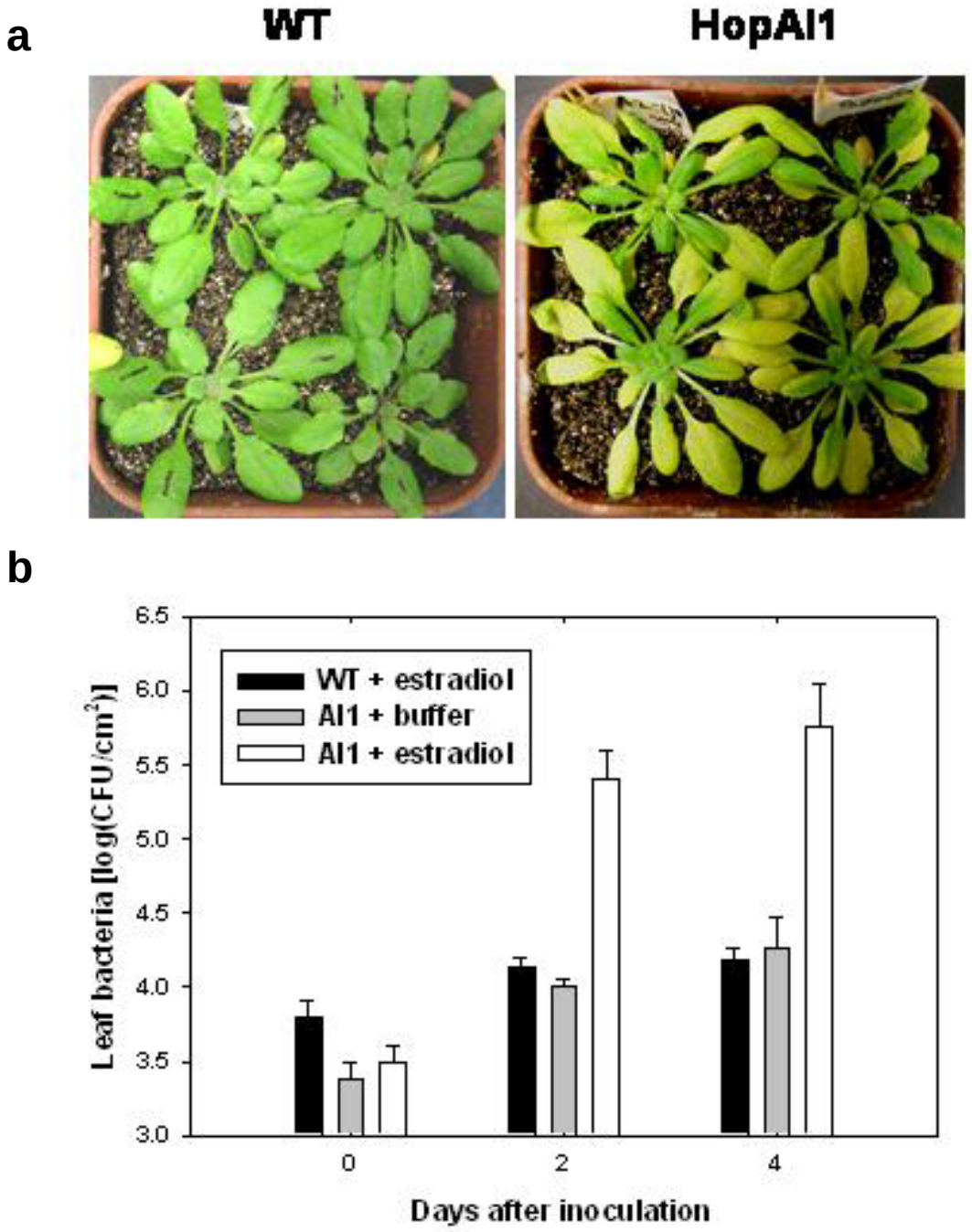

Figure 2-5 HopAI1 promotes virulence in plants

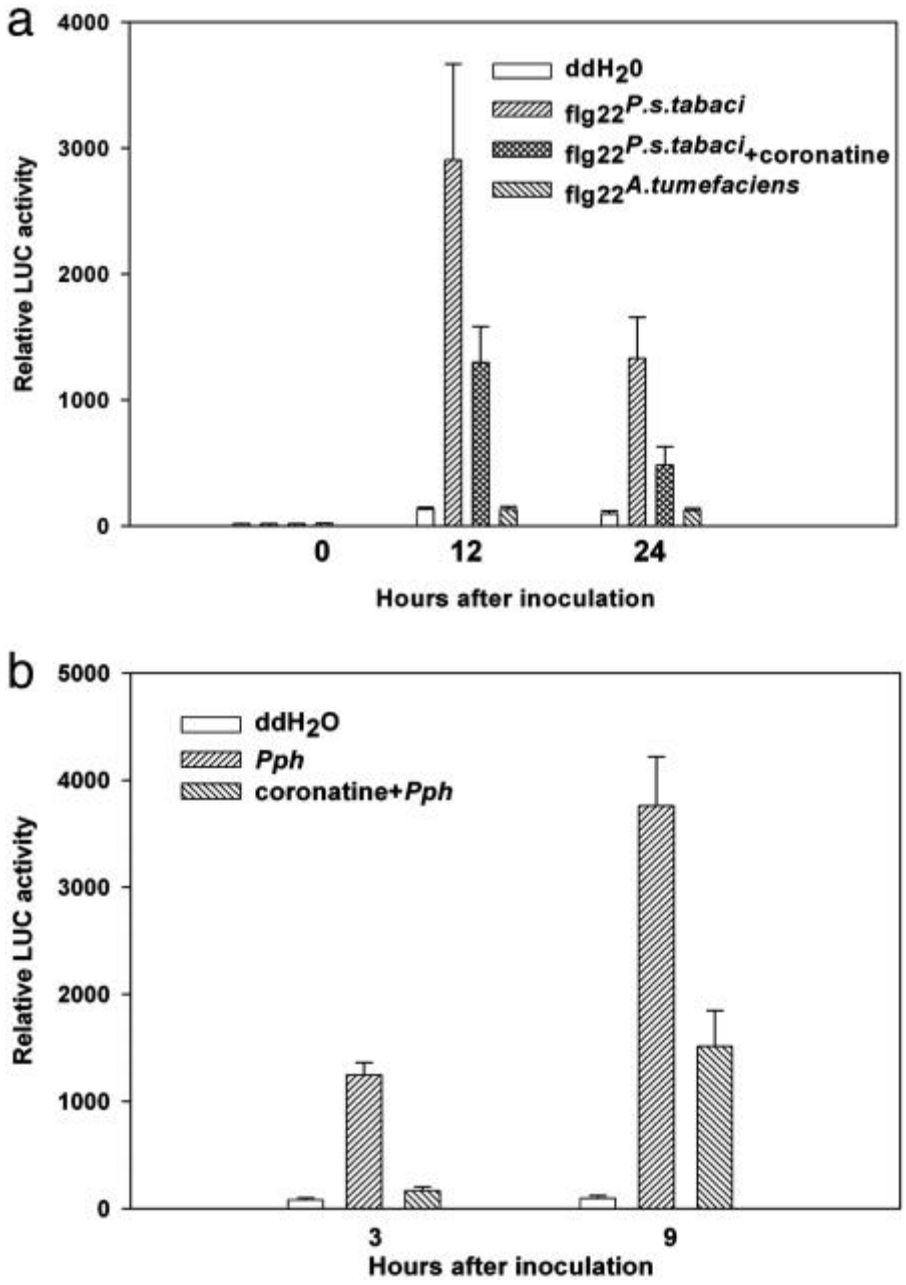

Figure 2-6 Coronatine partially suppresses *NHO1* expression

CHAPTER 3

RAR1, A SIGNALING MODULE IN HOST, NONHOST AND BASAL RESISTANCE, IS TARGETED BY *PSEUDOMONAS SYRINGAE* EFFECTOR AVRB

Some of the data here are published in *Proc Natl Acad Sci U S A.* 103:19200-19205

Note: Shang Y, Li X, Cui H, He P, Thilmony R, Chintamanani S, Zwiesler-Vollick J, Gopalan S, Tang X, and Zhou J contributed to the published paper. In this chapter, Shang Y. shares the credits of the AvrB dependent susceptibility in *rar1* and *coi1* mutant backgrounds; Cui H. contributed to the coimmunoprecipitation experiment.

Abstract

To explore the molecular mechanism for effector virulence activity, a screening for *Arabidopsis* mutants aiming at identifying AvrB virulence targets has been performed. Based on the expression level of *RAP2.6*, a reporter gene of AvrB virulence activity, six *rrb* (reduced response to *avrB)* mutants were isolated. One mutant, *rrb3* compromised AvrB specific *RAR2.6* induction and is more susceptible to DC3000 (*avrB*). The mutant allele *rrb3* was mapped to a 46 kb interval at the end of chromosome 5. *rrb3* carries a point mutation that convert H217 to Y in the conserved RAR1 CHORD II domain. RAR1, a co-chaperone for HSP90, is known to mediate resistance signaling of several CC-NBS-LRR proteins. However, the role of RAR1 in nonhost resistance, basal defense and bacterial virulence is not known. The studies here demonstrate that RAR1, together with NDR1, is involved in type II but not type I nonhost resistance. RAR1 participates in basal resistance against DC3000 by antagonizing COI1 activity. RAR1 coimmunoprecipitates with AvrB and is targeted by AvrB to trigger leaf chlorosis and promote nonpathogenic bacterial growth. The AvrB-dependent enhanced bacterial growth but not leaf chlorosis requires COI1, suggesting that AvrB targets the JA signaling pathway to promote parasitism.

Introduction

Plant resistance against bacterial pathogens can be classified into host, nonhost and basal resistance based on the level of specificity. The resistance conferred by plant resistance (R) genes upon recognition of avirulence (AVR) proteins in an adapted bacterium pathogen is called gene-for-gene resistance and is referred to as host resistance here. Often, host resistance is characterized by a visible hypersensitive response (HR) and is specific to avirulent pathogen strains carrying the corresponding AVR genes. Unlike the cultivar level host resistance, nonhost resistance is a species or higher taxonomic level resistance effective against all strains in a given pathogen species or pathovar that is not adapted to the nonhost plants. There are two types of nonhost resistance. Type I nonhost resistance does not trigger HR whereas type II nonhost resistance does (Mysore and Ryu, 2003). The molecular basis for nonhost resistance is not fully understood. However, emerging lines of evidence suggest that PTI (PAMP-triggered immunity) is a molecular mechanism of nonhost resistance (Felix and Boller; 2001; Fliegmann et al., 2004; Zipfel et al., 2006; Shimizu et al., 2003; Li et al., 2005). Signaling components involved in host resistance may also contribute to nonhost resistance (Peart et al., 2002; Yun et al., 2003; Lipka et al., 2005; Zhao et al., 2005). Besides host and nonhost resistance, plants also activate basal level resistance that is effective against all strains of a given pathogen (Nomura et al., 2005). The measurable basal resistance includes localized callose deposition, the induction of defense genes and the modulation of hormonal signaling pathways (Abramovitch and Martin, 2004).

To become a pathogen on plants, *Pseudomonas* bacteria developed sophisticated virulence mechanisms to subvert host, nonhost and basal resistance. For example, *Psueudomonas* bacteria use their type III secretion system (TTSS) to secrete effector proteins into plant cells to suppress defense responses (Collmer and Alfano, 2004). In some cases, the effector proteins are recognized by the hosts and trigger gene-for-gene resistance. One well accepted model explaining the recognition of effector proteins by plant R proteins is the guard hypothesis (Dangl and Jones, 2001). According to guard hypothesis, effector proteins target and modulate host components to promote parasitism. The perturbation of host component is then recognized by an R protein, which subsequently triggers disease resistance. Supporting

this model, four host targets of 6 different effector proteins have been identified (Mackey et al., 2002; Mackey et al., 2003; Axtell et al., 2003; Shao et al., 2003; Pedley and Martin, 2003; Mucyn et al., 2006; Kruger et al., 2002; Rooney et al., 2005). However, none of these targets have been associated with effector virulence activity. Based on the modified version of guard hypothesis, an effector protein could have more than one host target and the virulence targets are still to be identified (Belkhadir et al., 2004). The *P. syringae* effector protein AvrB enhances virulence on soybean and *Arabidopsis* plants lacking the corresponding resistance genes (Ashfield et al., 1995; Nimchuck et al., 2000), but triggers host resistance on soybean and *Arabidopsis* plants carrying *Rpg1* and *RPM1* respectively (Ashfield et al., 2004). The virulence and avirulence activity of AvrB has the same structural requirements, suggesting that they are intimately connected (Ong and Innes, 2006). Therefore, host proteins required for AvrB virulence function may provide a molecular link between effector virulence function and the elicitation of host resistance.

To identify possible host components involved in AvrB virulence activity, a screening for *Arabidopsis* reduced response to AvrB (*rrb*) mutant compromised in *RAP2.6* induction was performed (Chintamanani, 2005). RAP2.6 is an ethylene response factor (ERF) family transcription factor that is strongly induced by virulent *Pseudomonas* bacteria. *RAP2.6* induction was associated with bacteria pathogenicity and was modulated by individual type III effectors including AvrB (He et al., 2004). AvrB strongly induced RAP2.6 expression in the absence of *RPM1*. The induction of *RAP2.6* by AvrB is fully dependent on the jasmonic acid (JA) signalling pathway because AvrB fails to induce *RAP2.6* in *coi1* mutant background (He et al., 2004).

Using the AvrB-specific *RAP2.6* induction as a virulence indicator of AvrB, six *rrb* mutants were isolated based on their reduced response to AvrB (Chintamanani, 2005). One mutant, *rrb3* compromised AvrB-specific *RAP2.6* induction and is more susceptible to DC3000 (*avrB*), indicating that a host component involed in both AvrB virulence and avirulence activity was mutated. The *RRB3* gene was found to encode for RAR1, a cochaperone of HSP90 required for gene-for-gene resistance. Further experiments demonstrate a role of RAR1 in nonhost and basal resistance. Significanly, RAR1 is targeted by AvrB and

required for AvrB-dependent susceptibility and chlorosis, two indicators of AvrB virulence acitivity. AvrB-dependent susceptibility but not chlorosis requires COI1. The results suggest that AvrB targets RAR1 and modulates JA signalling pathway to promote parasitism.

Materials and methods

Construction of *rar1-29/rar1-29,rpm1/rpm1,avrB/avrB; rar1-20/rar1-20,rpm1/rpm1,avrB/+;* and *coi1/coi1,rpm1/rpm1,avrB/+* Plants

To construct *rar1-29/rar1-29, rpm1/rpm1, AvrB/AvrB* and *RAR1/RAR1, rpm1/rpm1, AvrB/AvrB* plants, the dexamethasone (Dex)-inducible *AvrB* transgenic line 1 (named Dex-*AvrB* here, Nd-0 background lacking the *RPM1* locus) was crossed with the *rar1-29* plants (Col-0 background carrying the *RPM1* resistance gene). The SSLP marker K17N15-19K (primers 5'-gactagagagtaagaacatgactc-3' and 5'-aagtcgaatcgttcacgcaataag-3') closely linked to the *RAR1* locus was used to identify the respective genotypes at the *RAR1* locus. Homozygous F4 plants with *rar1-29/rar1-29, rpm1/rpm1, AvrB/AvrB* and *RAR1/RAR1, rpm1/rpm1, AvrB/AvrB* genotypes were used for experiments. *rar1-20* (Tonerro et al., 2002) and *coi1-1* (Xie et al., 1998) mutants (Col-0 background, carrying the *RPM1* resistance gene) were similarly crossed with Dex-*AvrB*. *rar1-20/rar1-20, rpm1/rpm1, AvrB/+* and *RAR1/+, rpm1/rpm1, AvrB/+* plants, and *COI1/COI1, rpm1/rpm1, AvrB/+* and *coi1-1/coi1-1, rpm1/rpm1, AvrB/+* plants were identified from F2 plants by PCR. The genotype at the *COI1* locus was identified by using a CAPS maker as described (Xie et al., 1998). Primers 5'-atcttcaagtctcaaagtgtgc-3' and 5'-gattccacaagataacttgaagc-3' were used to determine the genotype at the *RPM1* locus (Nd-0 background lacking the *RPM1* locus). Plants carrying the *AvrB* transgene in F1 or F2 generations were confirmed with *AvrB*-specific primers 5'-atcaatgcttaattggtgcagc-3' and 5'-atcagaatctagcaagcttctg-3'. All the plants carry a chromosome segment from the Nd-0 ecotype and thus are *rpm1*-null.

Bacterial Growth and HR Assay

Five-week-old plants were infiltrated with bacteria suspension at 105 CFU/ml and bacterial number was counted at indicated time points. For AvrB dependent bacterial growth assay, plants were treated with 30 μM Dex solution containing 0.02% Silwet L-77 (Osi

Specialties, Friendship, WV) 2 days before bacteria inoculation. For HR assay, leaves were infiltrated with DC3000 (*avrB*) at 108 CFU/ml and HR was scored 10-12 hours after infiltration. Each experiment was repeated at least twice with similar results.

Mapping and Cloning of *rrb3* Gene

For mapping the *rrb3* gene, the *rrb3* mutant was crossed with the Nossen ecotype, and 5 week old F2 plants were inoculated with DC3000 (*avrB*) at 10^8 CFU/ml. Plants displaying delayed HR were scored as mutants. SSLP, INDEL and CAPS or SNP markers were used in fine mapping as described previously (Xiao et al., 2004).

CCD Imaging and Luciferace Activity Assay

Four to six week old plants were infiltrated with $2x10^6$ CFU/ml DC3000 (*avrB*) containing 0.02 mM luciferin. The inoculated leaves were then collected at different time points, sprayed with 1 mM luciferin containing 0.01% Triton X-100. The leaves were kept in dark for 6 minutes before luminescence images were captured. Quantitative luciferase assay was performed as described (He et al., 2004).

RNA Blot Analysis

For *RAP2.6* expression analysis, total RNA was extracted from plants at indicated time points after infiltration with 2 x 10^6 cfu/ml DC3000 (*avrB*), and the RNA blot was hybridized with the *RAP2.6* cDNA probe.

Western Blot Analysis

Anti-RAR1 antiserum was raised in rabbits using full-length recombinant RAR1 protein as antigen as described (Azevedo et al., 2002). Total protein was extracted from 5-week-old plants in a 1XPBS buffer containing 10 mg/mL leupeptin, 1 mM PMSF, 2 mM EDTA, 1 X proteinase inhibitor cocktail (Roche, Basel, Switzerland) and 1% Triton X-100. For AvrB protein detection, plants were sprayed with 30 μM Dex (Sigma, St. Louis, MO) for 24 h before protein extraction. Thirty μg protein samples were electrophoresed through a 12% or 15% SDS-PAGE. Protein was electrotransferred to an Immobilon P membrane (Millipore

Corp., Bedford, MA). Immunodetection was performed with a 1:2,500 dilution of anti-RAR1 antibodies, or a 1:10,000 dilution of anti-AvrB antibodies. The blot was then hybridized with a goat anti-rabbit or goat anti-mouse HRP-conjugated secondary antibody (Sigma, St. Louis, MO) and visualized with ECL western blotting detection reagents (Amersham, Piscataway, NJ) following the manufacturer's instructions.

Yeast Two-Hybrid Assay

The *RAR1, rar1-29, SGT1b,* and *HSP90.1* coding sequences were amplified from total cDNA of *Arabidopsis* Col-0 wild-type (*RAR1, SGT1b,* and *HSP90.*1) or *rar1-29* plants using gene specific primers 5'-aactctgaattcatggaagtaggatctgca-3' and 5'-aatctcgagctttgaatcgaaaatctcagg-3' (*RAR1* and *rar1-29*), 5'-gaattccctctgaaagaatcaatgg-3' and 5'-ctcgagagatcaatactcccacttc-3' (*SGT1b*), and 5'-gaattcctaaagttcgttgcgatgg-3' and 5'-ctcgagcttcatctcttagtcgac-3' (*HSP90.1*). PCR products were digested with *Eco*RI and *Xho*I and inserted into pJG4-5 (*RAR1* and *rar1-29*) or pEG202 (*SGT1b* and *HSP90.1*). The constructs were sequence-verified and co-transformed in pairs into the EGY48 yeast strain containing pSH18-34. At least six individual colonies from each transformation were tested for β-galactosidase activities on X-Gal plates following the protocol described (Golemis et al., 1997).

To determine if the RAR1 and *rar1-29* proteins accumulated to similar levels in yeast, total yeast protein was extracted by boiling equal amounts of yeast cells in 2× Laemmli sample buffer. The total protein was separated by 10% SDS-PAGE gel and transferred to immobilon membrane (Millipore Corp., Bedford, MA). The membrane was then hybridized with monoclonal mouse anti-HA antibody (Sigma, St. Louis, MO) and detected with the HRP-conjugated goat anti-mouse antibodies (Sigma, St. Louis, MO) and ECL regents (Amersham, Piscataway, NJ).

Co-Immunoprecipitation

Co-immunoprecipitation experiment was done as described (12). AvrB-3x-FLAG transgenic and nontransgenic *rpm1* plants were sprayed with 30μM estradiol (Sigma, St. Louis, MO) prior to protein extraction. The immune complex was precipitated with an

agarose-conjugated monoclonal anti-FLAG antibody (Sigma, St. Louis, MO). The presence of RAR1 and AvrB-FLAG in the complex was detected by using western blot.

Results

Mutant *rrb3* Compromises AvrB-Specific *RAP2.6* Induction and Gene-for-Gene Resistance

We showed previously that AvrB specifically induces *RAP2.6*, an ERF family transcription factor, in a COI1 dependent manner. *RAP2.6* induction correlates well with the pathogenicity of various *Pseudomonas* strains (He et al., 2004), suggesting that *RAP2.6* expression is a reliable virulence indicator. To identify host components required for AvrB virulence activity, *RAP2.6*-LUC transgenic lines developed in Col-0 background (He et al., 2004) was mutagenized with ethane methyl sulfonate (EMS). 15,000 M2 plants derived from 6,000 EMS-mutagenized M1 plants were screened and six *rrb* (reduced response to AvrB) mutants were identified (Chintamanani, 2005). One mutant, *rrb3* severely compromised AvrB specific *RAP2.6* induction (Fig. 3-1a and Fig. 3-4c) and showed a delayed HR induced by AvrB (Fig. 3-1b). Consistently, the *rrb3* mutant lost gene-for-gene resistance against the avirulent strain DC3000 (*avrB*) (Fig. 3-1c).

The Mapping and Cloning of *rrb3*

Genetic analysis indicated that *rrb3* is caused by a single recessive mutation (Shang et al., 2006). The F2 mapping population was constructed by crossing the mutant *rrb3* to Arabidospsis accession Nossen carrying a functional copy of *RPM1*. Out of a total of ~800 F2 plants, 43 plants showing the mutant phenotype, a delayed HR were selected for PCR analysis. *rrb3* was mapped to a ~2 cM interval between the flanking SSLP markers nga129 and MXC20 at the end of chromosome 5. Screening of another ~1,200 F2 plants generated another 60 recombinant plants showing a delayed HR. The *rrb3* gene was delimited to a 46 kb interval between the SNP markers K10D11-12K and MIO24-46K. Twelve genes were found in this area including *RAR1*, a well know signaling component required for gene-for-gene resistance. Allelism tests indicated that *rrb3* is a *rar1* allele, because *rrb3* x *rar1-20* F_1 plants displayed the mutant phenotype, a delayed HR (Table 3-1) in response to DC3000 (*avrB*). Transformation of

a wild-type copy of *RAR1* gene driven by its natural promoter into *rrb3* restored the normal HR (Fig. 3-2a). Sequencing analysis revealed a point mutation at the end of the CHORDII domain that led to a H217Y substitution (Fig. 3-2b). Twenty-eight *rar1* alleles had been reported prior to this study (Tornero *et al*., 2002). We therefore renamed *rrb3* as *rar1-29.* The mutant protein RAR1-29 is unstable *in planta* (Fig. 3-2c, contributed by Shang Y.). However, the stability of RAR1-29 expressed in yeast is not affected by the mutation (Fig. 3-2c). H217 is a highly conserved residue located at the end of CHORD II domain of the RAR1 family proteins. The CHORD II domain is known to be required for the interaction with SGT1, but not HSP90 (Takahashi et al., 2003; Liu et al., 2004). Indeed, a yeast two-hybrid experiment indicated that the RAR1-29 protein is unable to interact with SGT1b but interact normally with HSP90 (Fig. 3-2d).

RAR1 and NDR1 Are Required for Type II But Not Type I Nonhost Resistance

Based on the presence or absence of HR to a nonhost pathogen, plant nonhost resistance can be classified into two types (Mysore and Ryu, 2003). Type I nonhost resistance does not trigger HR whereas type II nonhost resistance does. According to this criterion, nonhost *Pseudomonas* bacteria strain *P. s.* pv. *tabaci* triggers type II nonhost resistance and *P. s.* pv. *phaseolicola* triggers type I nonhost resistance in *Arabidopsis* plants (Oh et al., 2006). To test the requirement of RAR1 in type I and type II nonhost resistance, we hand-infiltrated *P. s.* pv. *tabaci* and *P. s.* pv. *phaseolicola* into Col-0 and *rrb3* leaves. Interestingly, the *rrb3* mutant consistently supported *P. s.* pv. *tabaci* growth by 10 folds (Fig. 3-3a). The growth of *P. s.* pv. *phaseolicola* in *rar1-29* remained the same with that in wild type plants (Fig. 3-3b). Because RAR1 is known to participate in gene-for-gene resistance, we hypothesize that other signaling components may also affect type II nonhost resistance in *Arabidopsis*. To test this hypothesis, five mutants defective in *R* gene signaling were selected and checked for nonhost bacterial growth (Table 2). Two mutants, *rar1-20* and *ndr1* supported the growth of *P. s.* pv. *tabaci* but not *P. s.* pv. *phaseolicola* (Fig. 3-3c, Table 2). It has been shown that RAR1 and NDR1 contribute quantitatively to the function of a number of CC-NBS-LRR proteins (Torreto et al., 2002). However, the role of *R* genes or *R*-signalling genes in type II nonhost resistance is not clear. Recent microarray data showed that genes regulated by nonhost and avirulent bacteria

were overlapped (Navarro et al., 2004). Here we show that RAR1 and NDR1 are required for type II nonhost resistance, suggesting that gene-for-gene resistance and nonhost resistance share similar signaling components.

RAR1 Antagonizing COI1 Activity in Basal Resistance Against DC3000

COI1 is a well-known signaling component mediating JA response and bacterial virulence (Feys et al., 1994; Xie et al., 1996). A point mutation in COI1 led to reduced DC3000 growth (Kloek et al., 2001), indicating a role of JA signaling in DC3000 pathogenicity. The resistance of the *coi1* mutant to DC3000 is correlated with PR-1 activation and SA accumulation. Eliminating SA restored the susceptibility of *coi1* to DC3000 (Kloek et al., 2001), suggesting a crosstalk between JA and SA signaling pathways. Here we show that both *rar1-29* and *rar1-20* mutants supported 50 to 100 fold more DC3000 bacterial growth (Fig. 3-4a) and showed more severe disease symptoms (Fig. 3-4b). PR-1 expression upon DC3000 infection is down regulated (He P., Chintamanani S. and Zhou J.M.) whereas *RAR2.6* expression is up regulated (Fig. 3-4c) in the *rar1-29* mutant compared with the wild type plants. Microarray analysis showed that about 25% of the down-regulated genes in *coi1* upon DC3000 infection are up-regulated in the *rar1-29* mutant (Fig. 3-5a, Table 3), revealing an antagonism between RAR1 and COI1 in regulating DC3000 responsive genes. To determine the epistatic relationship between *rar1* and *coi1*, the *rar1/ coi1* double mutant was created and tested for DC3000 bacterial growth. As shown in Fig. 3-5b, DC3000 grow similarly in the *rar1/coi1* double mutant and the *coi1* mutant (Fig. 3-5b), suggesting that COI1 is epestatic to RAR1 and mediates *rar1* susceptibility to DC3000. Intriguingly, the JA response measured by root length inhibition in *rar1* is not significantly affected (Fig. 3-5c), suggesting that either RAR1 only affects COI1 activity triggered by DC3000 or the effect of RAR1 on JA response is masked by other signaling pathways. For example, SGT1b is involved in JA signaling (Gray et al., 2003). However, the effect of SGT1b in JA signaling is partially masked by ethylene signaling pathway and is more pronounced in *ein3* mutant background (Lorenzo and Solano, 2005; Solano, personal communication).

RAR1 is Required for AvrB-Dependent Susceptibility and Chlorosis

AvrB virulence activity is measured by leaf chlorosis in *Arabidopsis* and enhanced bacterial growth in bean plants (Ong and Innes, 2006). Dex-inducible AvrB expression in *Arabidopsis* contributes to enhanced bacterial growth of *P. s.* pv. *phaseolicola* (Fig.3-6a) as well as *P. s.* pv. *tomato DC3000 hrcC*- mutant strain (Fig. 3-6b, contributed by Yulei Shang), indicating *in planta* AvrB expression renders *Arabidopsis* plants more susceptible to *Pseudomonas* bacteria in a strain-nonspecific manner. To determine whether the ability of AvrB to cause leaf chlorosis and modulate bacterial growth is dependent on RAR1, the Dex-AvrB transgenic plants (in Nd-0 accession) were crossed to both the *rar1-29* and *rar1-20* plants. F2 plants with *AvrB//+, rpm1//rpm1, RAR1//rar1* genotype were selected and propagated to F3 and F4. *AvrB//AvrB, RAR1//RAR1, rpm1//rpm1* and *AvrB//AvrB, rar1//rar1, rpm1//rpm1* homozygous plants were tested for Dex-inducible leaf chlorosis as well as AvrB-dependent bacterial growth. As shown in Fig. 3-6c, leaf chlorosis induced by Dex treatment was abolished in both *rar1-29* (Fig. 3-7a) and *rar1-20* (Shang et al., 2006) mutant backgrounds, indicating that RAR1 is required for AvrB-dependent leaf chlorosis. Four independent F4 lines from two independent crosses were tested and showed consistent phenotypes (Fig. 3-7a). Besides a role in AvrB triggered leaf chlorosis, RAR1 is also required for AvrB enhanced DC3000 hrcC$^-$ bacterial growth (Fig. 3-7b, credit shared by Yulei Shang). Taken together, these data demonstrated AvrB requires RAR1 to mediate its virulence activity in *Arabidopsis* plants.

AvrB Reduces DC3000 Bacterial Growth in a RAR1-Dependent Manner

To test whether AvrB expression also enhances virulent bacterial growth in *Arabidopsis*, we inoculated DC3000 onto Dex-treated *AvrB//AvrB, RAR1//RAR1, rpm1//rpm1* and *AvrB//AvrB, rar1//rar1, rpm1//rpm1* homozygous plants. Surprisingly, AvrB expression reduces, instead of increases, the bacterial number of DC3000 (Fig. 3-8). The reduction of DC3000 bacterial number occurred before leaf chlorosis took place (data not shown), suggesting that it is not a side effect of leaf chlorosis. Interestingly, the AvrB dependent reduction of DC3000 growth is also mediated by RAR1 because Dex-induced AvrB expression no longer reduces DC3000 bacterial number in *rar1* background (Fig. 3-8). The reduction of

DC3000 bacterial growth triggered by AvrB is probably specific to the genetic background tested here because AvrB transgenic line in other genetic background did not significantly affect DC3000 bacterial growth (Makey, personal communication). Taken together, these results suggest that a cryptic *R* gene may exist in Nd-0 background, which recognizes AvrB and triggers disease resistance against DC3000.

RAR1 Is a Host Target of AvrB

Based on the guard hypothesis, effector proteins target host components to achieve virulence on host plants. Although we showed that RAR1 is required for AvrB dependent virulence activity, whether or not RAR1 is a host target of AvrB remains undetermined. To test whether AvrB directly interacts with RAR1, yeast two-hybrid experiment was carried out. As shown in Fig. 3-9a, there is no physical interaction between AvrB and RAR1 (Fig. 3-9a). However, AvrB and RAR1 co-immunoprecipitation experiments indicate they do exist in the same protein complex (Fig. 3-9b, contributed by Haitao Cui), suggesting that RAR1, or the protein complex containing RAR1 is targeted by AvrB. The interaction between AvrB and RAR1 could be indirect and may need other host protein (s).

COI1 Is Required for AvrB-Dependent Susceptibility

Because COI1 works downstream of RAR1 to mediate DC3000 virulence activity, we further tested the requirement of COI1 in AvrB dependent susceptibility and leaf chlorosis. The AvrB transgenic line was crossed to *COI1/coi1* heterozygous plants using *COI1/coi1* as the recipient. F1 plants with *COI1/coi1* genotype were forwarded to F2 and F3. *AvrB/+, COI1/COI1, rpm1/rpm1* and *AvrB/+, coi1/coi1, rpm1/rpm1* progenies were selected and analyzed for AvrB dependent bacterial growth and leaf chlorosis. As shown in Fig. 3-10, COI1 is required for AvrB dependent bacterial growth (Fig. 3-10a, credit shared by Yulei Shang) but not for AvrB dependent leaf cholosis (Fig. 3-10b, credit shared by Yulei Shang). How COI1 mediates AvrB virulence activity is not clear. It is likely that AvrB modulates COI1 activity to promote bacterial virulence. Supporting this hypothesis, seedlings with AvrB expression consistently exhibit better root length inhibition induced by MeJA (Fig. 3-10c).

Discussion

RAR1 is known to play a key role in gene-for-gene resistance to diverse pathogens (Shirasu et al., 1999; Takahashi et al., 2003). However, a role of RAR1 in nonhost resistance and bacterial effector virulence function has not been reported. Our results show that RAR1, together with NDR1, participate in type II but not type I nonhost resistance. Although it is not clear how RAR1 and NDR1 affect type II nonhost resistance, these two proteins do act synergistically in gene-for-gene resitance mediated by several CC-NBS-LRR proteins (Tornero et al., 2002). It is possible that RAR1 and NDR1 assist some unidentified CC-NBS-LRR proteins in mediating type II nonhost resistance. *R* genes and their signaling components are known to carry out species level resistance in several pathosystems (Peart et al., 2002; Zhao et al., 2005). More significantly, the *Pseudomons* effector AvrB targets RAR1 to mediate its virulence activity. In the *rar1* mutant background, both AvrB dependent leaf chlorosis and increase in bacterial growth are abolished. The AvrB dependent increase in bacterial growth but not leaf chlorosis also requires COI1, an F-box protein required for JA and wound-inducible responses.

How AvrB affects COI1 activity is not clear. It is likely that AvrB modulate COI1 activity through RAR1. It is known that SGT1, a RAR1 interactor, is involved in JA signaling (Lorenzo and Solano, 2005). SGT1 physically interacts with SKP1 and CUL1, subunits of the SCF (Skp1-Cullin-F-box) ubiquitin ligase complex (Azevedo et al., 2002) and is required for SCF (TIR1) mediated auxin responses (Gray et al., 2003). It is possible that RAR1, once targeted by AvrB, associates with SGT1 and SCF (COI1) to promote *Pseudomonas* pathogenicity. Supporting this model, a point mutation abolishing SGT1 interaction renders RAR1 totally unfunctional in mediating AvrB virulence activity (Fig. 3-2d, 3-6b and 3-6c). Moreover, we were able to detect a greater root length inhibition induced by JA in seedlings with AvrB expression (Fig. 3-9c), indicating that AvrB promotes JA signaling.

It is noteworthy that AvrB also reduces DC3000 growth in a RAR1-dependent, RPM1-independent manner. The resistance triggered by AvrB overexpression *in planta* may be a unique feature of the genetic lines used in this study because a similar overexpression of AvrB did not trigger any DC3000 resistance (Mackey, personal communication). A possible explanation is that a cryptic R protein (probably carried in the Nd-0 accession) recognized

AvrB and triggered the resistance against DC3000. Interestingly, this RPM1-independent, RAR1-dependent resistance is only triggered by AvrB overexpression in *Arabidopsis* plants. AvrB delivered by DC3000 failed to elicit an effective resistance response because DC3000 (*avrB*) grew similarly with DC3000 in *Arabidopsis* plants lacking the RPM1 resistance gene (Li X. and Zhou J.M., unpublished data). It is noteworthy that the bacterial growth of DC3000 *hrpL*$^{-}$ mutant is promoted, instead of inhibited by AvrB, suggesting that the AvrB-triggered, RPM1-independent and RAR1-dependent resistance may require other DC3000 effectors.

Besides a role in mediating type II nonhost resistance and AvrB virulence, RAR1 also functions in basal resistance against DC3000. We showed that the *rar1* mutant is more susceptible and that *rar1* susceptibility is carried out by COI1, indicating that RAR1 mediates basal resistance against DC3000 by antagonizing COI1 activity. Intriguingly, both RAR1 and COI1 are required for AvrB dependent bacterial growth. It is most likely that AvrB targets RAR1 and relieves the suppression on COI1 activity that eventually promotes AvrB virulence as illustrated in Fig. 3-11. RAR1 may also impose a positive regulation on SA pathway that is not negatively affected by AvrB targeting. Supporting this model, AvrB expression induces both JA and SA pathway genes in microarray analysis (Thilmony R., unpublished data).

The previously identified AvrB target protein RIN4 is required for effector-triggered resistance (Axtell et al., 2003; Mackey et al., 2002; Mackey et al., 2003) but not the effector virulence function (Belkhadir et al., 2004). It is suggested that other proteins associated with RIN4 might be required for the AvrRpt2 and AvrRpm1 virulence functions (Kim et al., 2005). Here we showed that RAR1 is required for AvrB virulence activity and coimmunoprecipitates with AvrB *in planta*. However, RAR1 and RIN4 do not exist in a same protein complex (Shang et al., 2006), suggesting that RAR1 may carry out AvrB virulence function independent of RIN4. Interestingly, both RAR1 and RIN4 negatively regulate flagellin induced cell wall defense (Kim et al., 2005; Shang et al., 2006), indicating that targeting negative regulators of PAMP-triggered immunity (Chisholm et al., 2006) could be a common strategy used by bacteria to suppress basal resistance.

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Table 3-1 HR complementation of *rrb3*

	LUC	HR
WT	+	3 (3)
rrb3	+	0 (7)
rar1-20	-	0 (4)
F1 (*rar1-20*×*rrb3*)	+	0 (5)
F1 (*rar1-20*×WT)	+	5 (5)

Table 3-2 The differential requirement of HR signaling components in type I and type II nonhost resistance

Arabidopsis mutants	Signaling components	*P. s.*pv. *phaseolicola* (Type I nonhost resistance)	*P. s.*pv. *tabaci* (Type II nonhost resistance)
rar1-20	RAR1	N	Y
ndr1	NDR1	N	Y
pbs1	PBS1	N	N
sgt1b	SGT1b	N	N
eds1	EDS1	N	N

Table 3-3 Selected gene list of *coi1*-downregulated and *rar1*-upregulated JA pathway genes

Array element	Locus	Description
249208_at	AT5G42650	allene oxide synthase (AOS) / hydroperoxide dehydrase / cytochrome P450 74A (CYP74A),
245928_s_at	AT5G24770 AT5G24780	vegetative storage protein 2 (VSP2), identical to SP:O82122 Vegetative storage protein 2 precursor {*Arabidopsis thaliana*}
255786_at	AT1G19670	coronatine-responsive protein / coronatine-induced protein 1 (CORI1), identical to coronatine-induced protein 1 (CORI1)
260399_at/ 261037_at	AT1G72520 AT1G17420	lipoxygenase, putative
265058_s_at	AT1G52030 AT1G52040	myrosinase-binding protein, putative (F-ATMBP),
265530_at	AT2G06050	12-oxophytodienoate reductase (OPR3) / delayed dehiscence1 (DDE1)

Figure Legend

Figure 3-1 The mutant *rrb3* compromises AvrB-specific activities. (a). *RAP2.6*-LUC reporter activity in both wild type and *rrb3* mutant plants in response to DC3000 (*avrB*). RAP2.6-LUC transgenic plants were inoculated with DC3000 (*avrB*) suspended in 0.2mM luciferin at $2X10^6$ cfu/ml. Luciferase activity was examined at the indicated time points. (b). HR response elicited by DC3000 (*avrB*). *Arabidopsis* wild type and *rrb3* mutant plants were infiltrated with DC3000 (*avrB*) at 10^8 cfu/ml. HR started to develop in wild type plants 6 hours after inoculation. Picture was taken 10 hours after inoculation. At least 6 individual plants were tested for HR and showed similar response as shown in the picture. (c). Disease symptoms induced by DC3000 (*avrB*) in both wild type and *rrb3* mutant plants. An inoculum of 10^6 cfu/ml was used. The picture was taken 6 days after inoculation. These experiments were repeated at least three times with similar results.

Figure 3-2 *RRB3* is a previously unidentified *RAR1* allele. (a). A wild type copy of *RAR1* driven by its natural promoter complemented the HR response in both *rrb3* and *rar1-20* mutants. *Arabidopsis* Col-0, *rrb3*, *rar1-20* plants and the *rrb3* or *rar1-20* T1 transgenic plants carrying the RAR1 transgene were inoculated with DC3000 (*avrB*) at 10^8 cfu/ml. Plants were examined for HR from 6 to 12 hours after inoculation. Picture was taken 10 hours after inoculation. From left to right: the wild type *Arabidopsis* plants, the *rrb3* mutant, the *rar1-20* mutant, the *rar1-20* mutant complemented by *RAR1* transgene, the *rrb3* mutant complemented by *RAR1* transgene. The number of leaves shown an HR is listed bellowed the picture. (b). *rrb3 (rar1-29)* mutant carries a point mutation at the end of CHORD II domain of the RAR1 protein. The filled boxes represent the CHORD I (cysteine-and histidine-rich domain), CCCH (Cys-Cys-Cys-His) and CHORD II domains of the RAR1 protein. (c). The stability of RAR1-29 protein in yeast (left) and plants (right). To express the proteins in yeast cells, *RAR1* and *RAR1-29* coding regions were constructed into pJG4-5 vector carrying an HA tag and transformed into the EGY48 yeast strain. Yeast total protein was extracted with the 2× Laemmli sample buffer and bloted with anti-HA antibody. The stability of RAR1-29 protein in *Arabidopsis* plants was determined with the anti-RAR1 antibodies. The total protein was

extracted from *Arabidopsis* wild type and *rar1-29* mutant plants. Equal amount of the total protein was electrophoresed through 12% SDS-PAGE gel and blotted with the anti-RAR1 antibodies. Protein bands were detected with the ECL detection reagents provided by Amersham, Piscataway, NJ. (e). The interaction with SGT1b but not HSP90.1 is abolished in RAR1-29 protein. RAR1 wild type and RAR1-29 coding sequences were cloned into the pJG4-5 vector carrying an HA tag. SGT1b and HSP90.1 coding sequences were constructed into the pEG202 vector. The corresponding contructs were transformed in pair into the EGY48 yeast strain containing pSH18-34. Protein interaction was tested on X-Gal plates. At least six individual transformants were examined for β-galactosidase activities. Two individual colonies were shown in the picture. These experiments were repeated at least twice with similar results.

Note: The western blot figure (Figure 3-2c, on the right) showing the RAR1-29 protein stability in *Arabidopsis* plants is contributed by Shang Y.

Figure 3-3 Both RAR1 and NDR1 contribute to type II nonhost resistance. (a) and (b). *rar1-29* mutant compromised nonhost resistance against *P. s.* pv. *tabaci* but not *P. s.* pv. *phaseolicola.* (c). *ndr1* compromised nonhost resistance against *P. s.* pv. *tabaci* but not *P. s.* pv. *phaseolicola.* Bacteria were inoculated at $2X10^5$ cfu/ml. Leaf discs were detached from the plants on 0, 2 and 4 days afer inoculation and counted for bacteria number. These experiments were repeated at least three times with similar results.

Figure 3-4 RAR1 plays a role in basal resistance against DC3000. (a) and (b). DC3000 bacteria grow more vigorously and cause more severe disease symptom in *rar1-29* and *rar1-20* mutant compared to wild type plants. Plants were dip-inoculated with $2X10^7$ cfu/ml DC3000 suspended in10mM $MgCl_2$ containing 0.02% Silwet L-77. The inoculated plants were covered with plastic dome for 1 hour before transforming to a plant growth chamber. For bacteria growth assay, *Arabidopsis* leaves were detached from the inoculated plants at the indicated time points, weighed and counted for bacteria number. For disease symptom analysis, the inoculated plants were encubated at 22°C in a plant growth chamber for symtom development. Picture was taken 6 days after inoculation (c). RAR1 mutation compromised AvrB-specific *RAP2.6* induction but enhanced *RAP2.6* expression upon DC3000 infection. Total RNA was extracted with 2XCTAB buffer from *Arabidopsis* plants inoculated

with DC3000 or DC3000 (*avrB*) at 2X10^6 cfu/ml. The RNA blot was hybridized with the *RAP2.6* cDNA probe.

Figure 3-5 RAR1 mediates *Arabidopsis* basal resistance against DC3000 through antagonizing COI1 activity. (a). RAR1 antagonizing COI1 in regulating DC3000 responsive genes. The wild type (Col-0), *rar1-29* and *coi1-1 Arabidopsis* plants were inoculated with DC3000 at 2X10^6 cfu/ml. Leaves were detached at 0 and 24 hours after inoculation. Total RNA was extracted with 2XCTAB buffer and purified with the RNeasy Mini Kit (QIAGEN Sciences, ML). Microarray experiments were carried out using the *Arabidopsis* Genome ATH1 Array containing 22,500 probe sets (Affymetrix, Inc). Each of the microarray experiments contains at least three independent replicates. Haitao Cui analyzed Microarray data with the Genespring 7.0 software. (b). *coi1-1* mutation complemented DC3000 susceptibility in *rar1-29* mutant. *Arabidopsis* Col-0, *coi1-1*, *rar1-29* and *coi1-1/rar1-29* plants were inoculated with DC3000 at 10^5 cfu/ml. Leaf bacteria number was counted at the indicated time points. This experiment was repeated twice with similar results. (c). The *rar1-29* mutation does not affect root length inhibition induced by MeJA *Arabidopsis* wild type, *rar1-20* and *rar1-29* seeds were surface sterilized and germinated on 1/2MS plates containing MeJA at the indicated concentrations. Root length was measured 9 dyas after germination. The relative root length was calculated by dividing the actual root length by the root length of control (Col-0) plants growing in 1/2MS without MeJA. This experiment was repeated twice with similar results.

Figure 3-6 AvrB expression in *Arabidopsis* enhances the bacterial growth of both nonhost and nonpathogenic bacteria. (a). AvrB expression enhances *P. s.* pv. *phaseolicola* bacterial growth. This experiment was done once. (b). AvrB expression enhances DC3000 (hrcC$^-$) bacterial growth. This experiment was repeated three times with similar results. AvrB transgenic plants were treated with Dex 2 days before bacterial inoculation. H_2O treatment was used as a control. Bacteria were inoculated at 10^5 cfu/ml. Leaf discs were detached from the inoculated bacteria plants and counted for bacterial number at the indicated time points.

Note: Fiugre 3-6b is contributed by Shang Y.

Figure 3-7 RAR1 is required for both AvrB dependent leaf chlorosis and bacterial growth. (a). Dex induced leaf chlorosis in *AvrB//AvrB, RAR1//RAR1, rpm1//rpm1* and

AvrB//AvrB, rar1//rar1, rpm1//rpm1 plants. Plants were inoculated with 30μM Dex to induce AvrB expression. Leaf chlorosis was examined 4 days after inoculation. Each genotype is represented by 4 independent F4 lines derived from two independent crosses. (b). DC3000 (*hrcC*-) bacterial growth in *AvrB//AvrB, RAR1//RAR1, rpm1//rpm1* and *AvrB//AvrB, rar1//rar1, rpm1//rpm1* plants. Bacteria were infiltrated at 10^5 cfu/ml into fully expanded *Arabidopsis* leaves 2 days after Dex treatment. Leaf bacteria number was counted at the indicated time points. These experiments were repeated at least two times with similar results.

Note: Shang Y. shares credit on Figure 3-7b.

Figure 3-8 RAR1 is required for AvrB dependent and RPM1 independent DC3000 resistance in *Arabidopsis* plants. Homozygous *AvrB//AvrB, RAR1//RAR1, rpm1//rpm1* and *AvrB//AvrB, rar1//rar1, rpm1//rpm1* plants were inoculated with 30μM Dex to induce AvrB expression. DC3000 bacteria were infiltrated at 10^5 cfu/ml into fully expanded *Arabidopsis* leaves 1 day after Dex treatment. Leaf bacteria number was counted at the indicated time points. This experiment was repeated at least twice with similar results.

Figure 3-9 AvrB and RAR1 do not interact in yeast but coimmunoprecipitate in plant protein extracts. (a). Yeast-two-hybrid analysis for AvrB and RAR1 interaction. AvrB and RAR1 (or RAR1-29) coding sequences were constructed into pJG4-5 and pEG202 respectively and transformed into EGY48 yeast strain containing pSH18-34. Six individual yeast transformants were tested for β-galactosidase activities on X-Gal plates. One representative clone was shown in the picture. RAR1-SGT1b interaction was used as a control. AvrB and SGT1b (in pJG4-5) proteins were detected with anti-HA antibody. (b). AvrB coimmunoprecipitated with RAR1. Total plant protein was extracted from AvrB-FLAG transgenic plants and immunoprecipitated with anti-FLAG antibodies. RAR1 protein was detected in the precipitates with the anti-RAR1 antibodies. These experiments were repeated at least twice with similar results.

Note: Fiugre 3-9b is contributed by Cui H.

Figure 3-10 AvrB expression enhances bacterial growth through COI1 and promotes JA response. (a). COI1 is required for AvrB dependent bacterial growth. *AvrB//+*,

COI1//COI1, rpm1//rpm1 and *AvrB//+, coi1//coi1, rpm1//rpm1* plants were pretreated with 30μM Dex and inoculated with DC3000 (*hrpL*$^-$) at 10^5 cfu/ml. Leaf bacterial number was counted at the indicated time points. (b). COI1 is not involved in AvrB dependent leaf chlorosis. *AvrB//+, COI1//COI1, rpm1//rpm1* and *AvrB//+, coi1//coi1, rpm1//rpm1* plants were sprayed with 30μM Dex to induce *AvrB* expression. Leaf chlorosis was examined 4 days after Dex treatment. (c). AvrB expression promotes root length inhibition mediated by exogenously applied MeJA. AvrB transgenic plants were germinated on 1/2MS plates with or without Dex. MeJA was added at the indicated concentration. Root length was measured 9 days after germination. Relative root length was calculated by dividing the actual root length by the root length of AvrB transgenic plants growing in 1/2MS without MeJA. These experiments were repeated at least twice with similar results.

Note: Shang Y. shares credit on Figure 3-10a and 3-10b.

Figure 3-11 A model illustrating roles of RAR1 and COI1 in both disease resistance and AvrB dependent virulence activity. In *Arabidopsis* plants without AvrB transgene, RAR1 suppress COI1 activity that mediated JA response and DC3000 susceptibility to play a role in basal resistance. With AvrB expression, RAR1 is recruited in a protein complex containing AvrB. The suppression on COI1 activity is thus relieved and downstream genes involving in pathogenicity are expressed.

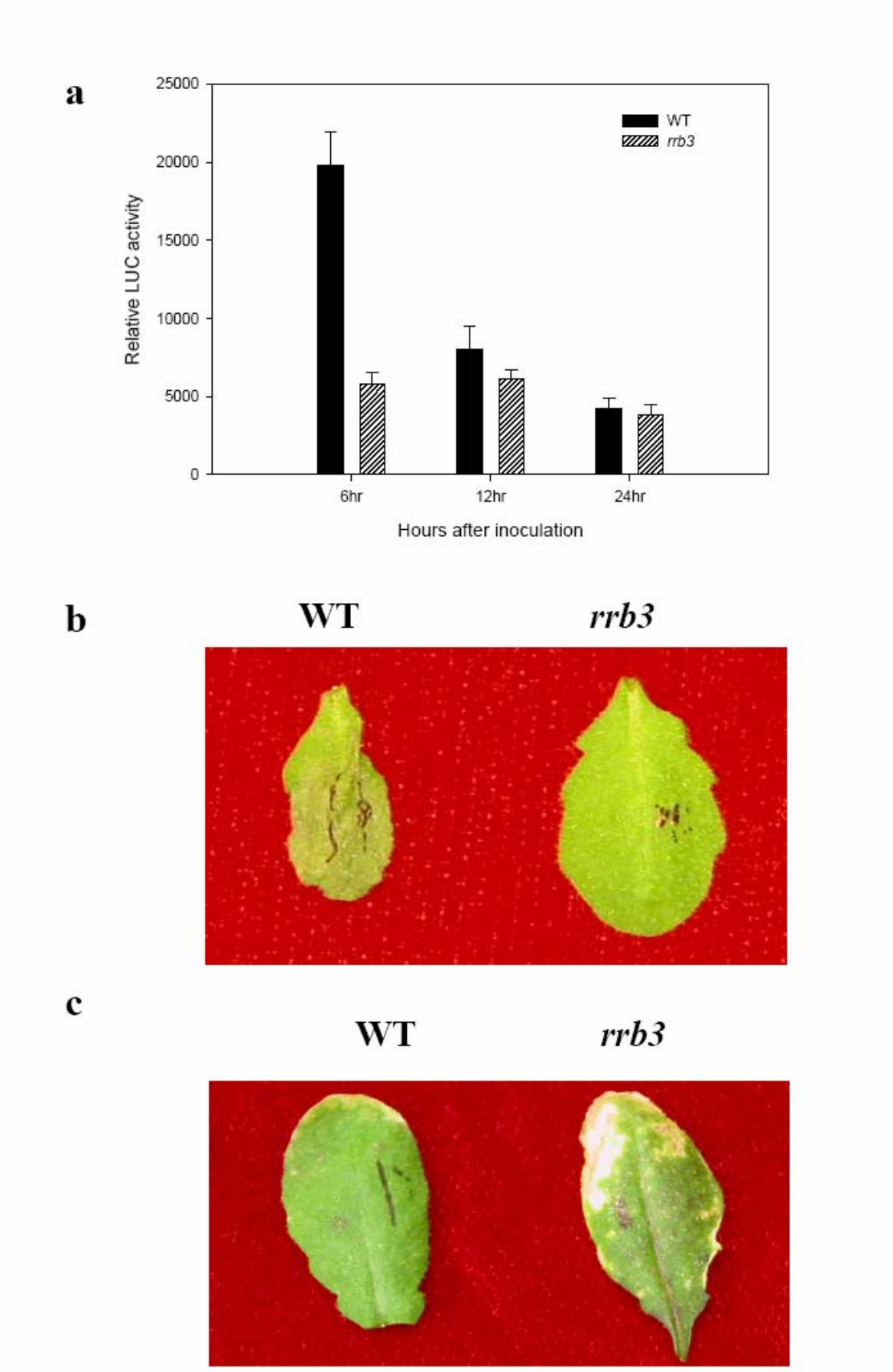

Figure 3-1 The mutant *rrb3* compromises AvrB-specific activities

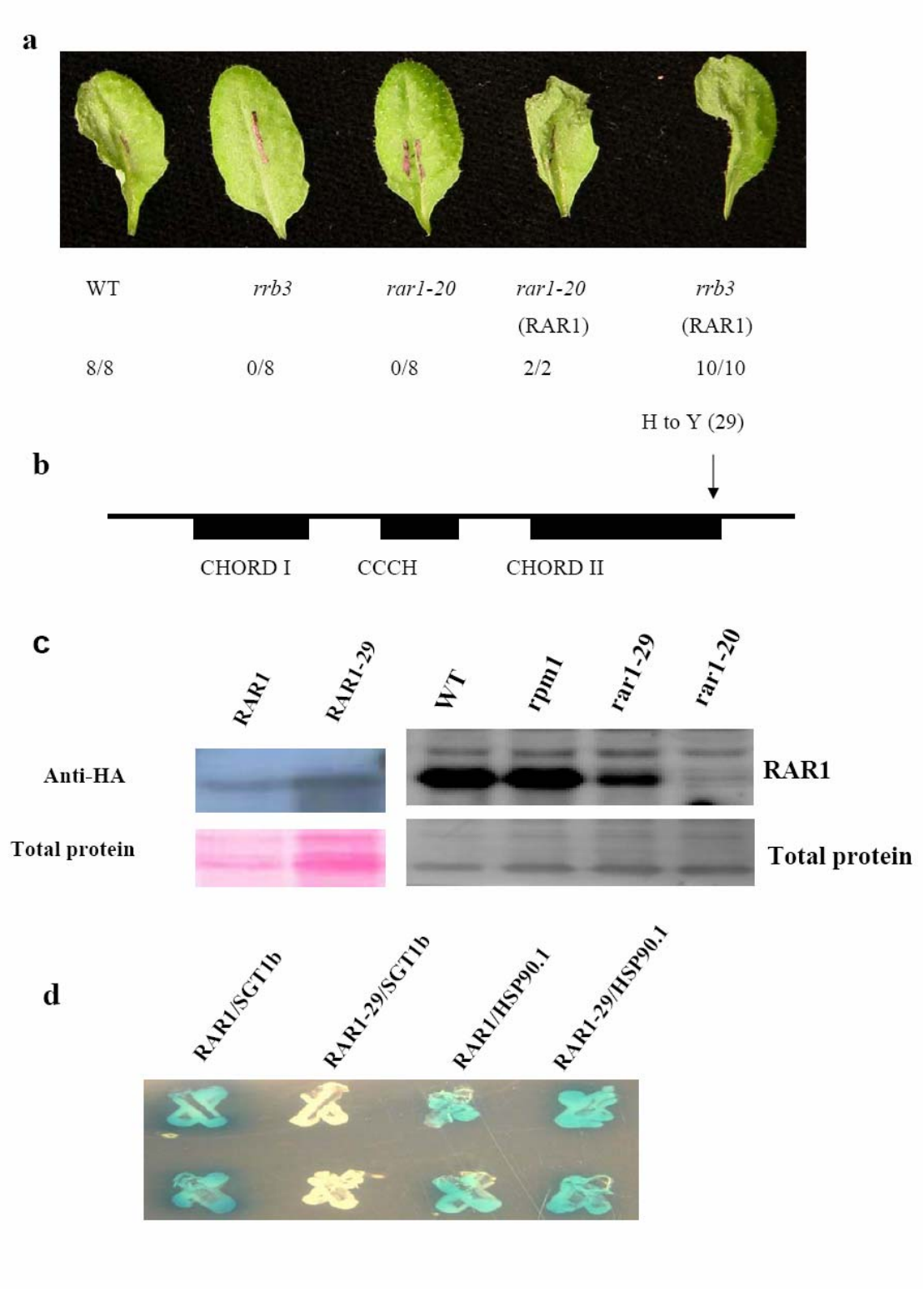

Figure 3-2 *RRB3* is a previously unidentified *RAR1* allele

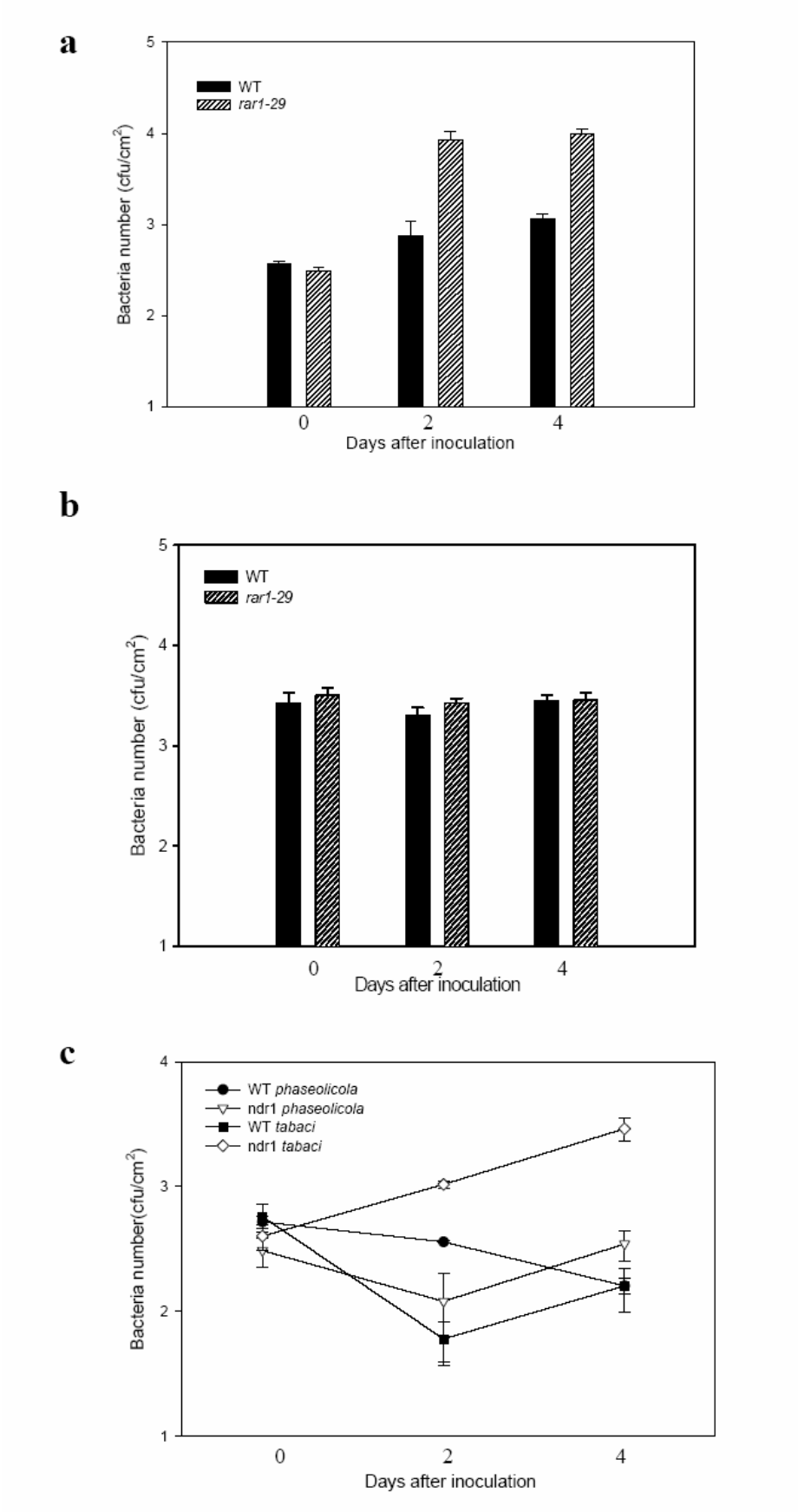

Figure 3-3 Both *RAR1* and *NDR1* contribute to type II nonhost resistance

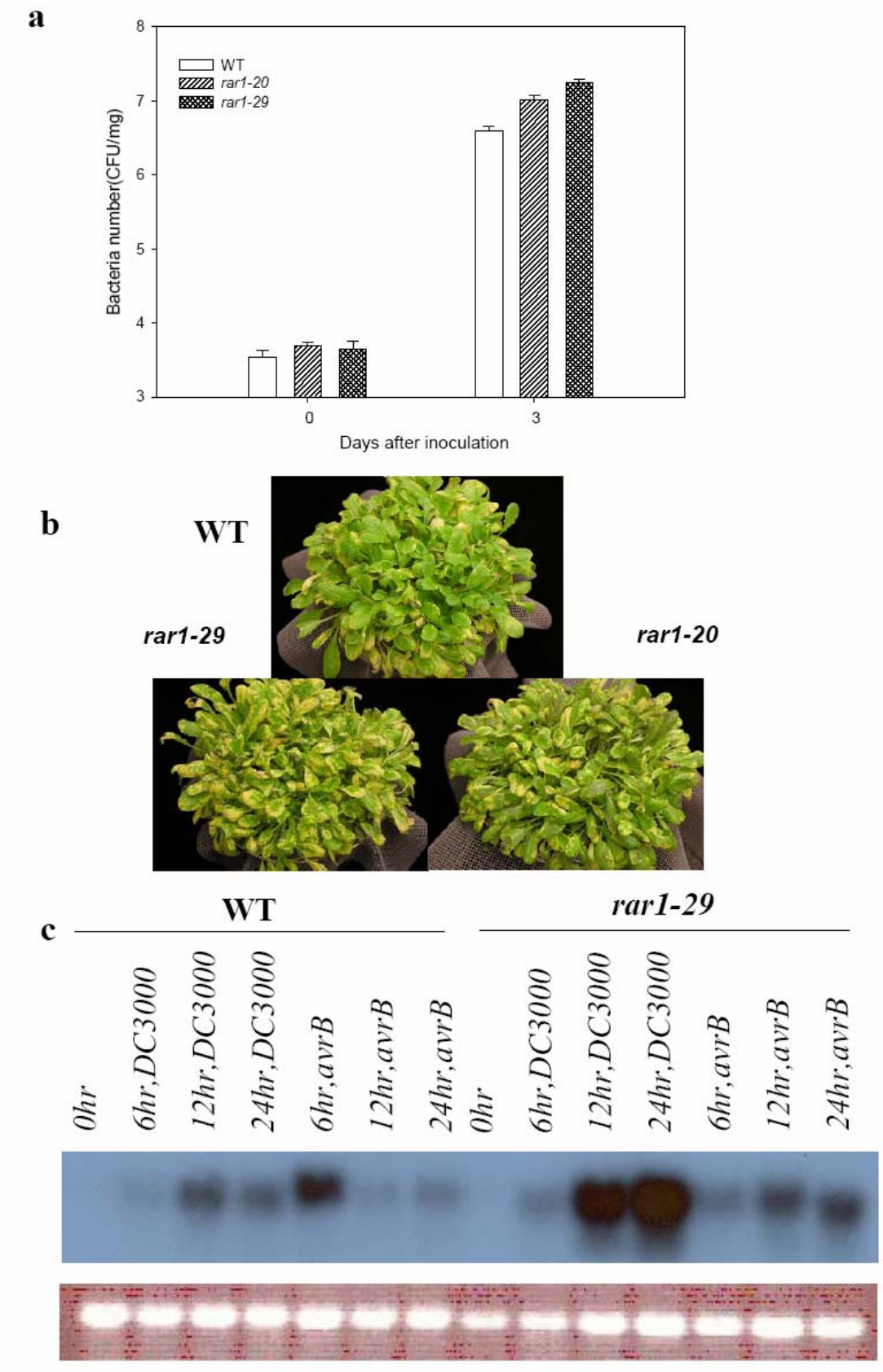

Figure 3-4 *RAR1* plays a role in basal resistance against DC3000

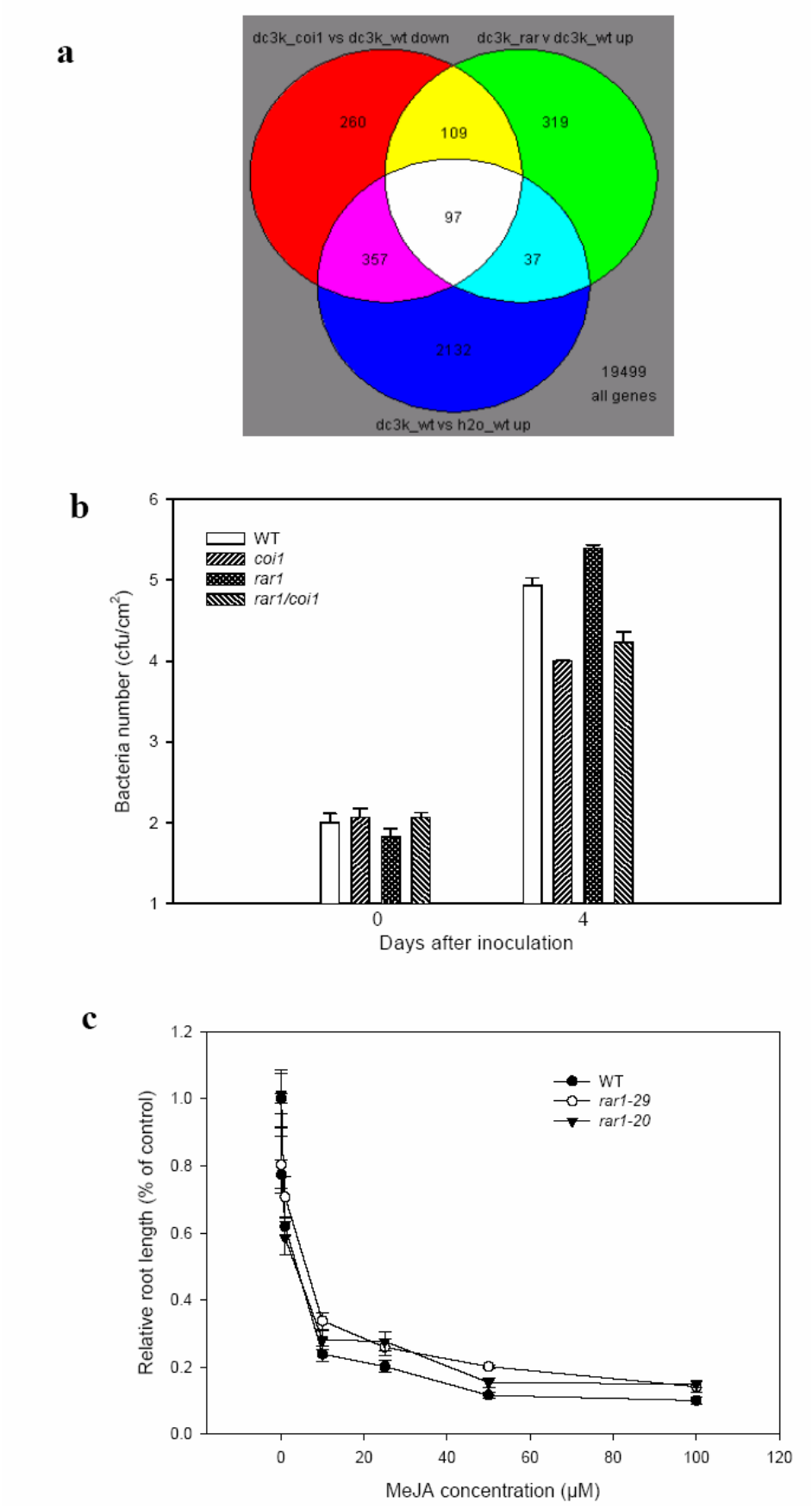

Figure 3-5 *RAR1* mediated basal resistance against DC3000 through antagonizing *COI1* activity

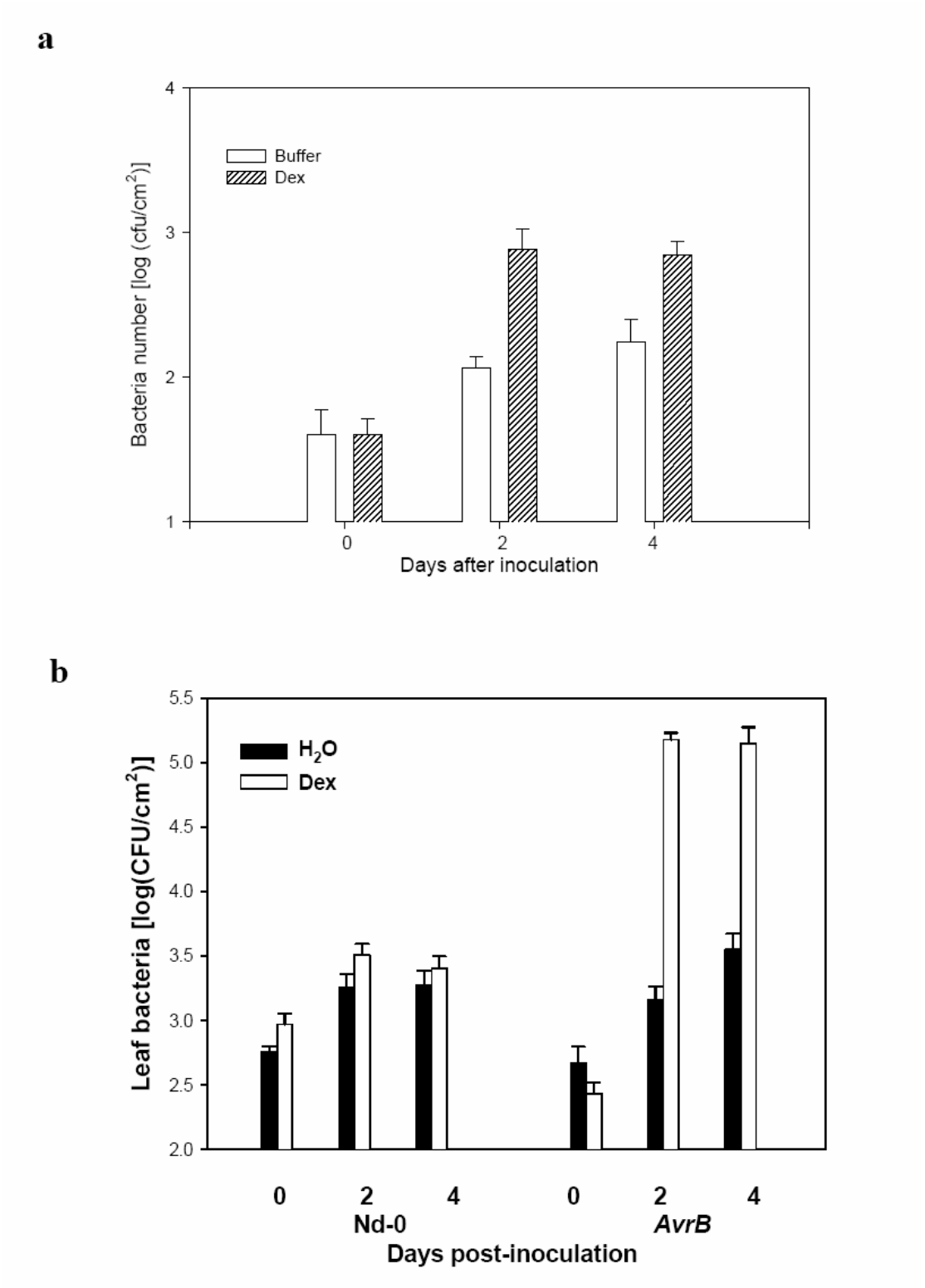

Figure 3-6 *AvrB* expression in *Arabidopsis* enhances the bacterial growth of both nonhost and nonpathogenic bacteria

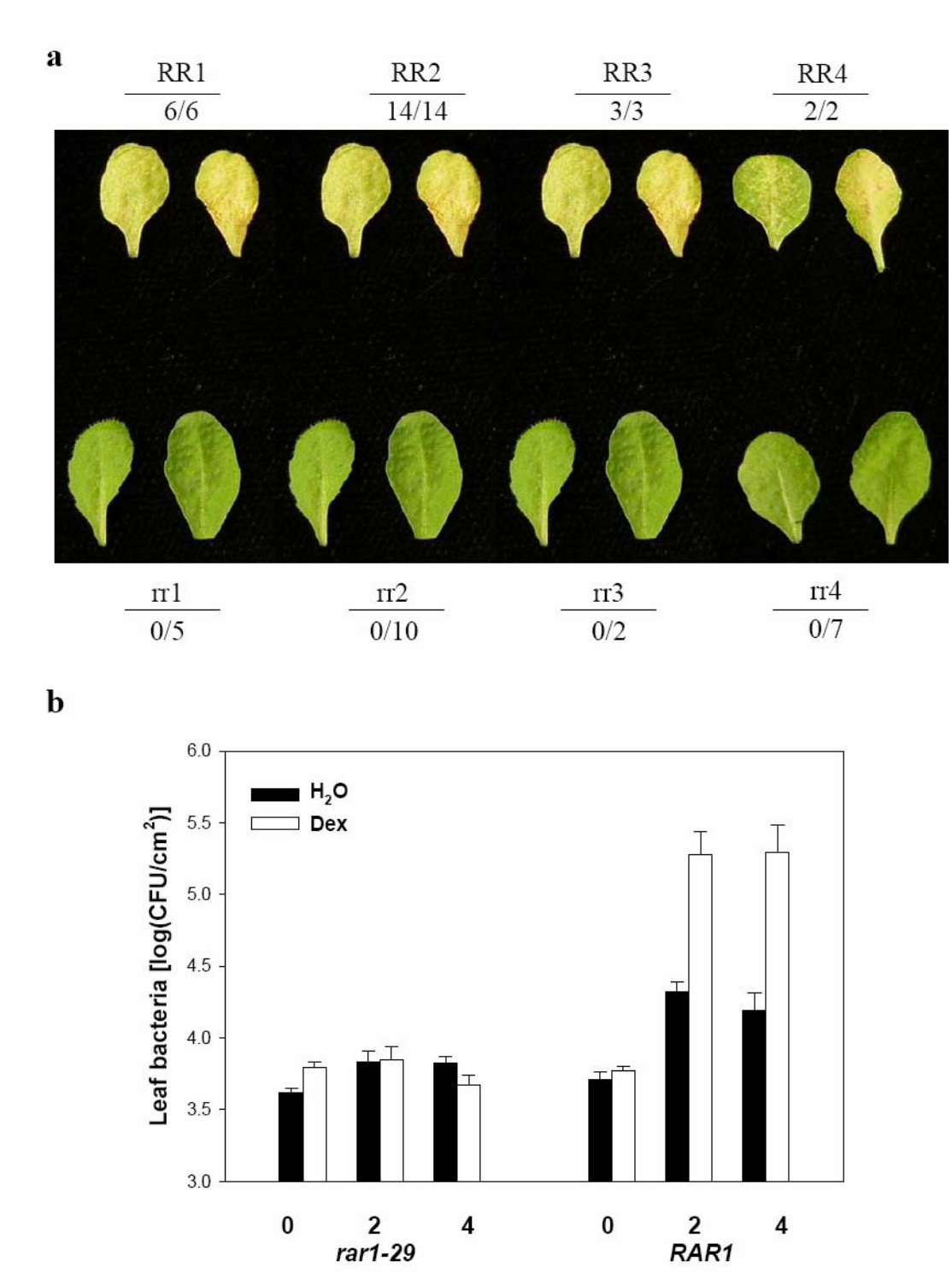

Figure 3-7 *RAR1* is required for both AvrB-dependent leaf chlorosis and bacterial growth

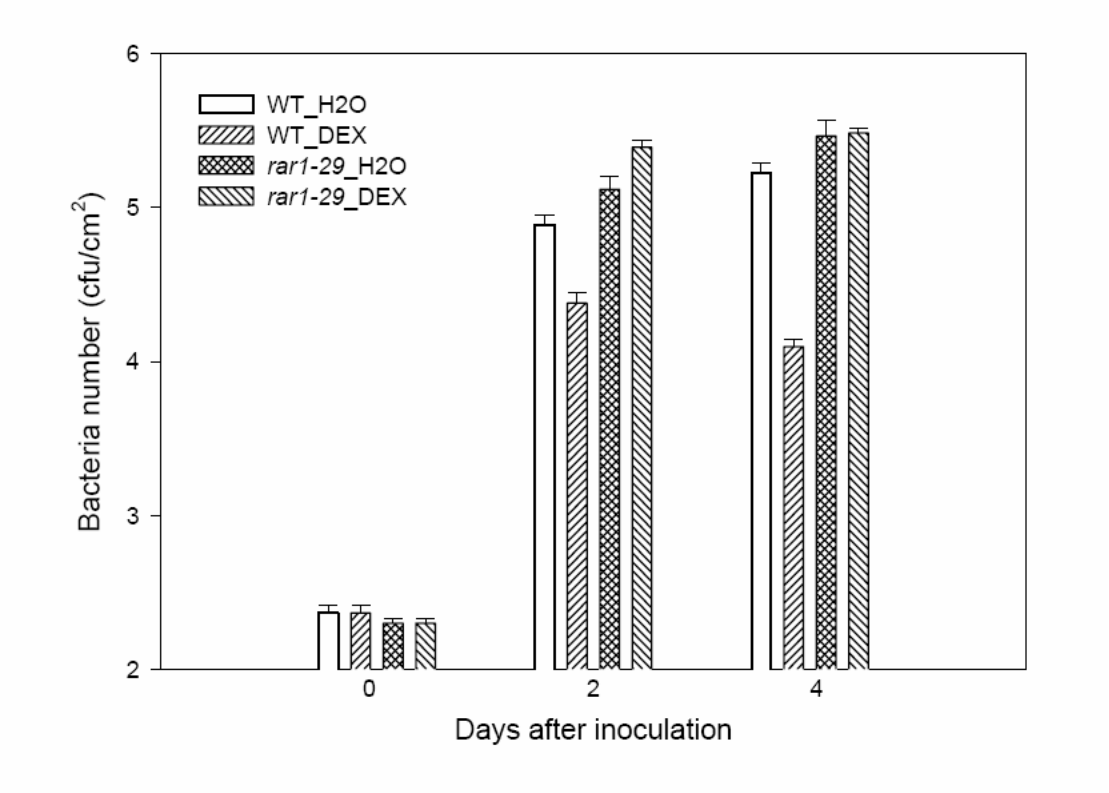

Figure 3-8 *RAR1* is required for AvrB-dependent and *RPM1*-independent DC3000 resistance in *Arabidopsis* plants.

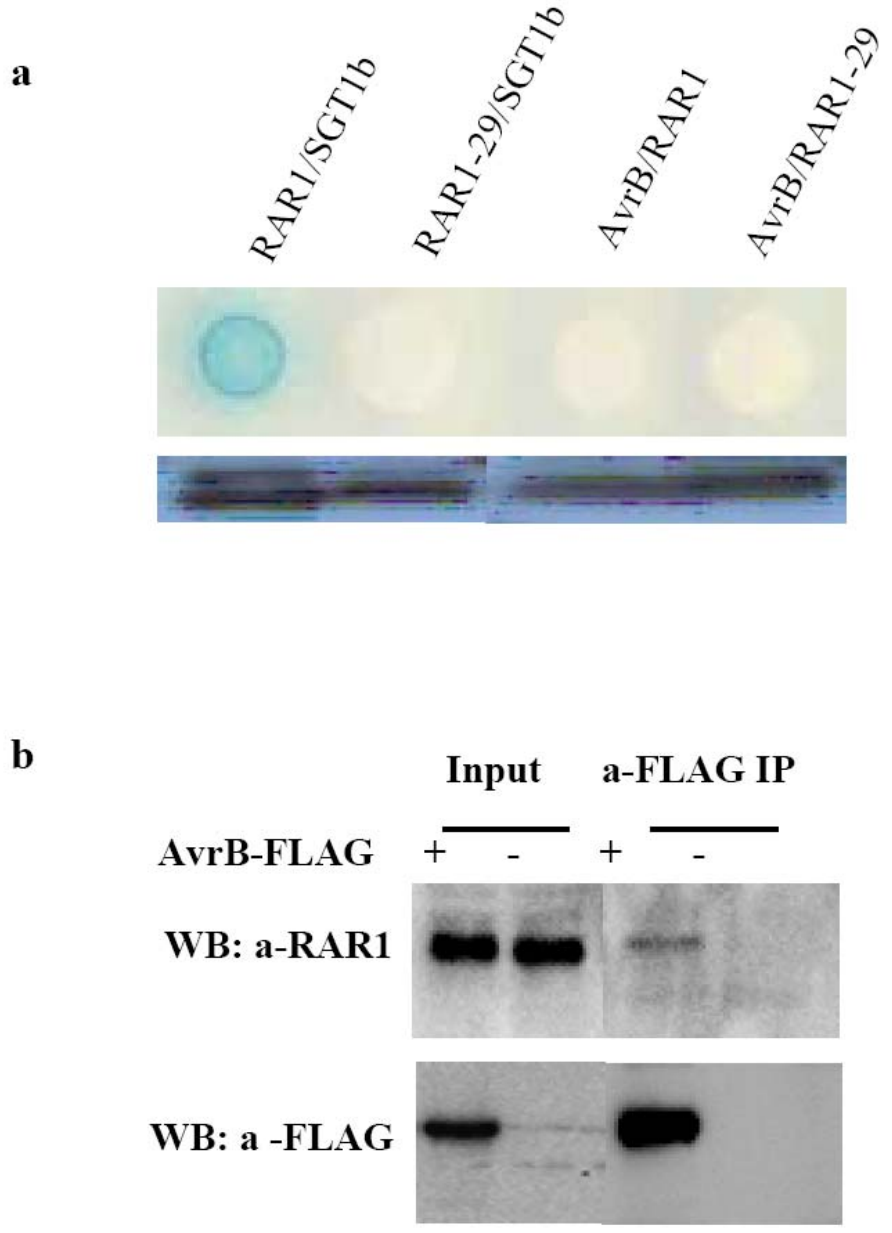

Figure 3-9 AvrB and RAR1 do not interact in yeast but coimmunoprecipitate in plant protein extracts

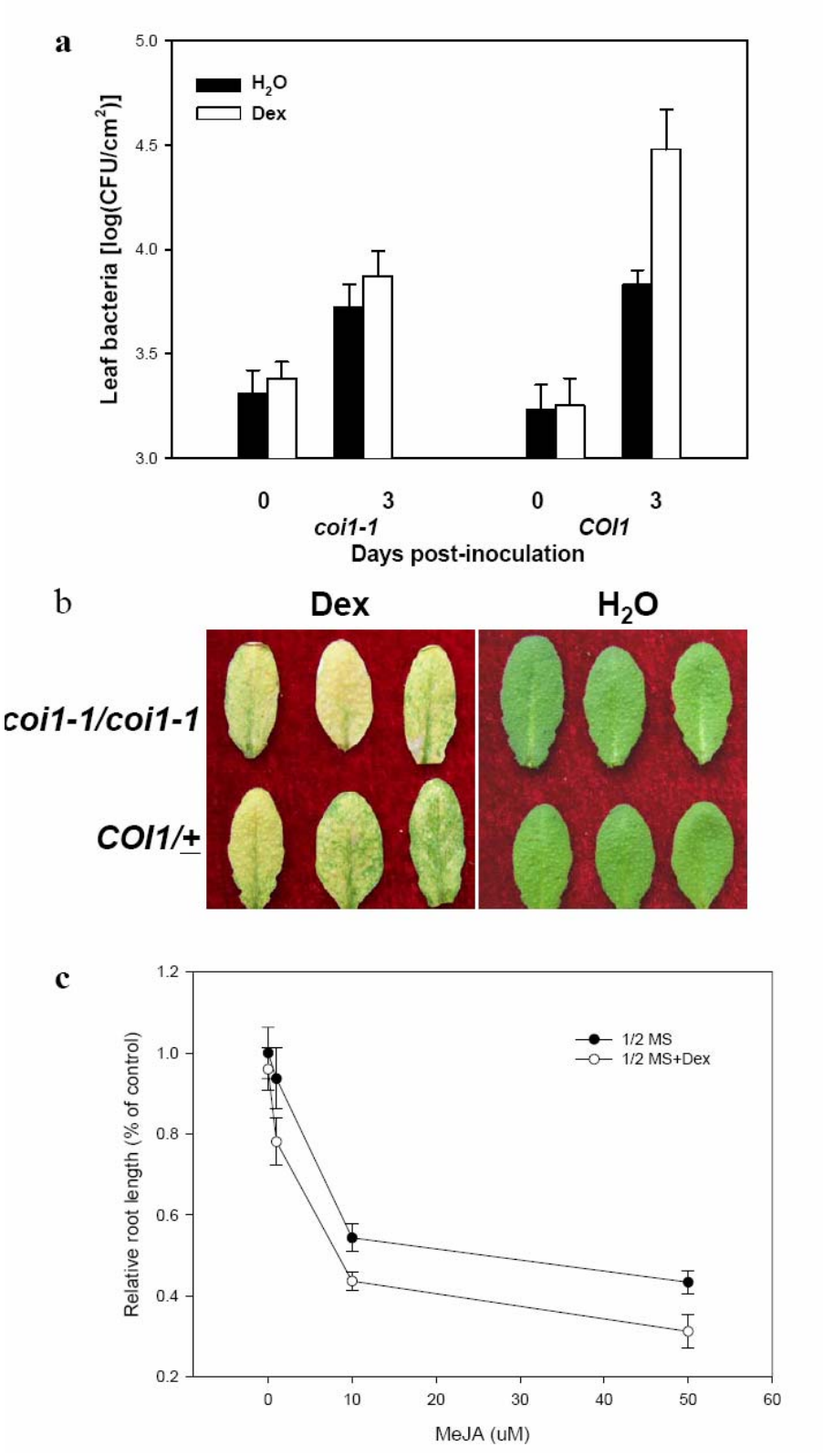

Figure 3-10 *AvrB* expression enhances bacterial growth through *COI1* and promotes JA response

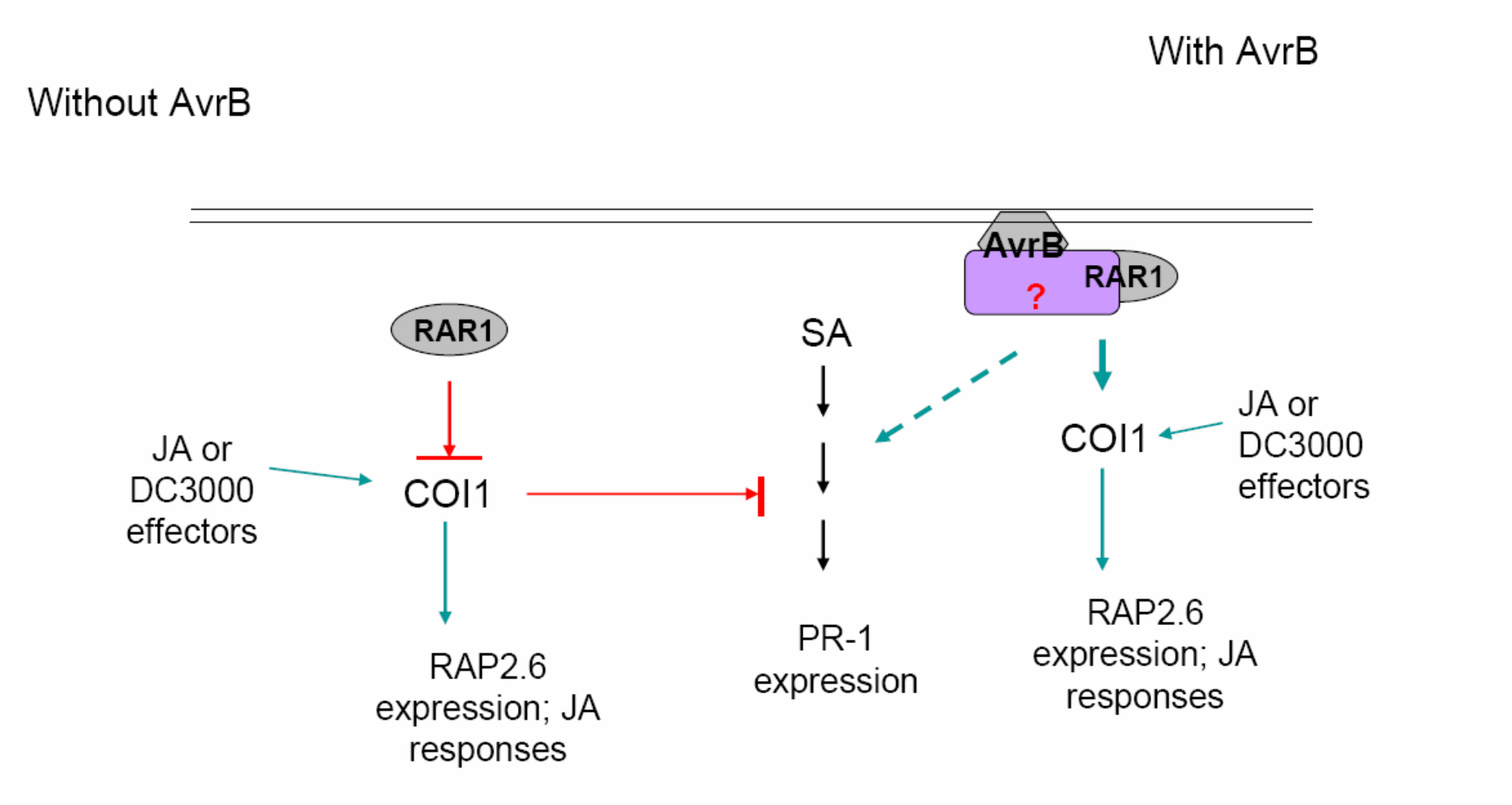

Figure 3-11 A model illustrating the roles of RAR1 and COI1 in both disease resistance and AvrB-dependent virulence activity

CHAPTER 4

GLYCEROL ACCUMULATION IN *NHO1* MUTANT PLANTS COMPROMISES ARABIDOPSIS NONHOST RESISTANCE AND REGULATES *PSEUDOMONAS* TYPE III SECRETION ACTIVITY

Note: Douglas Baker contributed to the construction of DC3000 transposon insertion mutant library and the screening of *glpF*$^{-}$ mutants.

Abstract

NHO1 encodes for glycerol kinase (GK) that converts glycerol to glycerol 3-phosphate. GK activity is abolished in *nho1* mutant plants, which leads to the accumulation of glycerol. The growth of nonhost *Pseudomons* strains in *nho1* plants requires intact glycerol uptake and alginate synthesis systems, indicating that *NHO1* functions by depriving glycerol from nonhost *Pseudomons* bacteria. The *NHO1* mediated glycerol deprivation works synergistically with *FLS2* mediated innate immunity in *Arabidopsis* nonhost resistance. Intriguingly, the glycerol uptake system in the virulent strain DC3000 imposes a negative regulation on both TTSS activity bacterial virulence. These studies demonstrate a role for glycerol uptake in regulating bacteria metabolism and virulence on plants.

Introduction

Nutrient availability is an important determinant in maintaining the biotrophic lifestyle of many obligate fungal pathogens (Hahn and Mendgen, 2001). In a typical biotrophic interaction, a penetration peg is developed to break through cell wall barriers and a haustorium is formed to obtain nutrients. Supporting a role of haustorium in nutrient uptake, genes involved in carbohydrates and amino acids transportation are specifically induced in haustorium isolated from living plant cells (reviewed in Panstruga, 2003; Biemelt and Sonnewald, 2005). Bacterial genes expressed in plants have been analyzed for type III regulation (Thwaites et al., 2004) but genes involved in nutrient uptake have received little attention. The type III secretion system (TTSS) has long been established as the most important bacterial virulence machinery (Alfano and Collmer, 1997). Bacteria use TTSS to secret effector proteins into the host cells to interfere with host defense responses and metabolism (Truman et al., 2006). For example, *Psudomonas syringae* pv. *tomato* DC3000 secretes more than 40 effectors into plant cells (Chang et al., 2004). About half of them are able to suppress at least one type of host defense response (reviewed in Grant et al., 2006) via various mechanisms. It has been shown that type III effectors are able to affect different aspects of plant metabolism including primary carbon metabolism, fatty acid biosynthesis, photosynthesis and cell wall metabolism (Truman et al., 2006; Thilmony et al., 2006) although little is know about how bacteria obtain nutrients from their hosts.

Pseudomonas species have more than 300 nutrient uptake systems responsible for metabolizing a large number of organic compounds. The ability to use diverse organic carbon sources including sugars, amino acids, glycerol and other carbon compounds, enables *Pseudomonas* bacteria to grow in a diverse range of hosts (reviewed in Tamber and Hancock, 2003). As an important carbon source, glycerol is supplied at a high concentration in liquid King's B medium which is widely used for *Pseudomonas syringae* growth *in vitro*. Glycerol uptake in *Pseudomonas* bacteria is mediated by a conserved glycerol transport system shared by many gram negative bacteria including E.coli (Schweizer et al., 1997). In E.coli, the glycerol facilitator (GlpF) and the glycerol kinase (GlpK) are encoded in the *glp* operon that is induced by glycerol or G3P but repressed by maltose or glucose (Weissenborn et al., 1992).

Glycerol uptaken by GlpF is phosphorylated to glycerol 3-phosphate (G3P) by GlpK and then incorporated into alginate synthesis and phospholipid metabolism (Schweizer et al., 1997).

Unlike bacterial membranes, plant plasma membrane is permeable to glycerol, which is only entrapped in the plant cytoplasm in its phosphorylated form. In *Arabidopsis*, *NHO1* (or *GLI1*, Eastmond, 2004) is the only gene coding for the glycerol kinase (GK) that catalyzes the phosphorylation of glycerol to G3P (Kang et al., 2003). An *Arabidopsis* mutant lacking the functional *NHO1* gene supports nonhost *Pseudomonas* bacterial growth, indicating a role of *NHO1* in plant defense (Lu et al., 2001). It has been suggested that blocking G3P synthesis may affect lipid metabolism that in turn compromise disease resistance in *nho1* mutants (Shah, 2005). Supporting this hypothesis, exogenous application of glycerol activates G3P biosynthesis, quenches 18:1 and induces PR1 expression in an *NHO1*-dependent manner (Kachroo et al., 2005). However, unlike *act1* mutation, *nho1* mutation did not affect *Arabidopsis* lipid profiling and was not able to suppress the resistance phenotype mediated by *ssi2* (Kachroo et al., 2005), indicating that other mechanisms are involved in *NHO1* mediated generol resistance. Besides blocking G3P synthesis, *NHO1* mutation also leads to the accumulation of glycerol that enhances resistance toward dehydration-associated abiotic stresses (Eastmond, 2004).

Here we show that the *nho1* mutation leads to the loss of GK activity and the accumulation of glycerol. Glycerol accumulation induces active glycerol uptake in the nonhost bacteria *Pseudomonas syringae* pv. *phaseolicola* (*Pph*). *Pph* growth in *nho1* mutant needs intact glycerol uptake systems, suggesting that *Pph* actively assimilate glycerol. The *NHO1* mediated nonhost resistance in *Arabidopsis* functions, at least partly, in depriving glycerol from invading bacteria cells and works synergistically with *FLS2* mediated innate immunity in the resistance against *P. s.* pv. *tabaci*. Interestingly, deficiency in glycerol uptake does not compromise DC3000 bacterial growth in *Arabidopsis*. Instead, glycerol uptake imposes a negative regulation on DC3000 virulence. Further experiments reveal that glycerol uptake negatively regulates DC3000 TTSS activity through G3P. Our results demonstrate a role of *Pseudomonas* bacteria glycerol uptake in both nonhost and host interactions for the first time.

Materials and Methods

Bacteria Strains and Bacterial Growth Assay

Bacteria strains used in this study include *Pseudomonas syringae* pv. *tomato* strain DC3000, *Pph* strain NPS3121 (Lu et al., 2001), *Pseudomonas syringae* pv. *tabaci* (*Ptab*) strain 6505 (Li et al., 2005), *Pseudomonas syringae* pv. *syringae* (*Pss*) 3525 and *Pss algL*$^-$ (Yun et al., 1999). Bacteria were cultured in King's B medium supplemented with appropriate antibiotics overnight. A bacteria suspension of OD value<2 was used for all experiments. For bacterial growth assay, bacteria were inoculated at 105 cfu/ml. Leaf discs were taken at the indicated time points and grounded in ddH2O or 10mM MgCl2. Bacterial number were counted as described before (He et al., 2004). All experiments were repeated at least twice with similar results. For disease development, bacteria were inoculated at 106 cfu/ml. Bean plants were grown in green house after inoculation to allow disease symptom development.

Transposon Insertion Mutant Screening

P. s. pv. *phaseolicola* (*Pph*) transponsome insertion mutant library was constructed as described before (Xiao et al., in press). A DC3000 mutant library was generated with an EZ-TzTM<KAN-2> transposon mutagenesis kit (Epicentre Technologies, Wiscosin, WS). *Pph glpF*$^-$ mutant was screened with two rounds of PCR reactions. The first PCR was carried out using the *glpF* diagonostic primer: 5'-ATGAATTCAGAGCCTTCAGATCAAGCGTTC-3' in the combination with TN-KAN-L1 primer or TN-KAN-R1 primer. The second round of PCR was carried out with the *glpF* diagonostic primer in combination with TN-KAN-L2 5'-GAATATGGCTCATAACACCCCTTGTATTAC-3' and TN-KAN-R2 5'-CAGACCGTTCCGTGGCAAAGCAAAAGTTC-3'

DC3000 *glpF*$^-$ mutant was screened with the diagonostic primer 5'-TGGTTTCACGCTGGTTGGTGATACCGATG-3' in combination with the TN-TET-L1 primer: 5'-TACCGGCATAACCAAGCCTATGCCTACAG-3' or the TN-Tet-R1 5'-CACATGGAACGGGTTGGCATGGATTGTAG-3' primer for the first round PCR. The second round PCR was carried out with the diagonostic primer and the TN-TET-L2:

5’-GAGGATGACGATGAGCGCATTGTTAGATTTC-3’ primer or the TN-TET-R2: 5’-CACTCCAAGAATTGGAGCCAATCAATTCTTG-3’ primer.

Construction of *Pph* TOPO: *algD* Mutant

A TOPO:*algD Pph* plasmid was constructed by cloning an *algD* partial ORF into the TOPO TA cloning vector (Invitrogen). The *algD*-F primer: 5’-ATCAACAAC GGCAAA TCAC-3’ and the *algD*-R primer: 5’-TTTCGTTGGCGAAAGTAACC-3’. were used for amplifying *algD* partial ORF. The TOPO:*algD Pph* plasmid was then introduced into *Pph* by electroporation. Colonies with kanamycin and ampamcilin resistance were shaked in King’B medium overnight and collected for genomic DNA extraction. *Pph* TOPO:*algD* mutant was confirmed by diagnosis PCR with the *algD*-F primer in combination with the T7 primer: 5’-TAATACGACTCACTATTGGG-3’ or the SP6 primer. 5’-TATTTAGGTGACACTATAG-3’.

Luciferase Activity Assay

For avrPto-LUC activity assay, bacteria overnight culture were harvested, washed twice with ddH2O and diluted to 0.2 OD_{600} with minimal (MM) and MM supplemented with fructose, glycerol or G3P at indicated concentration. Bacteria suspension in MM was culture for 6 hours at room temperature. Luciferin was added to the bacteria suspension at a final concentration of 0.01 mM. Luminescence images were captured with a low light imaging system, and relative luciferase activity was calculated with the WinView software (RoperScientific, Trenton, NJ; He et al., 2004).

Glycerol Kinase Assay and Glycerol Measurement

Fresh *Arabidopsis* leaves were ground in 200µl extraction buffer (50 mM Tris PH 7.6, 10 mM glycerol, 1 mM EDTA. 1 mM PMSF and 1 mM β-mercaptalethanol) with a hand drill. The total leaf protein extract was centrifuge at 13,000 rpm for 10 minutes. Supernatant was collected and used for glycerol kinase assay. For each eppendorf tube, 1,425 µl reaction buffer (750 µl Solution I, 37.5 µl 800 mM ATP, 15 µl 133 mM NAD and 15 µl glycerol-P dehydrogenase) and 75 µl protein extract was added. Solution I contains 0.6 M hydrazine, 0.33

M glycine, 0.33 M sodium carbonate, 40 mM $MgCl_2$ and 600 µl 25 mM glycerol). The mixture was mixed and incubated at 30°C dry bath for 5 minutes. Glycerol kinase activity was calculated based on NADH absorbance at OD_{340} (Hayashi and Lin, 1967). Glycerol was extracted and measured as described (Pinter et al., 1967) using a spectrophotometric method. Glycerol concentration was calculated by the reduction of NADH.

Results

NHO1 Mutation Leads to Accumulation of Glycerol in *Arabidopsis* Plants

It has been shown that *NHO1* confers resistance against both nonhost and virulent *Pseudomonas syringae* strains (Kang et al., 2003). Whereas the biochemical and physiological nature of this *NHO1* mediated resistance is not known. In *Arabidopsis* genome, At*NHO1* is the only gene encodes for glycerol kinase (GK) that converts glycerol to glycerol 3-phosphate (G3P) (Kang et al., 2003). Here we show that the GK activity is completely lost in nho*1* mutant (Fig. 4-1a). The loss of GK activity leads to the accumulation of glycerol to more than 200 mM, one hundred fold higher than that in wild type plants (Fig. 4-1b).

Glycerol Uptake Is Required for Nonhost Bacteria Ggrowth in *nho1* Plants

Glycerol is an important nutrient and carbon source for *Pseudomonas* bacteria. Hence we hypothesize that the susceptibility of *nho1* against *Pseudomonas* strains is caused, at least in part, by the availability of glycerol. If this is true, then a bacteria mutant that is unable to uptake glycerol will no longer grow in *nho1* plants. To test this possibility, we screened a *Pph* transposon insertion mutant library for a *glpF* mutant that lacks a functional glycerol transport system. In the *Pph* genome, *glpF* and *glpK* genes are organized in *glp* operon followed by *glpR*, a repressor of *glp* operon, and *glpD*, a G3P dehydrogenase (Fig. 4-2a). The screening of *Pph* transposon insertion mutant library yielded a TN:*glpF* mutant with TN:Km inserted 249 base pairs after the start codon (Fig. 4-2a). As expected, the *Pph* TN:*glpF* mutant is unable to grow in minimal medium (MM) supplemented with 50mM glycerol (Li X. and Zhou J.M., unpublished data).

To examine the bacterial growth of *Pph* TN:*glpF* mutant in *nho1* plants, we infiltrated the wild type and mutant strains into *nho1* plants. As shown in figure 4-2b, the wild type strain multiplied ~8 folds in *nho1* plants, whereas the bacterial growth of TN:*glpF* mutant was largely compromised (Fig. 4-2b), suggesting that a functional *glpF* gene is required for *Pph* growth in *nho1* plants. However, it is noteworthy that neither the wild type nor the mutant strain grew in Col-0 plants. This result is consistent with an extremely low concentration of glycerol in wild type plants (Fig. 4-1b). To further exploit the role of glycerol uptake in nonhost bacterial growth, we examined *glpF* promoter activity by fusing *glpF* promoter with the LUC reporter gene. As expected, *glpF*-LUC activity is induced in *nho1* but not in wild type plants (Fig. 4-2c). Taken together, these results demonstrate that glycerol uptake is actively induced and is required for nonhost bacterial growth in *nho1* plants.

NHO1 and *FLS2* Works Synergistically in Arabidopsis Nonhost Resistance

It has been suggested that plants activate layered defense responses to prevent pathogen infection (Thordal-Christensen, 2003; Yun et al., 2003; Lipka et al., 2005). Here we showed that *NHO1* functions in depriving glycerol from nonhost *Pseudomonas* bacteria. According to Thordal-Christensen, nutrient availability is an important layer of nonhost resistance (Thordal-Christensen, 2003). We demonstrated previously that flagellin sensing also plays a role in Arabdiospsis nonhost resistance. *Ptab* strain 6505 lacking the flagellin synthesis gene is able to grow in *Arabidopsis* that is a nonhost plant for *Ptab* (Li et al., 2005). Flagellin sensing is mediated by the receptor like kinase FLS2, which subsequently activates MAPK signaling cascade and WRKY transcriptional factors (Asia et al., 2002). To test whether there is a synergistic effect between *NHO1* mediated glycerol deprivation and *FLS2* mediated innate immunity, a *nho1/fls2* double mutant was constructed. As shown in Fig. 4-3, *nho1* and *fls2* single mutants supported 10-20 fold *Ptab* bacterial growth whereas the *nho1/fls2* double mutant supported more than 60 fold bacterial growth (Fig. 4-3), suggesting that *NHO1* and *FLS2* work synergistically in *Arabidopsis* nonhost resistance against *Ptab*.

Glycerol Uptake Is Not Required for *Pph* Growth on Bean Plants

To test whether glycerol uptake is an important determinant for *Pph* to grow on its host plants, both wild type *Pph* and TN:*glpF* mutant strains were inoculated into bean leaves via syringe infiltration. Our data show that both wild type and mutant strains grow similarly and cause indistinguishable disease symptom on bean plants (Fig. 4-4a and 4-4b), indicating that glycerol uptake is not required for *Pph* bacterial growth in compatible bean plants. Interestingly, a *Pph* mutant strain min41 (Xiao, 2005) deficient in fructose and mannose uptake grew less and failed to cause disease on bean plants (Fig. 4-4b). TTSS activity is downregulated in *Pph* min41 mutant (Xiao, 2005). So far, it is not clear whether the growth defect of *Pph* min41 mutant is caused by the lack of nutrient or by the downregulation of TTSS activity.

Alginate Production Is Required for Nonhost Bacterial Growth in *nho1* Plants

It has been shown that glycerol assimilation is incorporated into alginate synthesis in P. *aeruginosa* (Marty et al., 1992). Alginate production is a critical virulence determinant of P. *aeruginosa* in chronic lung infection of cystic fibrosis (CF) patients (Ramsey and Wozniak, 2005). A role for alginate in bacterial colonization of host plants has also been proposed (Yu et al., 1999; Keith et al., 2003). Supporting a role for alginate in facilitating nonhost bacterial growth in *nho1* plants, the TN:*algL* mutant of *Pss* strain 3525 (Yu et al., 1999) deficient in alginate biosynthesis failed to grow in *nho1* plants (Fig. 4-5a). However, it is noteworthy the TN:*algL* mutant had a general growth defect even in wild type Col-0 plants (Fig. 4-5a). To better illustrate the role of alginate in *Pseudomonas* virulence activity on plants, a *Pph* TOPO: Δ*algD* mutant was created by disrupting *algD* ORF with TOPO:Δ*algD* plasmid. The *Pph* TOPO:Δ*algD* mutant lost the mucoid phenotype on MG plates (Li X. and Zhou J.M., unpublished data). However, the bacterial growth of *Pph* TOPO: Δ*algD* on bean plants was not affected (Fig. 4-5c), suggesting alginate is not a critical virulence determinant in *Pph* pathogenicity on its host plants.

Glycerol Uptake Negatively Regulates DC3000 Virulence on Arabidopsis Plants

To test whether glycerol serves as a carbon source for compatible bacteria strains in *Arabidopsis* plants, we screened a DC3000 transposon insertion library for a DC3000 TN:*glpF* mutant (Fig. 4-6a) and tested its bacterial growth in *nho1* and Col-0 plants. Consistent with our previous results, DC3000 grew similarly in the *nho1* mutant and the Col-0 plants (Lu et al., 2001). However, the DC3000 TN:*glpF* mutant strain grew much better in both Col-0 and the *nho1* mutant plants compared to the wild type DC3000 strain (Fig. 4-6b), suggesting that glycerol uptake negatively regulates DC3000 virulence activity on *Arabidopsis* plants. Interestingly, although DC3000 grew similarly on both Col-0 and *nho1* plants, a significant difference in DC3000 TN:*glpF* bacterial growth on Col-0 and *nho1* plants was consistenly observed.

Glycerol Uptake Suppresses DC3000 TTSS Activity through G3P

Because TTSS is the most important virulence determinant for bacteria pathogenesis, we further tested whether glycerol uptake affects type III gene expression. Consistent with the previous report (Xiao et al., in press), *avrPto* promoter activity is induced by MM supplemented with 10mM fructose (Fig. 4-7a). No induction was observed when fructose was removed from MM (Fig. 4-7a), indicating that fructose is the major inducer of *avrPto*-LUC activity. Glycerol is able to induce *avrPto* promoter activity when supplied as the sole carbon source in MM (Fig. 4-7a). However, in the presence of fructose, supplement of glycerol inhibits *avrPto*-LUC activity that is induced by fructose (Fig. 4-7b). The suppression imposed by glycerol is abolished in DC3000 *glpF*$^{-}$ mutant (Fig. 4-7b), indicating that active glycerol uptake system is needed. It is noteworthy that the suppression of avrPto-LUC activity by glycerol is sensitive to glycerol concentration. Glycerol supplemented at as low as 10mM is able to suppress *avrPto*-LUC by about 1.5 folds. The suppression is most obvious when glycerol was added at 50mM. Intriguingly, increasing the glycerol concentration to 100mM abolished the suppression and brought the avrPto-LUC activity back to normal.

Because glycerol is converted to G3P upon uptake, we hypothesized that the suppression of TTSS activity by glycerol is actually mediated by G3P. Supporting this

hypothesis, G3P is able to suppress *avrPto*-LUC activity in both DC3000 wild type and *glpF*$^-$ mutant strains (Figue 4-7c), suggesting that G3P is responsible for TTSS repression. Consistent with the TTSS activity in MM, *avrPto*-LUC activity in planta is also negatively regulated by glycerol uptake (Fig. 4-8). Taken together, these results demonstrated that glycerol accumulation in *nho1* mutant imposed a negative regulation on TTSS in a glycerol uptake dependent manner.

Discussion

Glycerol is an important carbon source for *Pseudomonas* bacterial growth. Glycerol taken up by bacteria cells is converted to dihydroxyacetone phosphate (DHAP) and used for lipid biosynthesis, alginate production and cellular metabolism (Schweizer et al., 1997). Here we showed that glycerol uptake is required for *Pph* bacterial growth in *nho1* plants, suggesting that glycerol availability determines the outcome of nonhost bacterial growth. However, it is noticeable that the bacterial growth of *Pph* TN*:glpF* mutant in *nho1* plants was not completely abolished (Fig. 4-2b). Whether or not other mechanisms are also involved in *Arabidopsis* nonhost resistance to *Pph* remains an open question. The abolishment of cytosolic G3P by *NHO1* mutation could somehow affect *Arabidopsis* lipid metabolism, which has been assigned a role in plant defense response against *Pseudomonas* bacteria (Shah, 2005).

A synergistic effect is detected between *NHO1* and *FLS2*, both of which contribute to *Arabidopsis* nonhost resistance against *Ptab*. *NHO1* is induced by a *Pseudomonas* PAMP flg22 in *FLS2*-dependent manner (Li et al., 2005; Zhang J. and Zhou J.M., unpublished data). The inability of a *Ptab flic*$^-$ mutant to induce *NHO1* was associated with its gain-of-virulence on Arabidopsis plants (Li et al., 2005). These data suggest that flg22 induced *NHO1* expression contributes to the *Arabidopsis* nonhost resistance against *Ptab*. *FLS2* was identified to play a role in *Arabidopsis* nonhost resistance because the *fls2* mutant supported *Ptab* bacterial growth (Fig. 4-3). How do *FLS2*-mediated innate immunity and *NHO1*-mediated nonhost resistance work together is not clear. It is possible that *FLS2* and *NHO1* defend bacteria at different infection stages. For example, *nho1* mutant allowed nonhost bacteria multiplication and the

formation of bacterial colonies in the intercellular spaces (Lu et al., 2001). *FLS2*, instead, may limit the movement and spread of bacterial cells upon the recognition of flagellin.

Glycerol is a good energy source for alginate synthesis. A role of alginate in *Pseudomonas* bacterial virulence on plants is arguable. Keith (2003) observed a reduction in *Pss* TN:*algL* pathogenicity on bean plants. The *Pss* TN:*algL* mutant strain was not able to produce alginate (Yu et al., 1999), revealing a correlation between alginate production and *Pss* pathogenicity. A growth deficiency of *Pss* TN:*algL* on both *Arabidopsis* wild type and the *nho1* mutant plants were also observed in this study. However, alginate production was not a virulence determinant in *P. aeruginosa* (Yorgey et al., 2001) and *P. s.* pv. *tomato* strain DC3000 (Li X. and Zhou J.M., unpublished data) pathogenicity on Arabidopsis. Moreover, a *Pph algD* mutant unable to synthesize alginate grew normally on its host bean plants. Further experiments are needed to determine the bacterial growh of *Pph algD* mutant on its nohost *Arabidopsis* plants. The result on this experiment will help us to address the role of alginate in *Pph* growth on *nho1* plants.

Although glycerol uptake is important for *Pph* bacterial growth on nonhost *Arabidopsis* plants, it seems despensible for *Pph* bacterial growth on host bean plants. It is possible that glycerol availability is noncritical in compatible interactions when a number of other nutrients including various carbohydrates are accessable. For example, the most important photosynthesis product, sucrose can be broken down into two hexoses, fructose and sucrose. Fructose seems to be an important carbon source for *Pph* growth in bean plants because *Pph min41* mutant unable to uptake fructose failed to cause disease (Fig. 4-4b). It will be interesting to examine whether fructose is also an important carbon source for DC3000 growth in *Arabidopsis* plants.

Besides a role in nutrient absorbance, the glycerol uptake system seems to negatively regulate TTSS activity in *P. s.* pv. *tomato* strain DC3000. DC3000 *glpF* mutant that was unable to take up glycerol exhibited higher TTSS activity and grew better on *Arabidopsis* plants. Glycerol also suppresses TTSS activity in MM supplemented with fructose in a concentration-sensitive manner. Although it is difficult to explain why glycerol at 50mM instead of 100mM suppresses TTSS induction by fructose, the regulation of TTSS seems

consistent with DC3000 bacterial growth in *Arabidopsis* plants. DC3000 grew to a same level in both Col-0 and *nho1* plants (Lu et al., 2001). However, in the *nho1* mutant plants, glycerol accumulated to about 200mM (Fig. 4-1b), a concentration at which TTSS is not suppressed (Fig. 4-7b) and DC3000 growth is not affected. The TTSS regulation in DC3000 *glpF* mutant also correlates well with the *in planta* bacterial growth (Fig. 4-7b).

Glycerol is converted to G3P, which forms the triglycerol backbone for bacterial fatty acids biosynthesis (Schweizer et al., 1997). Like glycerol, G3P similarly suppresses TTSS activity in MM supplemented with fructose. Commercially available C16 and C18 fatty acids including palmitic acid, oleic acid and stearic acid are also active suppressors of TTSS activity (Xiao et al., 2004). Based on these results, bacterial glycerol uptake and lipid biosythesis seem to impose a negative regulation on TTSS activity. Intriguing questions are then raised: why would bacteria suppress their own virulence machinery upon glycerol uptake? What is the message carried by glycerol that is sensed by the bacterial cells? How would this message modulate bacterial lipid metabolism and TTSS activity?

A model illustrating the role of bacteria glycerol uptake in bacteria metabolism and TTSS regulation is proposed (Fig. 4-9). In compatible interactions, the *NHO1* expression and other plant metabolism genes are suppressed by type III effectors (Li et al., 2005), allowing the accumulation of various nutrients in the intercellular spaces. Unlike the role of glycerol uptake in nonhost interactions, glycerol uptake in compatible interactions may act as a sensitive tuner to adjust the balance between bacterial cellular metabolism and TTSS activity. Glycerol itself may not be an important nutrition in compatible interactions. However, glycerol availability could be an indicator of the overall status of nutrient avalaibility in the plant intercellular spaces. When glycerol is available, it may indicate a nutrient rich environment. In such a case, glycerol slightly inhibits TTSS activity through G3P to allow bacterial multiplication. However, when glycerol uptake system is mutated, the TTSS will be derepressed and more type III effectors will be secreted to suppress host defense genes and to obtain nutrients.

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Figure Legend

Figure 4-1 Loss of glycerol kinase activity and glycerol accumulation in *nho1* mutant plants. a. Glycerol kinase (GK) activity is abolished in *nho1* mutant plants. b. Glycerol accumulation in *nho1* mutant plants. Fresh Arabidopsis leaves were deatched from 5-week old Col-0 and *nho1* plants. The total leaf protein and glycerol were extracted as described in materials and methods. Glycerol kinase activity and glycerol content was calculated based on the change of NADH absorbance at OD_{340}.

Figure 4-2 Active glycerol uptake is required for nonhost bacterial growth in nho1 plants. a. Organization of *Pph* glycerol uptake genes in *glpF* operon. *glpF*: glycerol fercilitator; *glpK*: glycerol kinase; *glpD*: glycerol 3-phosphate dehydrogenase; *glpR*: putative *glp* repressor; *glpT*: membrane associated glycerol 3-phosphate permease. TN represents the transposon insertion at the beginning of *glpF* open reading frame in *Pph glpF*$^-$ mutant. b. Glycerol uptake system is required for *Pph* bacterial growth in *nho1* mutant plants. Bacteria were cultured in King' B medium supplied with 1mM glucose. Bacterial number was counted at indicated time points. Each data point represents four independetn replicates. This experiment was repeated at least two times with similar results. **c.** *Pph* glycerol uptake is activated by glycerol accumulated in *nho1* mutant plants. *Pph* bacteria carrying pPLT:*glpF*-LUC plasmid (construct made by Xiao F.) was inoculated at 0.2 OD_{600} on 5-week old *Arabidopsis* plants. Leaves were detached at 6, 12 and 24 hours after inoculation and examined for LUC activity. The picture shown here was taken 24 hours after inoculation. This experiment was repeated three times with similar results.

Figure 4-3 *NHO1* and *FLS2* work synergistically in Arabidopsis nonhost resistance. Bacteria were inoculated at 10^5 cfu/ml. *Arabidopsis* leaves were detached at the indicated time points and surface sterized with 75% alcohol before grinding with a hand drill. Each data point represents 4 replicates. This experiment has been done once.

Figure 4-4 Glycerol uptake is not a virulent determinant in *Pph* on host bean plants. a. Glycerol uptake is not required for *Pph* growth on its host bean plants. Bacteria were inoculated at 10^5 cfu/ml. Leaf discs were detached and analyzed for bacterial growth at

indicated time points. Each data point contains 4 independent replicates. b. Disease symptoms caused by *Pph* wild type ad *glpF*$^-$ mutant strains. Bacteria were inoculated at 10^6 cfu/ml. Picture was taken 7 days after inoculation.

Figure 4-5 Alginate is required for *Pss* growth in *nho1* mutant plants but not required for *Pph* growth in bean plants. a. Bacterial growth of *Pss* and *algL*$^-$ mutant strains in *Arabidopsis* plants. b. Bacterial growth of *Pph* and *algD*$^-$ mutant strains in bean plants. Bacteria were inoculated at 10^5 cfu/ml. Leaf discs were detached and analyzed for bacterial growth at the indicated time points. Each data point represents 4 independent replicates. These experimnets have been repeated at least two times with similar results.

Figure 4-6 Glycerol uptake negatively regulates DC3000 bacterial virulence. a. Organization of DC3000 glycerol uptake genes in *glpF* operon. *glpF*: glycerol fercilitator; *glpK*: glycerol kinase; *glpD*: glycerol 3-phosphate dehydrogenase; *glpR*: putative *glp* repressor; *glpT* : membrane associated glycerol 3-phosphate permease. TN represents the transposon insertion at the beginning of *glpF* open reading frame in DC3000 *glpF*$^-$ mutant. b. Bacterial growth assay of DC3000 wild type and *glpF*$^-$ mutant strains in *Arabidopsis* plants. Bacteria were inoculated at 10^5 cfu/ml. Leaf discs were detached and analyzed for bacterial growth at the indicated time points. Each data point represents 4 independent replicates. This experimnet has been repeated at least two times with similar results.

Figure 4-7 Glycerol uptake negatively regulates DC3000 TTSS activity through G3P. a. Induction of *avrPto*-LUC activity in MM supplied with fructose or glycerol. b. Glycerol suppresses *avrPto*-LUC activity induced by fructose in DC3000 wild type but not *glpF*$^-$ mutant strain. c. G3P suppresses *avrPto*-LUC activity in both DC3000 wild type and *glpF*$^-$ mutant strains. Bacteria were cultured overnight in King'B medium, washed with ddH2O and encubated in MM supplied with the corresponding carbon source for 6 hours before examining for LUC activity. These experiments have been repeated at least twice with similar results.

Figure 4-8 Glycerol uptake negatively regulates TTSS activity in *nho1* mutant plants. Bacteria were inoculated at 0.2 OD_{600} on 5-week old *Arabidopsis* plants. Leaves were

detached at the indicated time points and examined for LUC activity. Each data represents at least 5 replicates.

Figure 4-9 A model of glycerol regulation on *Pseudomonas* bacterial metabolism and TTSS activity. In compatible interactions, the *NHO1* is suppressed by type III effectors and glycerol is available for bacterial cellular metabolism. To allow enough energy for bacterial multiplication and alginate biosynthesis, glycerol uptake slightly inhibits TTSS activity through G3P. However, when glycerol uptake system is mutated, the TTSS will be derepressed and more type III effectors will be secreted to suppress host defense genes and get nutrients.

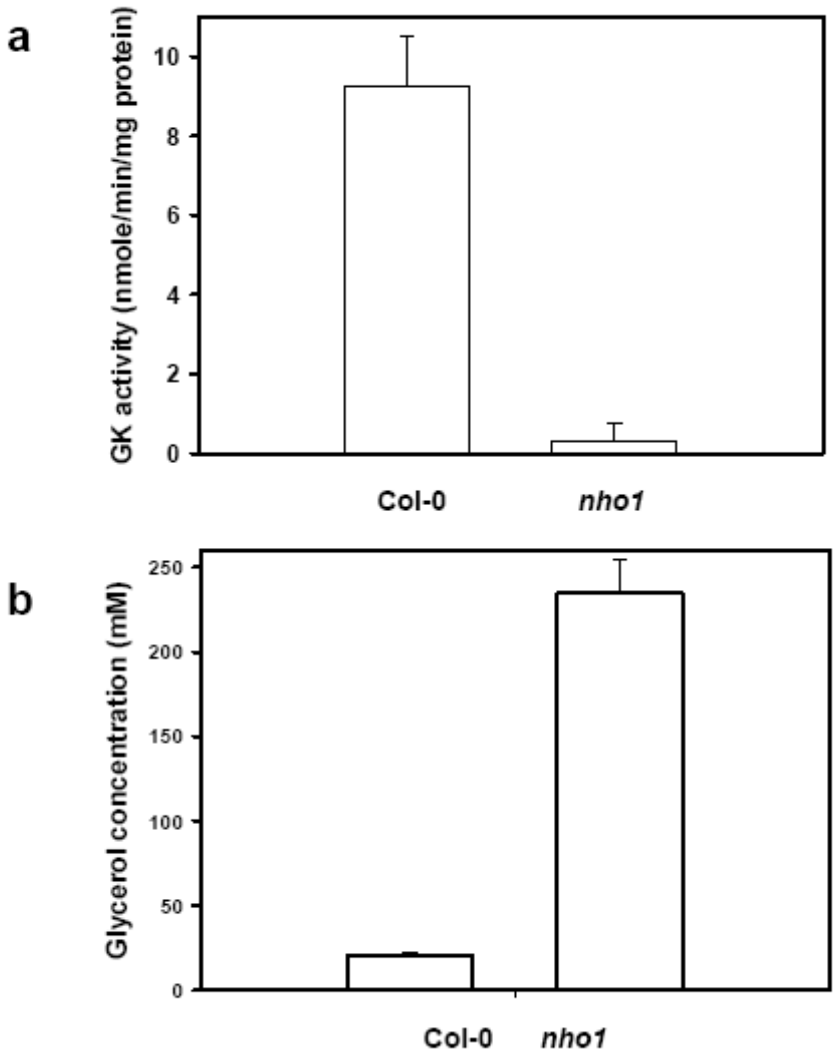


Figure 4-1 Loss of glycerol kinase activity and glycerol accumulation in nho1 mutant plants

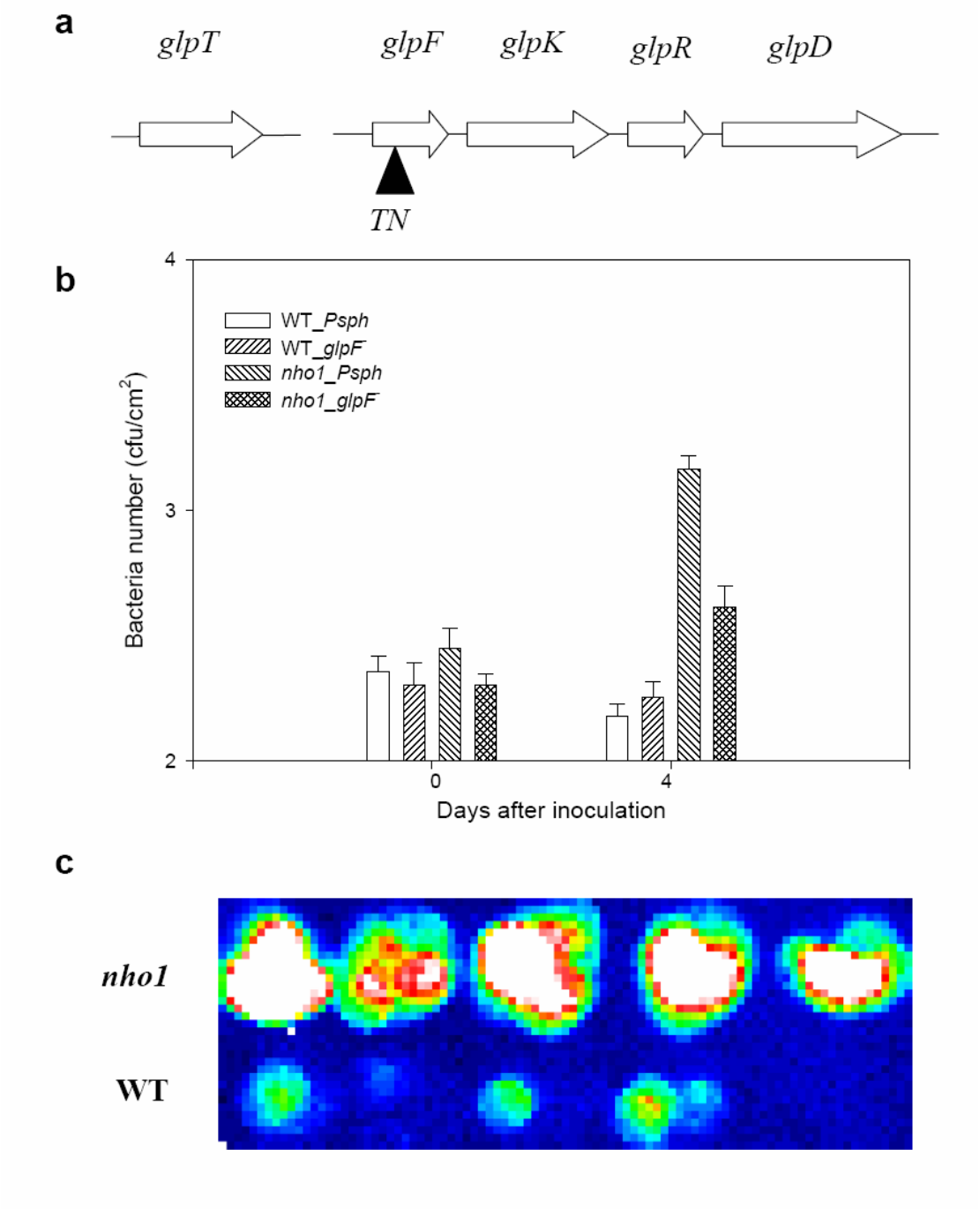


Figure 4-2 Active glycerol uptake is required for nonhost bacterial growth in nho1 plants

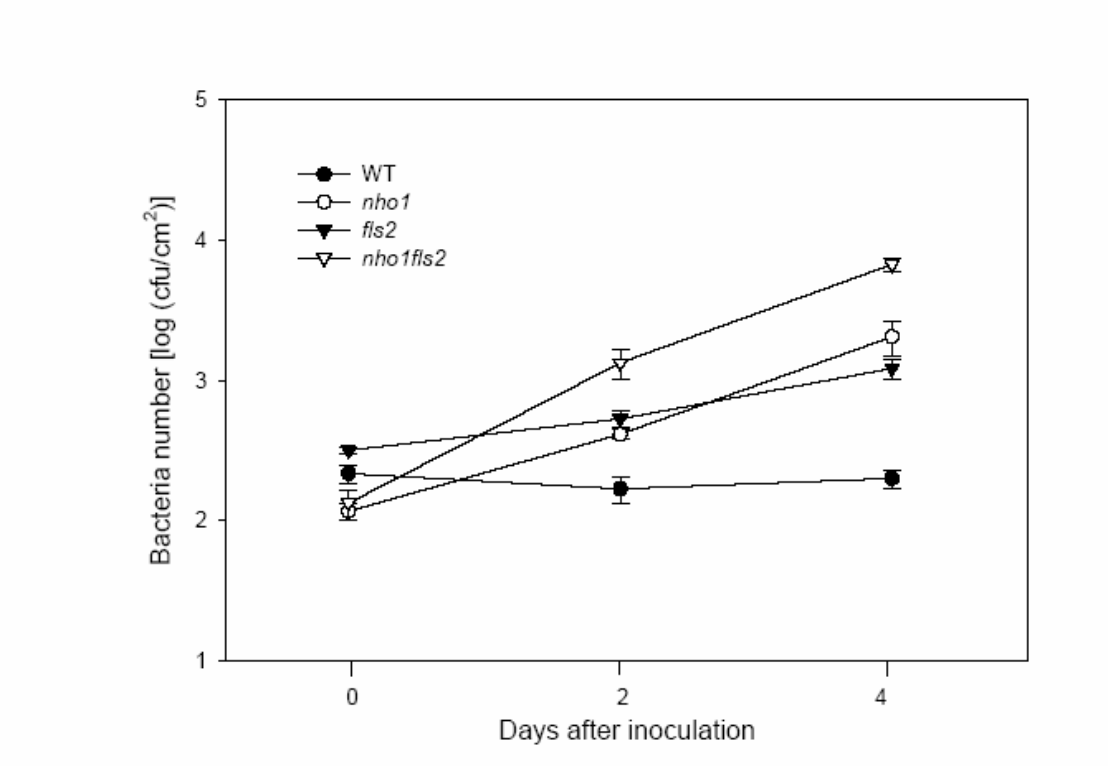


Figure 4-3 *NHO1* and *FLS2* work synergistically in *Arabidopsis* nonhost resistance

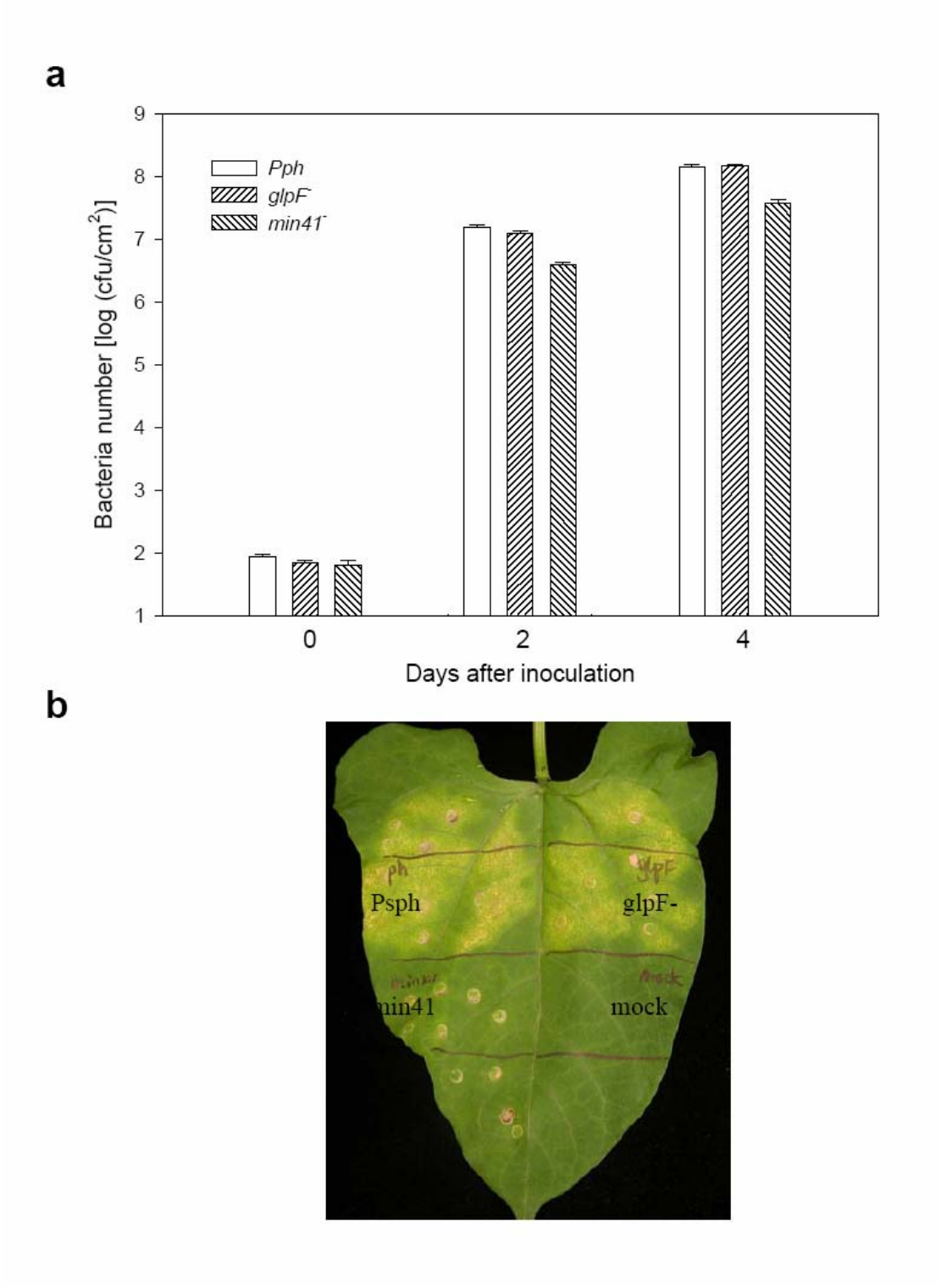


Figure 4-4 Glycerol uptake is not a virulent determinant in *Pph* on host bean plants

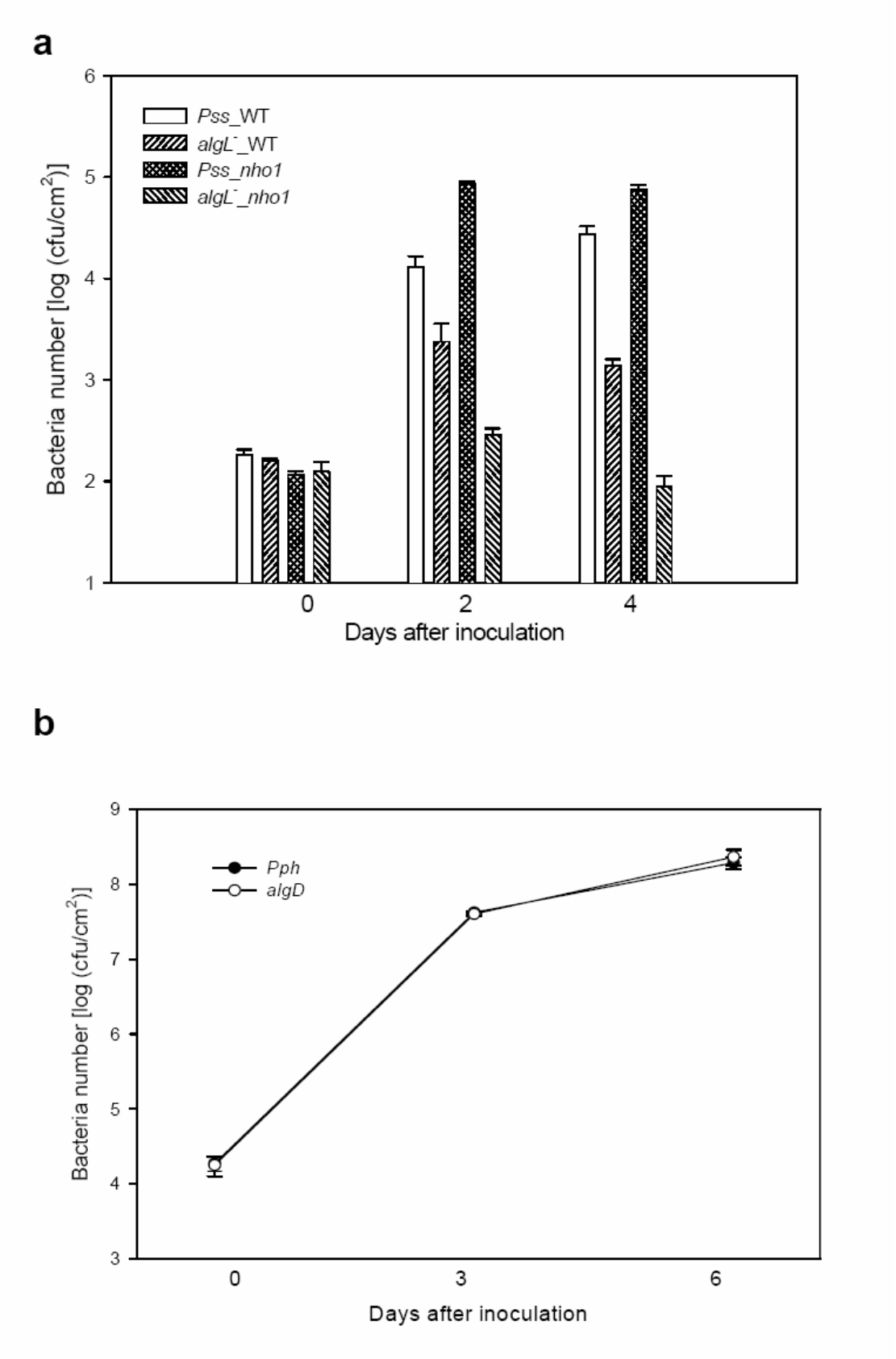


Figure 4-5 Alginate is required for *Pss* growth in *nho1* mutant plants but not required for *Pph* growth in bean plants

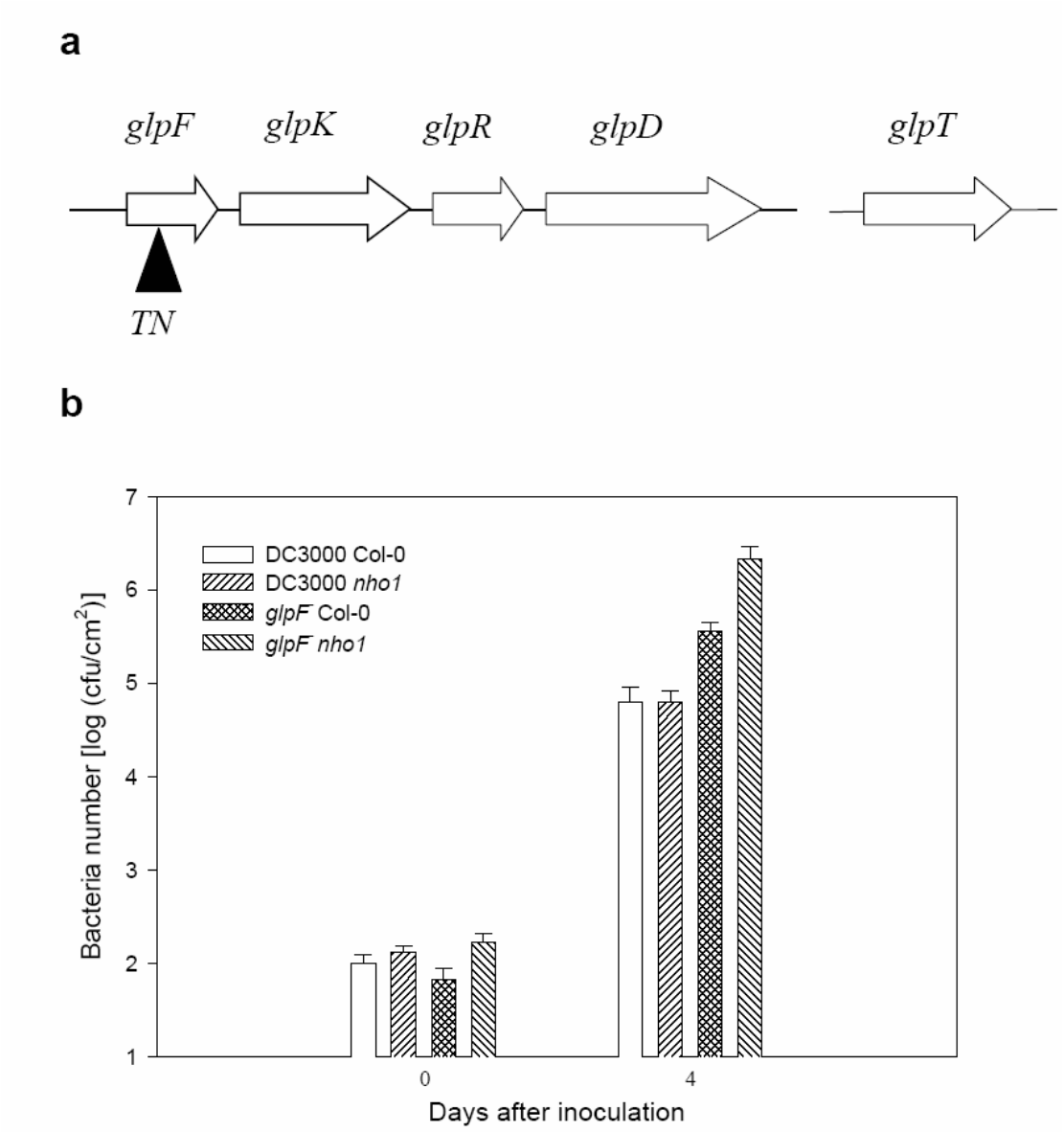


Figure 4-6 Glycerol uptake negatively regulated DC3000 bacterial virulence

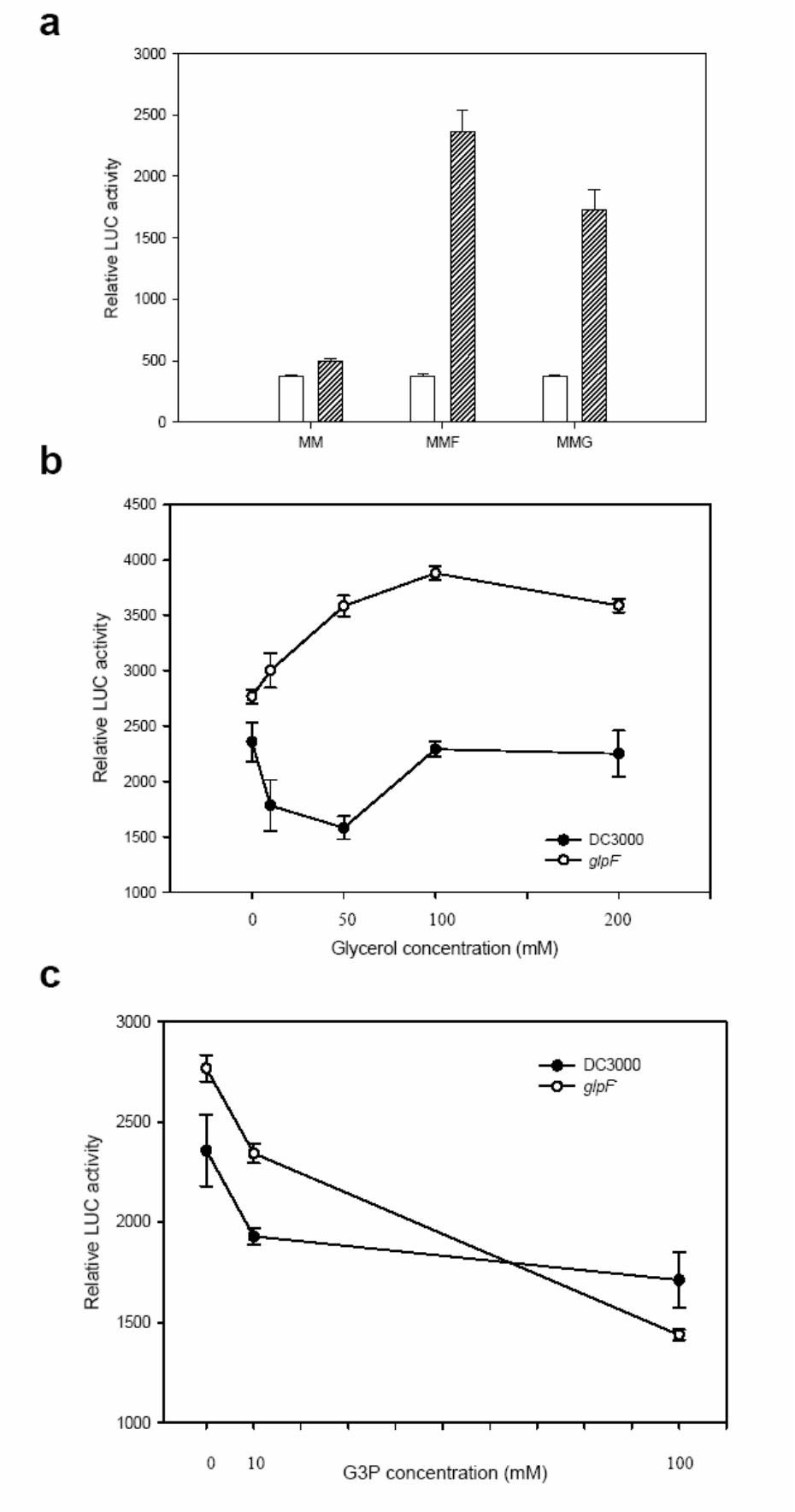


Figure 4-7 Glycerol uptake negatively regulates DC3000 TTSS activity through G3P

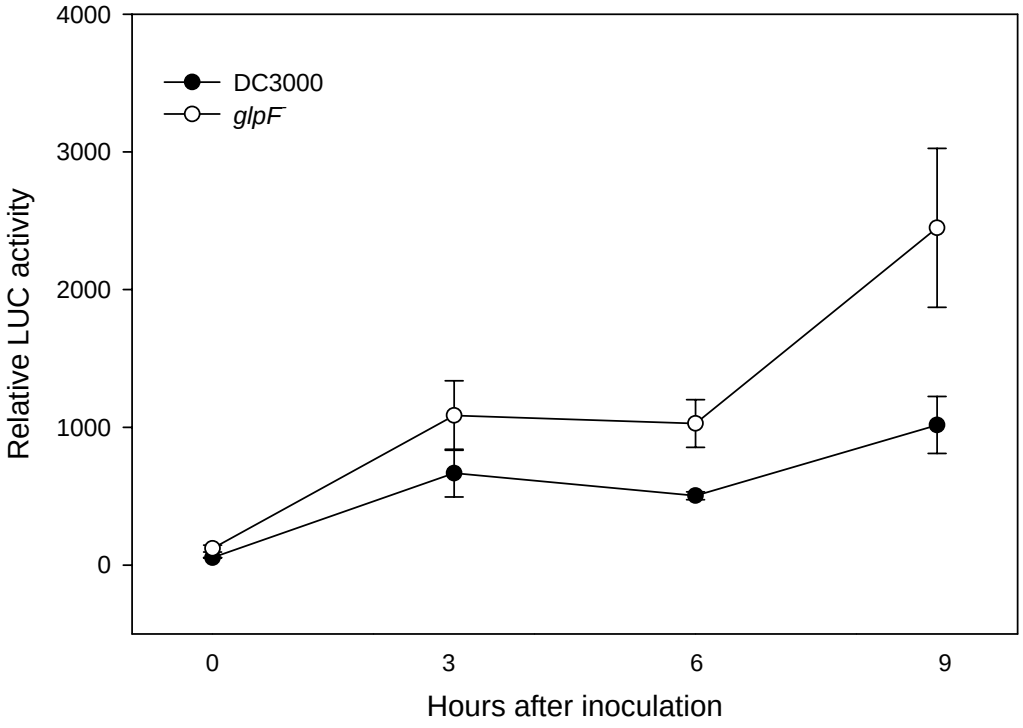


Figure 4-8 Glycerol uptake negatively regulates TTSS activity in nho1 mutant plants

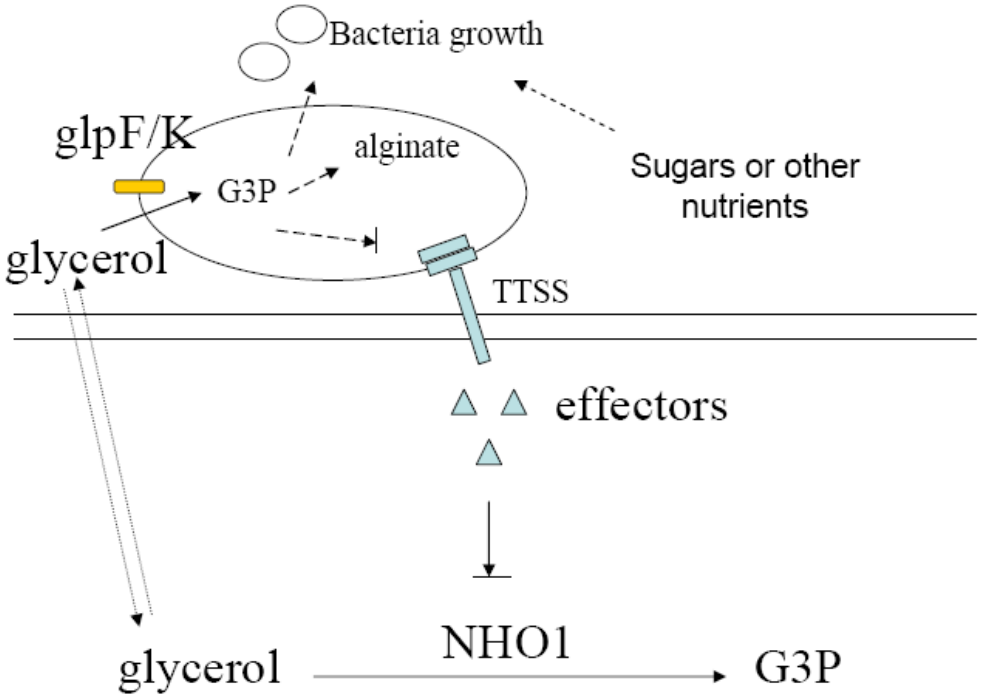


Figure 4-9 A model of glycerol regulation on *Pseudomonas* bacteria nutrient uptake, growth and virulence

Appendix A Supporting Information

Detection of HopAI1-FLAG in plants

Transgenic plants carrying the *hopAI1-FLAG* transgene were sprayed with 25 mM estradiol for 24 h. Total protein was extracted from leaves and approximately 100 □g protein was fractionated through SDS Page, and protein blot was detected with the mouse anti-FLAG M-2 monoclonal antibody (Sigma, St. Louis, MO) and the ECL kit from Amersham Biosciences (Piscataway, NJ).

Reference

Ronald, P.C., Salmeron, J.M., Carland, F.M. & Staskawicz, B.J. (1992) *J. Bacteriol*. 174, 1604-1611.

Appendix B Definition of Abbriviations

APAF1: apoptosis protease activating factor 1

AtROC1: *Arabidopsis* single-domain cyclophilin

AVR: avirulence

BRI1: brassinosteroid-insensitive 1

CAPS: cleaved amplified polymorphic sequence

CC: coiled-coil

CEL: conserved effector loci

CHORD I: cysteine-and histidine-rich domain

CLV: CLAVATA1

COI1: coronatine insensitive

DEX: dexamethasone

EDS1: enhanced disease susceptibility

EFR: EF-Tu receptor

EF-Tu: elongation factor Tu

ETI: effector-triggered immunity

FLS2: flagellin sensing 2

GEBP: GE binding protein

GE : β-glucan elicitor

HR: hypersensitive response

HSP90: Heat shock protein 90

INDEL: insertion-deletion

ISR: induced systemic resistance

JA: jasmonic acid

LBP: soluble LPS-binding protein

LII: localized innate immunity

LPS: lipopolysaccharide

LRR: leucine-rich repeats

MAPK: mitogen-activated protein kinase

NBS: nucleotide binding site

NDR1: nonrace-specific disease resistance

NHO1: nonhost resistance 1

NO: nitric oxide

NOD: nucleotide-binding oligomerization domain

NOS: nitric oxide synthase

NPG1: no pollen germination1

NPR1: nonexpressor of pathogen-related gene 1

PAD4: phytoalexin-deficient 4

PAMP: pathogen associated molecular pattern

PBS1: required for AvrPphB/RPS5-mediated resistance

PCD: program cell death

PEN: penetration

PRR: pattern recognition receptor

PRS2: resistance to *Pseudomonas syringae*

PTI: PAMP-triggered immunity

R: resistance

RAR1: required for Mla12 resistance

Rcr3: required for Cf-2 mediated resistance

RIN4: RPM1 interacting protein 4

RLK: receptor-like kinase

RLP: receptor like protein

RPG1: resistance to Pseudomonas syringae pv. glycinea

RPM1: resistance to Pseudomonas syringae pv. maculicola

RPW8: resistance to powdery mildew 8

Rxo1: reaction to Xanthomonas oryzicola 1

SA: salicylic acid

SAG101: senescence-associated gene101

SAR: systemic acquired resistance

SCF: Skp1-Cullin-F-box

SGT1: suppressor of the G2 allele of Skp1

SIR: systemically induced resistance

SNARE: soluble N-ethylmaleimide-sensitive factor attachment protein receptor

SNP: single nucleotide polymorphism

SSLP: simple sequence length polymorphism

TIR: Toll/interlucin-1 receptor

TLR: Toll-like receptor

TMV: tobacco mosaic virus

TTSS: type III secretion system

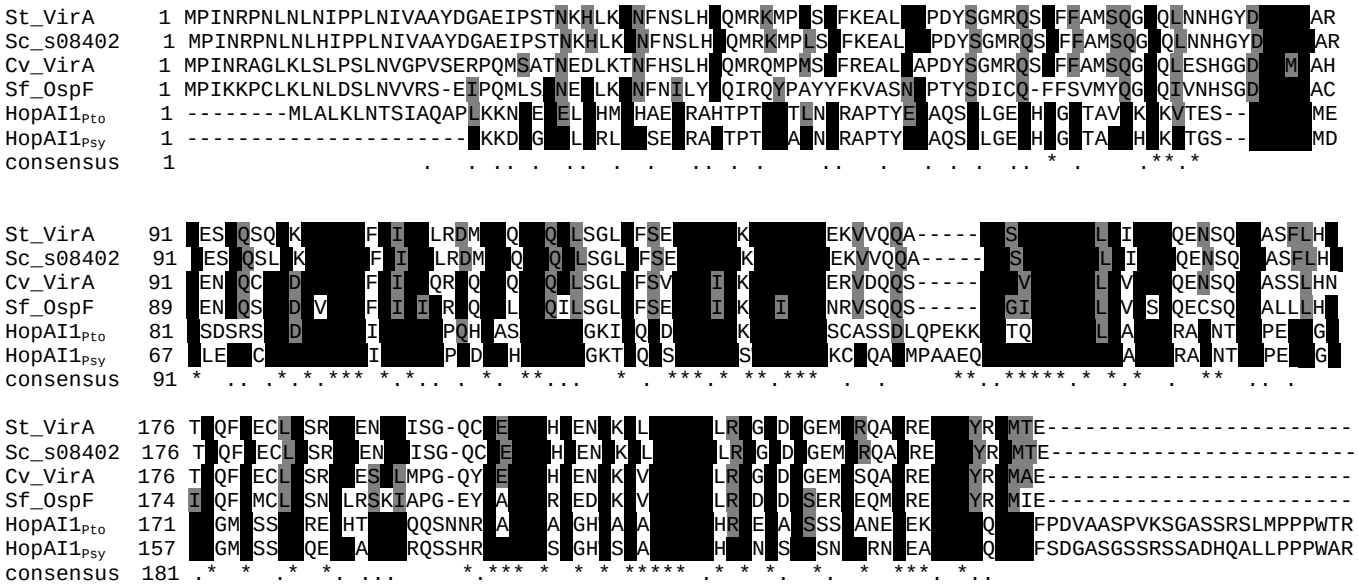
WIR: wound-induced resistance

Supporting Table 2-1 Primers used for effector transient expression constructs

Construct	Forward primer	Reverse Primer	Effector Digestion	Note
35S-AvrPto	ttggtaccctcgagatgggaaatatatgtgtc	actcgtcgacatagttcattgccagttacggtac	XhoI/SpeI	
35S-HopAI1-FLAG	aactcgagcgatagaaagcaggaaaacaac	ttttcgaagcgagtccagggcggtggcatc	XhoI/Csp45I	
35S-HopAQ1-FLAG	aactcgagcgtaaaaatgaggactgcag	ttttcgaatgcactgccaccagcaatcgag	XhoI/Csp45I	
35S-HopQ1-2-FLAG	aactcgagcgtgtaatcagcacttcctc	ttttcgaaatccgggactgccgtcgac	XhoI/Csp45I	
35S-HopT1-2-FLAG	ttgtcgacgataagtggcacgtcgatttc	ttttcgaagcctacccgatcagcggctgaat	SalI/Csp45I	
35S-HopS1-FLAG	ttctcgagtgctctgatgcgataaaaagag	ttttcgaagaccttcccaagctctg	XhoI/Csp45I	
35S-HopS2-FLAG	aactcgaggccttcatgaaaaagtctg	ttttcgaagccagatgagctcgccacag	XhoI/Csp45I	start codon GTG changed to ATG
35S-HopAS1-FLAG	aactcgagccgatggagcccgctcatgaccttaag	ttttcgaaagaaaactcggctttctgttcaacctg	XhoI/Csp45I	
35S-HopJ1-FLAG	aactcgagcaacaggcggcgttcgtcaaggaaag	aaatcgataaccttgcgcttgagtggctcgccttcgaatg	XhoI/ClaI	from old annotation, missing first 36 aa
35S-HopAA1-1-FLAG	aagtcgactgttcaaacttccgtagaacgagag	aaatcgatcgaccgcataggccgaaacggtatttc	SalI/ClaI	
35S-HopC1-FLAG	aactcgagtcagggaactgaaccgcttatg	ttttcgaagtgtatttttgaagcgaatacttgaac	XhoI/Csp45I	
35S-HopF2-FLAG	ttctcgaggaaaccattatgggtaatatttgc	ttttcgaagacccttttcgaccggcactttc	XhoI/Csp45I	start codon ATA changed to ATG
35S-HopAF1-FLAG	ccgctcgagcggatggggctatgtatttca	cccttcgaattgtgcgaccagatgttt	XhoI/Csp45I	
35S-HopK1-FLAG	ccgctcgagcggatgaatcgcatttcaaccagc	cccttcgaagcagtagagcgtgtcgcgac	XhoI/Csp45I	
35S-HopL1-FLAG	aactcgagaaactacagctctacaccatgttg	ttttcgaatctcgctttgaacgcctggatgac	XhoI/Csp45I	
35S-HopQ1-1-FLAG	ccgctcgagcggatgcatcgtcctatcacc	cccttcgaaatctggggctaccgtcga	XhoI/Csp45I	
35S-HopU1-FLAG	ccgctcgagcggatgaatataaatcgacaactg	cccttcgaaaatctgacttaatacaaataaatg	XhoI/Csp45I	
35S-HopO1-2-FLAG	ttctcgagacgctgtgtatgaatatc	aaatcgattctcgttcaaatcgacgtg	XhoI/ClaI	
35S-HopT1-1-FLAG	aactcgagtgactcatatgatgaaaacag	ttttcgaatgacttttgagccgcctgcctgac	XhoI/Csp45I	

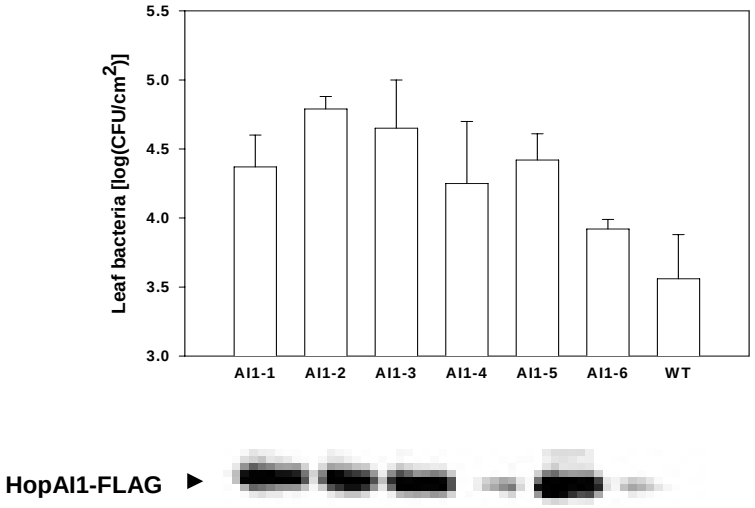
The *P. syringae* pv. *tomato* T1 strain carrying the pPTE6 plasmid (1) was used as template for *avrPto*-amplification.
Other effector genes were amplified from DC3000 genomic DNA.

Supporting Figure 2-1 HopAI1 is a conserved effector in both animal and plant pathogenic bacteria



Supporting Figure 2-1. HopAI1 is a conserved effector in both animal and plant pathogenic bacteria. Amino acid sequence of DC3000 HopAI1$_{Pto}$ (accession AA05440) was aligned with homologous sequences from other bacteria using CLUSTAL W. Homologous amino acid sequences are shaded by using BOXSHADE. St_VirA, *Salmonella typhimurium* VirA (accession A41481); Sc_s08402, *Salmonella choleraesuis* virulence-associated protein (accession S08402); Cv_VirA, *Chromobacterium violaceum* VirA (accession AAQ58983); Sf_OspF, *Shigella flexneri* OspF (accession AAP78969); HopAI1$_{Psy}$, HopAI1 from *P. syringae* pv. *syringae* (accession ZP00128143). Asterisks and dots denote invariant and conserved residues, respectively. Strong homology between the two *Pseudomonas* HopAI1 and animal bacterial proteins exists throughout the entire protein, except for the N-terminus where the presumed type III secretion signal resides.

Supporting Figure 2-2 *HopAI1* expression in primary transgenic plants enhances bacterial growth in plants

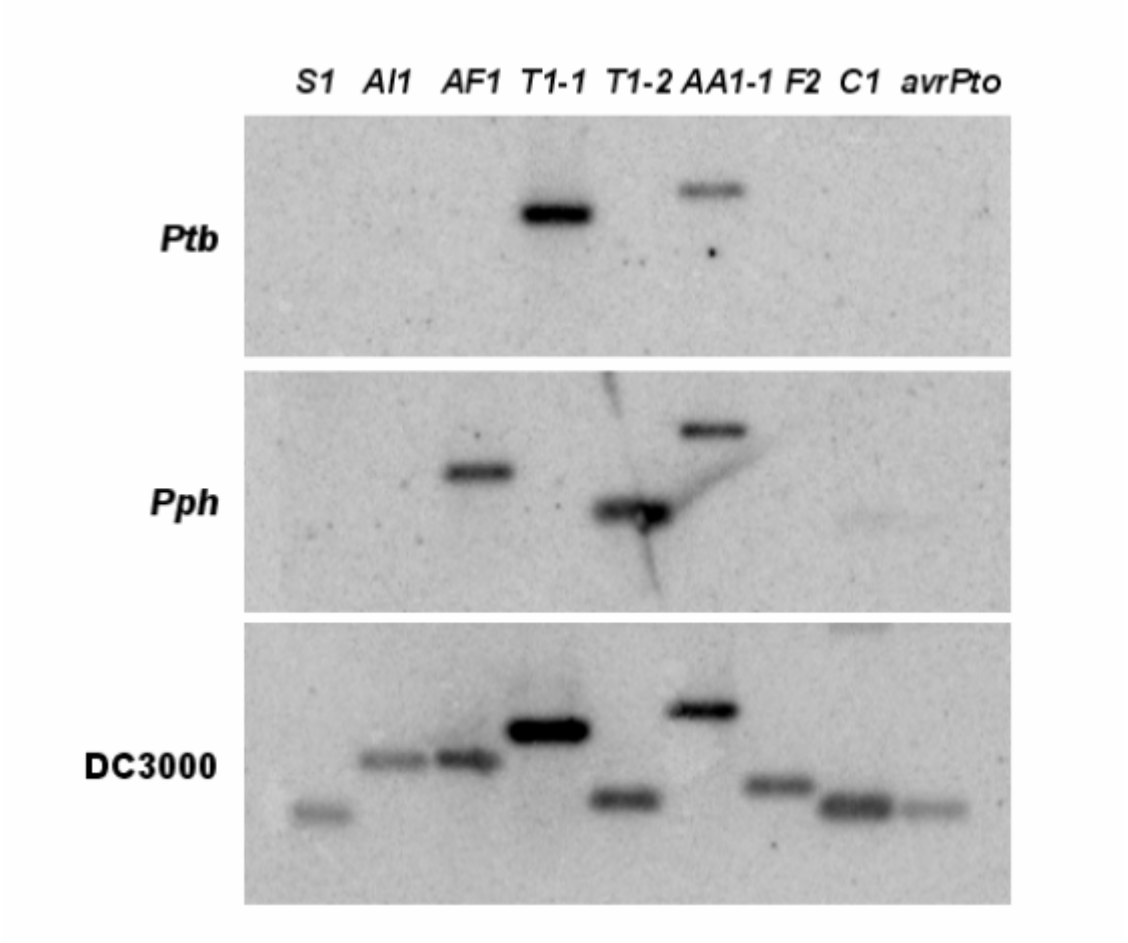


Supporting Figure 2-2. HopAI1 expression in primary transgenic plants enhances bacterial growth in plants. Six primary transgenic plants were sprayed with 50 □M estradiol. 24 h after the spray, *hrpL*$^{-}$ mutant DC3000 bacteria were inoculated into leaves, and bacterial population was determined 4 days later. Error bars indicate standard error. Western blot below the graph shows HopAI1-FLAG protein in individual transgenic plants one day after estradiol spray. This figure is contributed by Zhang J.

Supporting Figure 2-3 DC3000 wild type and *flic*$^-$ mutant strains cause similar disease symptoms on *Arabidopsis* plants

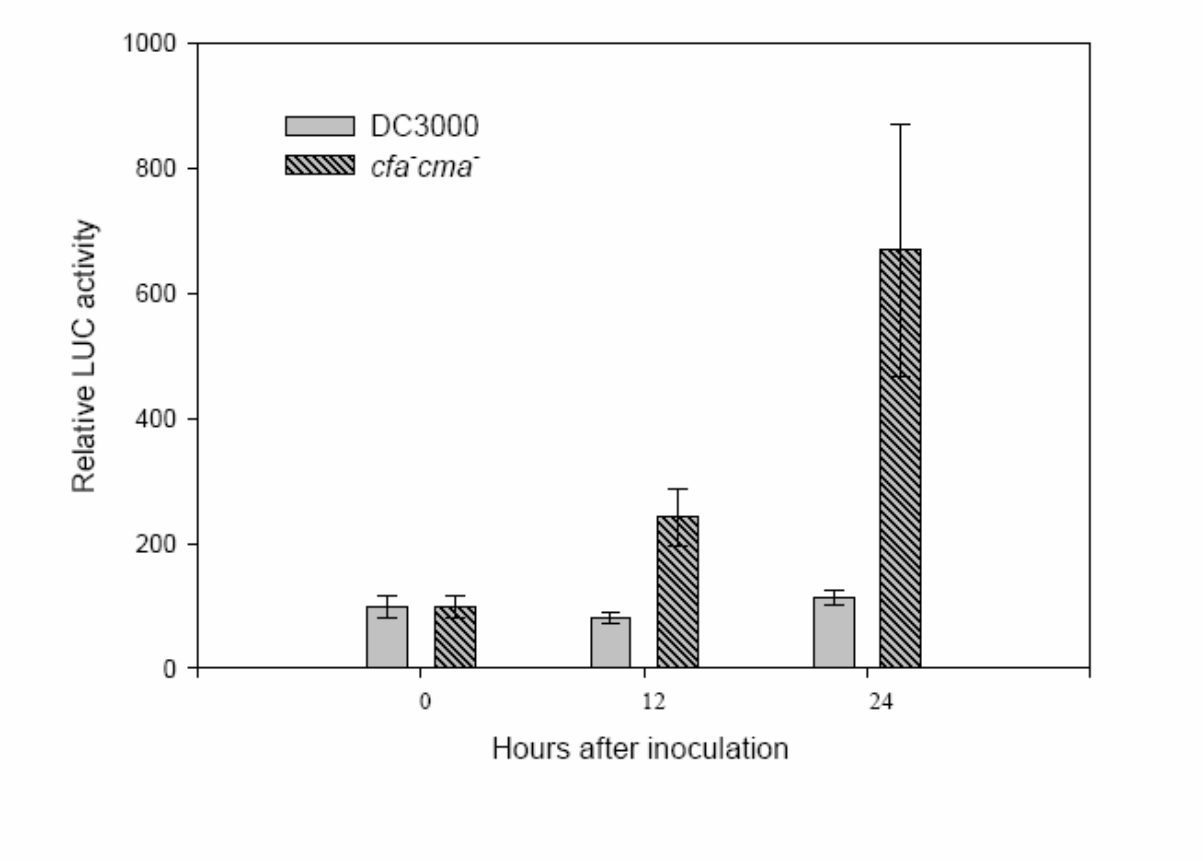
Supporting Figure 2-3. DC3000 wild type and *flic*$^-$ mutant strains cause similar disease symptoms on Arabidopsis plants. DC3000 (left) and *flic*$^-$ mutant (right) strains were inoculated at 10^6 cfu/ml into *Arabidopsis* Col-0 plants. The inoculated plants were encubated in a 22ºC plant growth chamber to allow symptom development. Picture was taken 6 days after inoculation. This experiment was repeated two times with similar results.

Supporting Figure 2-4 Southern blot analysis of effector sequences in *Ptab*, *Pph*, and *DC3000*



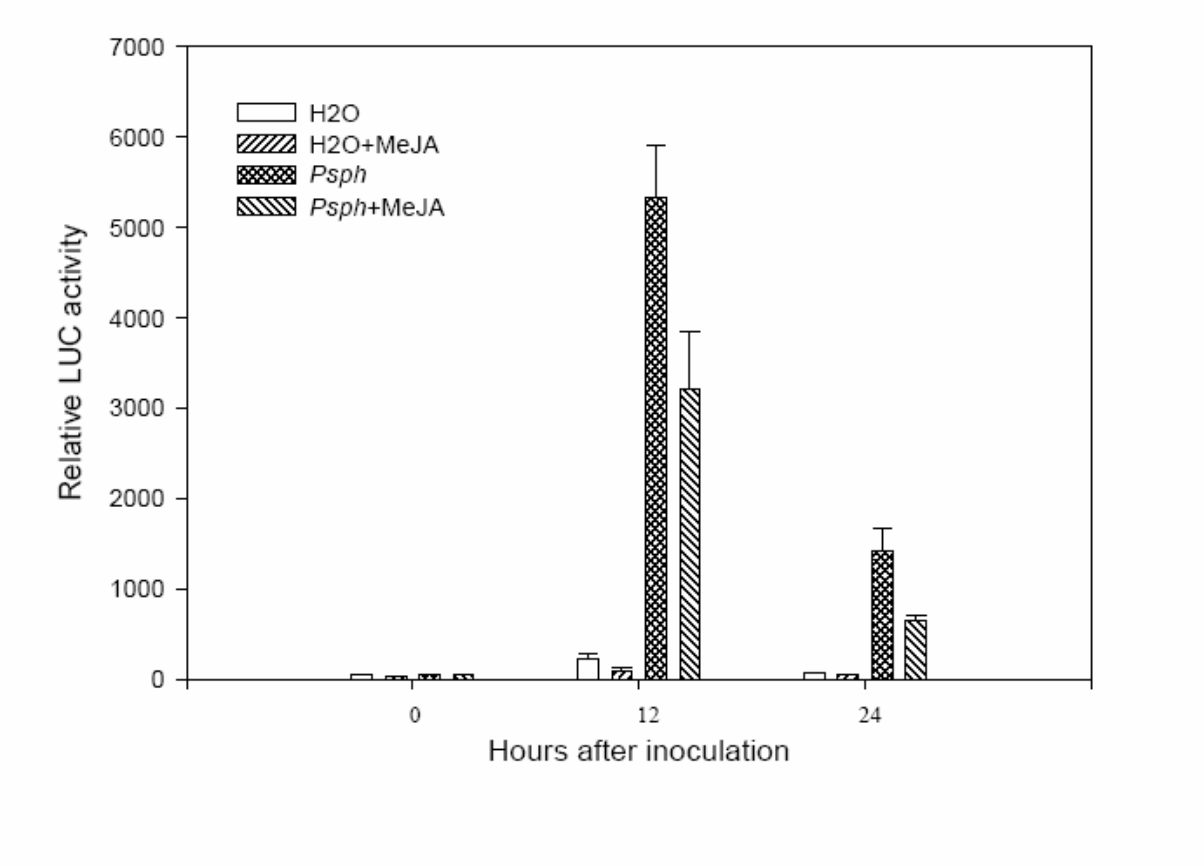
Supporting Figure 2-4. Southern blot analysis of effector sequences in *Ptab, Pph*, and DC3000. A total of 200 ng of plasmid DNA containing *hopS1*, *hopAI1*, *hopAF1*, *hopT1-1*, *hopT1-2*, *hopAA1-1*, *hopF2*, *hopC1*, and *avrPto* were digested with *Xho*I and *Spe*I to release the effector sequences and transferred to the membrane. Triplicated membranes containing equal amounts of DNA were hybridized with radio-labeled genomic DNA isolated from the indicated bacterial strains. Standard hybridization was carried out at 65°C, and membranes were washed to 0.5 × SSC at 65°C. This figure is contributed by Lin H.

Supporting Figure 2-5 DC3000 *cfa*⁻*cma*⁻ double mutant deficient in coronatine systhesis slightly compromises *NHO1*-LUC suppression



Supporting Figure 2-5 DC3000 *cfa*⁻*cma*⁻ double mutant deficient in coronatine systhesis slightly compromises *NHO1*-LUC suppression. *NHO1*-LUC transgenic *Arabidopsis* plants were inoculated with DC3000 wild type and *cfa*⁻*cma*⁻ mutant strains resuspended in 0.2mM luciferin at 10^7 cfu/ml. *Arabidopsis* leaves were detached and sprayed with 1mM luciferin containing 0.01% Triton X-100. Luminescence images were captured with a low light imaging system. Relative luciferase (LUC) activity was calculated with the WinView software provided by RoperScientific, Trenton, NJ.

Supporting figure 2-6 MeJA partially suppresses the *Pph*-induced *NHO1*-LUC activity



Supporting Figure 2-6 MeJA partially suppresses the *Pph*-induced *NHO1*-LUC activity. *NHO1*-LUC transgenic *Arabidopsis* plants were encubated in a sealed 47-liter glass chamber. 50µl 10% MeJA were added to a cotton swab inside the chamber. Same amount of ethonal were used as control. *Arabidopsis* leaves were detached at the indicated time points and sprayed with 1mM luciferin containing 0.01% Triton X-100. Luminescence images were captured with a low light imaging system. Relative luciferase (LUC) activity was calculated with the WinView software provided by RoperScientific, Trenton, NJ.