APPLICATION OF A FAST-FORWARD GENETIC TOOL TO IDENTIFY PLANT GENES INVOLVED IN THE PERCEPTION OF CORONATINE, A PHYTOTOXIN PRODUCED BY *PSEUDOMONAS SYRINGAE* PV. *TOMATO* DC3000

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CHAPTER I

INTRODUCTION

Many pathogenic variants of *Pseudomonas syringae* cause diseases in plants and induce a wide variety of symptoms including blights, cankers and leaf spots (Alfano and Collmer, 1996; Hirano and Upper, 2000). The specificity of *P. syringae* to cause disease on particular host plants led to investigations focusing on the molecular basis of host specificity (Staskawicz *et al*., 1984; Lindgren *et al*., 1986). Multiple bacterial genes are induced during pathogenesis and are required for pathogenicity (Staskawicz *et al.,* 1984; Lindgren *et al.,* 1986; Collmer et al., 2000) or contribute to disease severity (e.g., virulence factors) (Mittal and Davis, 1995; Kloek *et al.,* 2001).

Pseudomonas syringae pv. *tomato* (*Pst*) causes bacterial speck of tomato, an economically important disease. The pathogen causes necrotic lesions on the leaves, stems, and fruit of tomatoes (Goode and Sasser, 1980). Foliar lesions are generally surrounded by a yellow chlorotic halo (Peñaloza-Vázquez, *et al.,* 2000). In *Pst* strain DC3000 (*Pst* DC3000), the type III secretion system (TTSS) determines pathogenicity (He, 1998; Galan and Collmer, 1999; Hutcheson *et al.,* 2001), and coronatine (COR) is an important component of *Pst* DC3000 virulence (Bender *et al.,* 1999). The TTSS is a protein secretion complex used by many Gram-negative plant pathogenic bacteria to promote pathogenesis (Arnold *et al.,* 2003). COR is a phytotoxin that contributes to the virulence of *Pst* DC3000 (Brooks *et al.,* 2004; Uppalapati *et al.,* 2005; Elizabeth and Bender, 2007).

Bacterial speck disease of tomato is largely regarded as a foliar disease (Preston, 2000), and studies of the *Pst* DC3000-tomato interaction have primarily been conducted in the leaves of 3-4 week old tomato plants. Foliar assays generally require high relative humidity to enable pathogen entry and development of typical disease symptoms following inoculation (Lund *et al.*, 1998; Peñaloza-Vázquez *et al*., 2000; Zhao *et al*., 2003; Uppalapati *et al*., 2007). Unfortunately, these assays require extensive growth chamber or greenhouse space and are labor-intensive. Thus a part of my study was focused on the development of a reliable seedling assay for *Pst*-tomato interactions that could reduce both the time and space needed for virulence assays.

COR has structural and functional resemblance to methyl jasmonate (MeJA), and related derivatives known as the jasmonates (Feys *et al.,* 1994; Weiler *et al.,* 1994). MeJA is a plant growth hormone that plays a key role in plant defense to biotic and abiotic stress (Howe *et al*., 1996; McConn *et al*., 1997; Vijayan *et al*., 1998; Truman *et al*., 2007). COR also play a major role in disease symptom production (Brooks *et al.,* 2004).

During a compatible interaction with a host, *Pst* DC3000 infection results in the activation of the JA signaling pathway (Zhao *et al*., 2003; Laurie-Berry *et al*., 2006). This results in suppression of the SA pathway because of mutual antagonism with the JA pathway (Kloek *et al*., 2001; Kunkel and Brooks, 2002). It has been proposed that the suppression of the SA pathway during the *Pst* DC3000-host interaction is caused by COR, which acts as a molecular mimic of JA (Feys *et al.,* 1994; Bender *et al.,* 1999; Staswick and Tiryaki, 2004).

Our present understanding of COR function does not clearly explain how chlorosis impacts or benefits pathogen virulence. Furthermore, the identity of host molecular

targets for COR and the downstream signaling cascades that ensue are not well understood. Based on similarities between COR and JA in terms of structure and function (Feys *et al*., 1994; Uppalapati *et al*., 2005), it seems likely that COR and JA interact with at least one common host receptor.

Therefore, another part of my study has focused on the identification and characterization of plant proteins that are the molecular targets of COR. I explored the utility of virus-induced gene silencing (VIGS) as a fast-forward genetics tool to screen a cDNA library of *Nicotiana benthamiana*, with the aim of identifying plant genes that are involved in COR-mediated chlorosis.

CHAPTER II

REVIEW OF LITERATURE

Pseudomonas syringae

Pseudomonas syringae is a Gram-negative plant pathogenic bacterium that causes a wide variety of symptoms on plants, including blights, galls and leaf spots (Alfano and Collmer, 1996). *P. syringae* is divided into approximately 50 pathovars (pv.) based on its host range (Hirano and Upper, 2000). For example, *P syringae* pv. *phaseolicola* causes halo blight in bean (Webster *et al.,* 1983), and *P. syringae* pvs. *glycinea* and *maculicola* cause blight on soybean (*P. syringae* pv. *glycinea*) and leaf spots on tomato and *Brassica* spp. (*P. syringae* pv. *maculicola*) (Wiebe and Campbell, 1993). The specificity of the host-pathogen interaction in different pathovars of *P. syringae* is a potential tool to understand various aspects of the host-pathogen relationship. To this end, genomes of *P. syringae* pv. *tomato* DC3000, pv. s*yringae* B728a and pv. *phaseolicola* 1448a have been sequenced (Buell *et al.,* 2003; Feil *et al.,* 2005; Joardar *et al.,* 2005) Information from annotations and comparative sequence analysis of the genomes are being used to understand the molecular basis of the host-pathogen interaction (Vencato et al., 2006).

The importance of virulence in pathogens arises from the fact that they require nutrients and congenial environmental conditions that are essential for their successful establishment. The genetic basis for pathogenicity and virulence of *P. syringae* includes various factors including phytotoxins (Bender *et al.,* 1999), the type III secretion system

(TTSS; encoded by the *hrp/hrc* cluster) (He, 1998; Galan and Collmer, 1999; Hutcheson *et al.,* 2001), exopolysaccharides (Yu *et al.,* 1999; Keith *et al.,* 2003), and global regulatory proteins such as GacA and GacS (Hrabak and Willis, 1992; Rich *et al.,* 1994; Chatterjee *et al.,* 2003). Furthermore, the presence of flagella and the ability to synthesize extracellular polysaccharides are also important for epiphytic colonization and pathogenicity in *P. syringae* (Hatterman and Ries, 1987; Yu *et al.,* 1999; Keith *et al.,* 2003). Also, multiple genes are induced during pathogenesis and function as pathogenicity factors (Collmer *et al.,* 2000; Preiter *et al.,* 2005; Fu *et al.,* 2006; Sreedharan *et al.,* 2006; Tang *et al.,* 2006). In *P. syringae*, pathogenicity is host specific; in other words, the pathogen produces effector proteins that are released into the plant host cells. If the host possesses the corresponding resistance (*R*) gene, the interaction generally results in a hypersensitive response (HR), followed by activation of defense signal transduction pathways in the host plant. Plant hosts that lack the corresponding *R* gene are susceptible to the pathogen (Badel *et al.,* 2003).

P. syringae **pv.** ***tomato*****, a model organism for bacterial-plant interactions**

P. syringae pv. *tomato* DC3000 (*Pst* DC3000), a pathogen of tomato, *Brassica* spp. (collard, turnip), and *Arabidopsis thaliana* (Moore *et al.,* 1989; Whalen *et al.,* 1991; Wang *et al.,* 2002; Elizabeth and Bender, 2007) has become a model strain for investigating plant–microbe interactions, largely because of its genetic tractability, pathogenicity on *Arabidopsis*, and the availability of its genomic sequence (www.tigr.org). The *Pst* DC3000 genome (6.5 megabases) consists of a circular chromosome and two plasmids, which collectively code for 5763 ORFs, including 298

virulence genes (Buell *et al.,* 2003). In *Pst* DC3000, the TTSS and coronatine (COR) play a major role in symptom development (Figure 1; see Brooks *et al.,* 2004; Jin *et al.,* 2003). A number of regulatory proteins have also been implicated in the virulence of *Pst* DC3000 including GacA, HrpL, CorR, and CorS (Figure 1; Fouts *et al.,* 2002; Chatterjee *et al.,* 2003; Jin *et al.,* 2003; Sreedharan *et al.,* 2006). In the following sections, I describe some of the classic literature along with some recent discoveries on symptom development, biology, epidemiology, and disease development with respect to *Pst.*

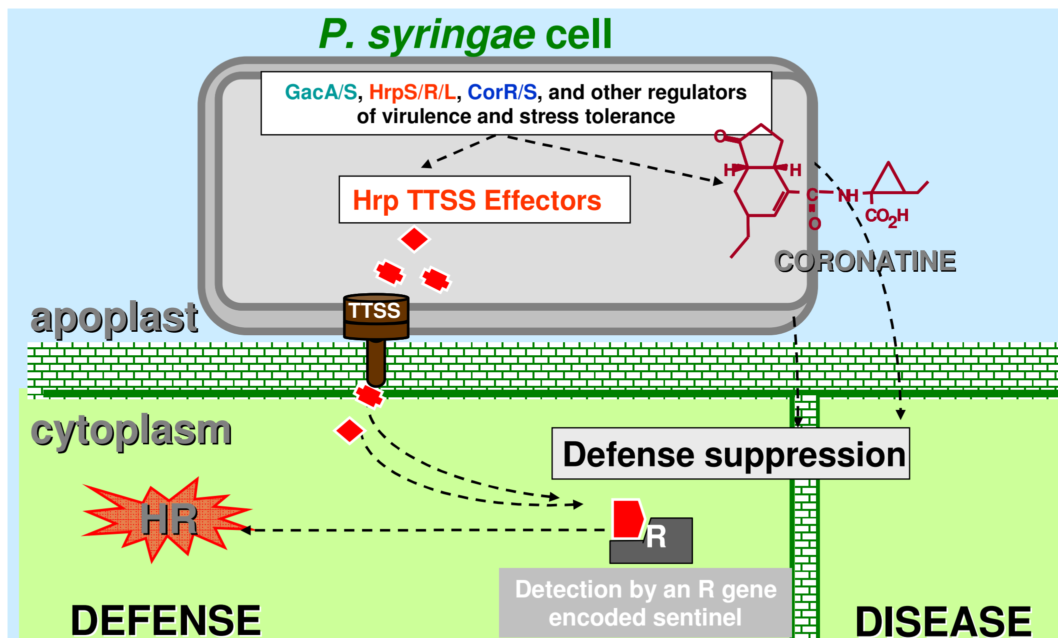

Figure 1: Schematic overview of *P. syringae*–plant interactions. Biosynthesis of the TTSS and small molecules such as coronatine are controlled by several regulatory factors. The central pathogenic process is the injection of multiple effector proteins into plant cells by the TTSS, which is depicted as the brown structure traversing the bacterial inner and outer membranes, plant cell wall, and plasma membrane. The effectors may suppress defenses and promote nutrient and water accumulation in the apoplast unless any one of them is detected by a resistance (*R*) gene-encoded sentinel, in which case strong host plant defenses associated with the hypersensitive response (HR) are triggered. Coronatine (COR) is a non-host specific phytotoxin that presumably suppresses the host defense system. Figure adapted from Buell *et al*. (2003).

1. Symptoms

One of the most obvious phenotypic changes observed in tomato as a result of *Pst* infection is necrosis on leaves and fruit. Typical symptoms observed on tomato leaves are necrotic lesions, which are generally surrounded by chlorotic halos. Infected fruits show dark brown specks with a dark green halo around them. Specks (which are usually superficial on fruit) form sunken lesions on ripened fruits.

Formation of necrotic lesions on tomato leaves infected by *Pst* (formerly known as *P. tomato*) has been studied for several decades. Bashan *et al*. (1980) reported that the appearance of necrotic lesions correlated with a pH increase from 6.5 to 8.0 in *Pst*-infected leaves. Furthermore, the increased incidence of necrotic lesions was associated with elevated electrolyte leakage 120 h after inoculation; this correlated with a high concentration of accumulated ammonia (Bashan *et al.,* 1980).

In general, the process of plant infection by phytopathogenic bacteria is via wounds and natural openings. Although the significance with respect to pathogenesis is not clear, several plant cell wall degrading enzymes have been detected during the infection of tomato by *Pst.* For example, high levels of cutinase activity were reported on both susceptible and resistant tomatoes 48 h post inoculation (hpi; Bashan *et al.,* 1985). Interestingly, genes encoding cutinase activity were not identified in the genome of *Pst* DC3000 (Buell *et al.,* 2003), and a role for cutinase activity in pathogenesis has not been investigated using a genetic approach in *P. syringae*. Among the pectinolytic enzymes, polygalacturonase, pectin lyase, pectate lyase and pectin-methylesterase activities were also observed during *Pst*-tomato interaction (Bashan *et al.,* 1985). More recently, a functional annotation of the *Pst* DC3000 genome revealed genes encoding cell-wall

degrading enzymes including a pectin lyase, a polygalacturonase and three enzymes with cellulolytic activity (Buell *et al.,* 2003).

2. <u>Biology & epidemiology</u>

Transmission of *Pst* over long distances in the absence of a host plant largely depends on survival and fitness of the pathogen. Bashan *et al*. (1982) reported that *Pst* survived on infested seeds for a period of 20 years. The infested seeds developed into seedlings that either showed disease symptoms or contained a high population of the pathogen. Furthermore, artificially infested seed lots that were stored up to six years also contained the pathogen. Although there is some controversy regarding how *Pst* overwinters, several reports show that seeds, weeds, and volunteer tomato plants can facilitate the survival of the pathogen, and these may also serve as inoculum sources in tomato fields (Chambers & Merriman, 1975; Jardine *et al.,* 1988; Schneider and Grogan, 1977; McCarter *et al.,* 1983). The extent to which *Pst* establishes in the field depends on several environmental factors and cultural practices. For example, airborne *Pst* occurred in relatively high numbers above the *Pst*-inoculated tomato fields after clipping the plants, before irrigation, and after harvest (McInnes *et al.,* 1988). Lindemann and Upper (1985) found that the upper flux of epiphytic bacteria in bean plants was higher during sunny days when the leaves were dry. In addition to suggesting the influence of wind and rain, followed by dry, sunny days, the authors also propose that large positive electrostatic charges may be correlated with the high upper flux of bacteria. In another study, Yunis *et al*. (1980) observed a positive correlation between cool temperatures (13-28°C), high relative humidity (>80%), and disease severity, which agrees with the earlier

results of Pohronezny *et al*. (1979). Host factors also influenced disease development with higher yield losses occurring in plants that were infected in earlier developmental stages (Yunis *et al.,* 1980). For example, infection occurred in immature, green tomato fruit but not in ripened, red fruit (Yunis *et al.,* 1980). This was attributed to the lower pH of the red fruit skin (5.2) and red flesh (4.0) compared to that of green fruit skin (6.3) and green flesh (5.0). Although *Pst* is known primarily as a foliar pathogen, it also causes very mild symptoms in tomato roots (Bashan, 1998).

3. Infection

For successful establishment and infection, phytopathogenic bacteria enter plant hosts via natural openings including stomata, hydathodes, lenticels and trichomes (Bashan *et al.,* 1981; Getz *et al.,* 1983a, b; Hugouvieux *et al.,* 1998). Scanning electron and light microscopy indicate that 48 h after spray-inoculation with 10^6 CFU/ml *Pst*, only a few randomly-dispersed bacteria were observed. However, stomata, trichome bases and intercellular regions below the epidermal cells were completely covered with bacteria (Bashan *et al.,* 1981). A similar phenomenon was observed by Boureau *et al.* (2002) where *Pst* DC3000 expressing green fluorescent protein was used to monitor pathogen growth on tomato leaves. There was a drastic reduction of the epiphytic population of *Pst* DC3000 48 hpi on leaves spray-inoculated with 10^7 CFU/ml of the pathogen. However, the endophytic population in the substomatal cavities and intercellular spaces increased approximately 100-fold in susceptible plants. Although natural openings on the plant surface such as stomata are viewed as passive openings for the entry of plant pathogenic bacteria, recent evidence suggest pathogen-associated molecular patterns (PAMPs) such

as exopolysaccharides and flagella trigger stomatal closure as part of the host basal defense response (Melotto *et al.,* 2006). The pathogen attempts to intervene in this process by producing virulence factors that induce the re-opening of guard cells (Melotto *et al*., 2006; Underwood *et al*., 2007).

Infection of tomato fruit resulting in bacterial speck symptoms depends largely on the presence of trichome bases that are opened as a result of trichome loss (Getz *et al.,* 1983a). On small, developing tomato fruits (e.g. <3 cm diameter), trichomes are lost leaving the base open. This enables the pathogen to invade the open trichomes and multiply subepidermally (Getz *et al.,* 1983a). In ripening fruits (e.g. >3 cm diameter), the aperture at the base of the trichome gradually closes; when the fruit is fully ripened, the trichome bases are completely covered with a cuticular layer (Getz *et al.,* 1983a). In addition to changes in pH, alterations in the apertures of the trichomes may also explain why green fruits are more susceptible to *Pst* infection.

The Type III Secretion System (TTSS)

Another critical component in the pathogenicity of *Pst* is the type III secretion system (TTSS). The TTSS, which is a protein delivery mechanism employed by some Gram-negative pathogenic bacteria, functions to secrete proteins known as the ‘effectors’ into host cells (Cornelis and Van Gijsegem, 2000; Tang *et al*., 2006). The TTSS consists of a needle-like structure that forms on the bacterial cell envelope and acts as a conduit for the delivery of effector proteins into the host (Cornelis and Van Gijsegem, 2000; Jin and He, 2001; Cornelis, 2002). In plant pathogens, a large cluster of genes that encode the TTSS are designated as the *hrp* genes (for *h*ypersensitive *r*esponse and *p*athogenicity) due to the inability of the *hrp* mutants to cause disease on susceptible host plants and failure to elicit

an HR on nonhost plants and the resistant cultivars of susceptible plants (Lindgren, 1997). In *P. syringae,* some of the effector proteins are designated as 'Avr' proteins because of their detection through the gain-of-function avirulence phenotypes. Other effectors are named 'Hop' proteins (Hrp outer proteins) based on their ability to translocate across the TTSS machinery (Alfano and Collmer, 2004). The importance of effector proteins arises from the fact that they play key roles in promoting pathogenesis as well as eliciting defense responses (Alfano and Collmer, 2004; Mudgett, 2005). An example of an effector protein that interacts directly with a host *R* gene product is the AvrPto protein secreted by *Pst.* AvrPto interacts with the corresponding *R* gene product, Pto (Tang *et al*., 1996), in a classical gene-for-gene manner (Keen, 1990), which results in a HR. However, interactions exist where the *R* gene-mediated defense reaction that elicits the HR does not involve a direct interaction of *Avr* and *R* gene products. For example, the products of *AvrRpm1* and *AvrB* interact with RIN4 (RPM1-INTERACTING PROTEIN4), and RIN4 then becomes phosphorylated. The product of the Arabidopsis *R* gene *RPM1* then interacts with phosphorylated RIN4, and this results in a defense response (Mackey *et al.,* 2002; Desveaux *et al.,* 2007; reviewed in Mudgett, 2005).

Hypersensitive cell death occurs through the induction of ion fluxes and oxidative bursts that result in the generation of extracellular reactive oxygen species, which cause membrane damage to host cells (Heath, 2000; Greenberg and Yao, 2004). In susceptible plants, *Avr* gene products may suppress HR-mediated defense in various ways. For example, the *Pst* DC3000 effector AvrPtoB can inhibit the cell death induced by oxidative stress (Abramovitch *et al*., 2003). Another *Pst* DC3000 effector, AvrRpt2,

suppresses the AvrRpm1-mediated HR by cleaving the RIN4 protein, thus resulting in suppression of *RPM1*-mediated defense (Mudgett, 2005; Nomura *et al.*, 2005).

Regulatory factors

Several regulatory genes that impact virulence in *Pst* DC3000 are associated with the TTSS. The *hrpL* gene is required for the expression of several transcripts in the *hrp* gene cluster and encodes a sigma factor related to the extracellular factor family of alternate sigma factors (Xiao *et al.,* 1994). *hrpS* and *hrpR* encode response regulator members of the two-component regulatory system (TCRS) and are responsible for the transcriptional activation of *hrpL* (Xiao *et al.,* 1994). *hrpR* and *hrpS* are expressed as a single operon and then they interact to form a stable heterodimeric complex that positively regulates the σ^{54}-dependent *hrpL* promoter (Hutcheson *et al.,* 2001). A study by Bretz *et al*. (2002) reported that the TTSS in *P. syringae* is negatively regulated by Lon protease, an ATP-dependent serine protease, which is involved in the degradation of several regulatory proteins. Regulation involves Lon protease-mediated degradation of HrpR, which is reduced when *hrp* gene expression is induced. Thus, Lon protease negatively regulates the TTSS in *P. syringae* by the degradation (proteolysis) of HrpR.

The GacA and GacS proteins also comprise a TCRS, where GacA is a response regulator and GacS is the sensor kinase. GacS presumably senses environmental signals, is autophosphorylated at a conserved histidine residue, and phosphorylates GacA, which then activates the transcription of target genes (Rich *et al.,* 1994). GacA acts as a central regulator that controls an assortment of transcriptional and posttranscriptional factors (Chatterjee *et al.,* 2003). *Pst* DC3000 *gacA* mutants show reduced virulence on both

tomato and Arabidopsis (Chatterjee *et al.,* 2003). Another TCRS that was recently discovered in *P. syringae* consists of RhpR and RhpS. In this interaction, autophosphorylated RhpR is a negative regulator of *hrpL*, *hrpR* and *avrPto* genes, whereas RhpS induces the TTSS by reversing the autophosphorylation of RhpR and thus promotes pathogenesis (Xiao *et al.*, 2007).

Phytotoxins

Plant pathogens produce a number of metabolites that are toxic to plant cells. However, only those metabolites that fulfill the following criteria are considered phytotoxins: (i) a purified toxin should reproduce the disease symptoms; (ii) there should be a direct correlation between pathogenicity and toxin yield; (iii) the toxin should be produced during the active growth of the pathogen *in planta*; and (iv) a non-toxigenic strain should be reduced in virulence (Bender *et al.,* 1999). Several strains of *P. syringae* produce phytotoxins that generally induce chlorosis (coronatine, phaseolotoxin, and tabtoxin) (Gnanamanickam, 1982; Mitchell, 1976; Levi, 1986) or necrosis (syringomycin and syringopeptin) (Paynter and Alconero, 1979; Iacobellis *et al.,* 1992). Most phytotoxins produced by *P. syringae* are considered non-host specific because they cause disease symptoms on plants that are non-host for the phytotoxin producing pathogen.

1. Coronatine

Pst DC3000 produces the phytotoxin coronatine (COR), which contributes to virulence in Arabidopsis, tomato, collard and turnip (Brooks *et al.*, 2004; Uppalapati *et al.*, 2005; Elizabeth and Bender, 2007). COR also acts as a virulence factor in other *P.*

syringae pathovars such as pv. *atropurpurea*, *glycinea*, *maculicola*, and *morsprunorum*, which infect ryegrass, soybean, crucifers, and *Prunus* spp., respectively (Bender *et al*., 1999). The structural components of COR consist of the polyketide coronafacic acid (CFA) and the cyclized isoleucine derivative coronamic acid (CMA) (Parry *et al*., 1994). While CFA is synthesized from precursor metabolites that include three units of acetate, one unit of pyruvate and one unit of butyrate, CMA is a product of isoleucine that has undergone an isomerization and a cyclization (Bender *et al.,* 1999). CFA and CMA are linked together via amide bond to form COR (Figure 2a; Bender *et al.,* 1999).

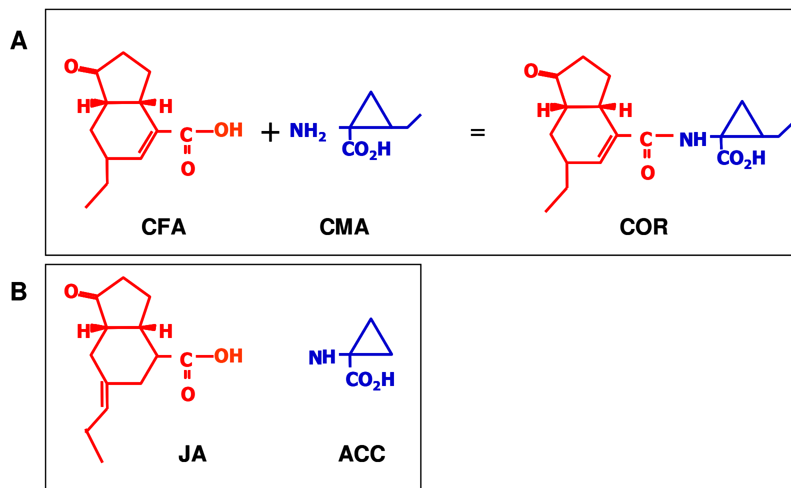

Figure 2: Structural resemblance of COR and plant metabolites. A) Structure of COR, showing the coronafacic acid (CFA) and coronamic acid (CMA) components linked by an amide bond. B) The biochemical structure of the CFA component of COR has a high degree of similarity to the plant hormone jasmonic acid (JA), whereas 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor metabolite for ethylene, resembles CMA.

2. Mode of action

COR is a non-host specific phytotoxin that induces chlorosis on several plants including tomato and soybean (Gnanamanickam *et al*., 1982) COR has structural and functional properties that are similar to jasmonic acid (JA; Figure 2a, b) and its

derivatives (Feys *et al*., 1994; Weiler *et al*., 1994). A recent high-throughput transcriptome analysis indicated that COR regulates about 35% of the methyl jasmonate induced genes (Uppalapati *et al*., 2005). JA is a plant growth regulating hormone that plays a key role in plant defense response against biotic and abiotic stresses (Howe *et al*., 1996; McConn *et al*., 1997; Vijayan *et al*., 1998; Truman *et al*., 2007).

3. *Pst* DC3000 and coronatine production.

Unlike many *P. syringae* pathovars where the coronatine (COR) genes are clustered and plasmid-encoded, the COR genes in *Pst* DC3000 are chromosomally encoded. In *Pst* DC3000, the genes encoding CFA and CMA, are separated by a 26-kb region (Brooks *et al.,* 2004; Buell *et al.,* 2003). Boch *et al.* (2002) used in vivo expression technology (IVET) to identify bacterial genes that are specifically induced during the infection of *A. thaliana* by *Pst* DC3000. Approximately 15% of the genes induced during infection were localized to the CFA operon and included *cfl*, *cfa1*, *cfa6*, *cfa7*, *cfa8*, and *cfa9*. These results are consistent with a previous study where a *cor*::*inaZ* transcriptional fusion was used to identify plant factors that stimulated *cor* gene expression in *Pst* DC3000. COR production by *Pst* DC3000 is induced in the presence of the host. Malic, citric, shikimic, and quinic acids were identified as compounds that stimulate COR production, and these compounds were present in leaf extracts and apoplastic fluids of tomato (Li *et al*., 1998).

Current understanding on function of COR in virulence

Genetic screens for COR/JA insensitivity have resulted in the identification of the *coronatine insensitive1* (*coi1*) and *JA insensitive1* (*jai1*) genes in Arabidopsis and tomato,

respectively (Staswick *et al.,* 1992; Feys *et al.,* 1994; Berger *et al.,* 1996; Li *et al.,* 2003; Li *et al.,* 2004). Studies employing these mutants suggest that COR may have biochemical functions similar to JA. For example, during a compatible interaction with a host, *Pst* DC3000 infection results in the activation of the JA signaling pathway (Zhao *et al*., 2003; Laurie-Berry *et al*., 2006). This leads to the suppression of the salicylic acid (SA) pathway owing to the antagonistic relationship between the JA and SA pathways (Kloek *et al*., 2001; Kunkel and Brooks, 2002). The suppression of the SA pathway during the *Pst* DC3000-host interaction is thought to be caused by COR, which functions as a molecular mimic of JA (Feys *et al.,* 1994; Bender *et al.,* 1999; Staswick and Tiryaki, 2004). More recently, COR has been shown to induce stomatal opening to promote entry of bacteria into the host (Melotto *et al*., 2006).

Despite the studies described above, the molecular mechanisms involved in COR-mediated chlorosis and the precise mode of action of COR are unknown. Therefore, one objective of the current work was to use virus-induced gene silencing (VIGS) as a fast-forward genetics tool for identifying plant genes associated with COR-mediated chlorosis.

Virus induced gene silencing (VIGS)

VIGS, also known as post-transcriptional gene silencing, is a mechanism adopted by plants to defend against viruses through RNA-mediated viral gene suppression (Baulcombe, 1999). It involves the synthesis of the viral dsRNA, by an RNA-dependent RNA polymerase, following viral entry into the host cell (Dalmay *et al.,* 2000). The

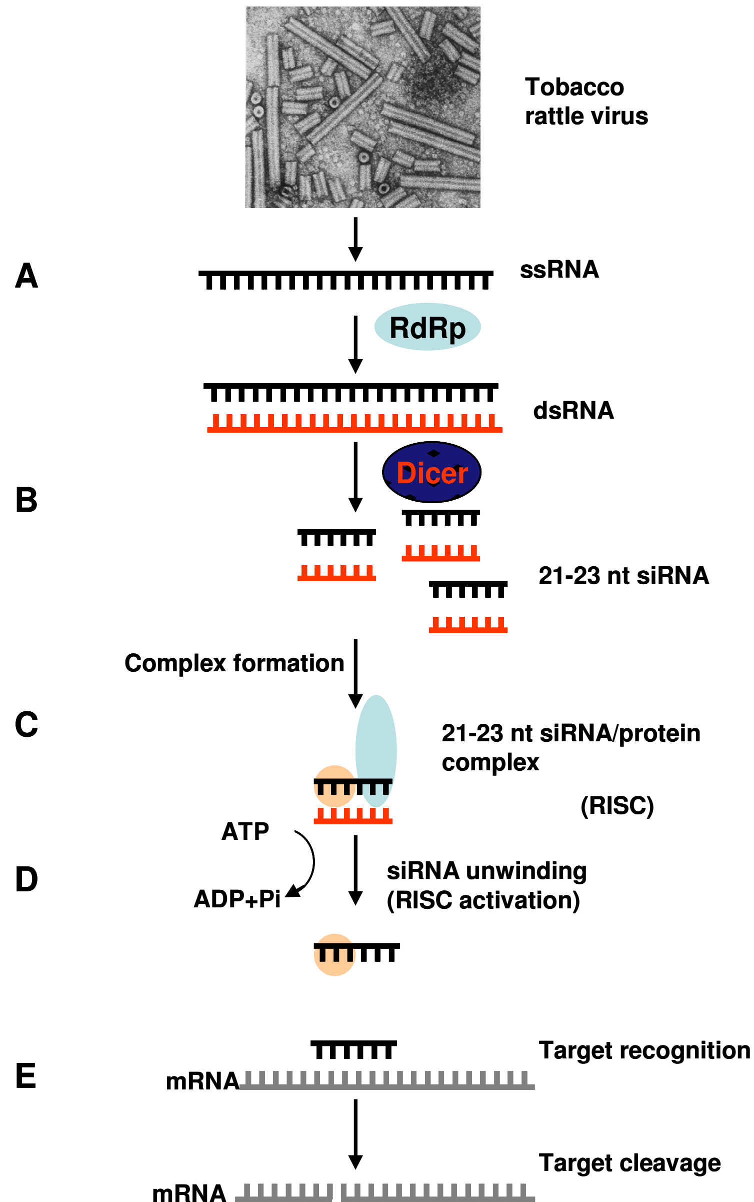

Figure 3: A model for virus-induced gene silencing pathway showing the *Tobacco rattle virus* as an example. A) Following its entry into the host cell, the virus is uncoated. The naked single stranded RNA (ssRNA) is synthesized into dsRNA by an RNA-dependent RNA polymerase (RdRp) which may be encoded by both the viral and the host genome. B) The dsRNA thus formed serves as a target for DICER, an enzyme that specifically cleaves dsRNAs. This results in the formation of several 21-23 bp dsRNAs known as the short interfering RNAs or siRNAs. C) The siRNAs then form a protein-RNA complex known as the RNA-induced silencing complex (RISC). D) An ATP-dependent helicase activity in the complex unwinds the siRNA into single stranded RNA. E) These RNA molecules serve as guides and facilitate the cleavage of mRNA molecules by binding to homologous target mRNAs resulting in their degradation. (Figure adapted from Nykanen *et al.,* 2001).

dsRNA thus formed serves as a target for DICER, an endonuclease that specifically cleaves dsRNAs (Figure 3; Bernstein *et al.,* 2001). This results in the formation of several 21-23 bp dsRNAs known as the short interfering RNAs or siRNAs. These RNA molecules serve as guides and facilitate the cleavage of homologous mRNA molecules (Zamore *et al.,* 2000). The siRNAs are then recognized by a protein-RNA complex known as the RNA-induced silencing complex (RISC) (Hammond *et al.,* 2000; Nykanen *et al.,* 2001). An ATP-dependent helicase activity in the complex unwinds the siRNA into single stranded RNA, and this binds to homologous target mRNAs resulting in their degradation (Nykanen *et al.,* 2001).

Tobacco rattle virus (TRV) as a VIGS Vector

VIGS has been manipulated as a technique for transient suppression of host gene transcripts or "knockdown" of gene expression. This is accomplished when a recombinant virus vector containing a partial sequence from the host gene is introduced into the plant (Baulcombe, 1999; Liu *et al*., 2002b). This approach was initially used with *Tobacco mosaic virus* to silence *phytoene desaturase* (*PDS*), a gene that encodes an essential enzyme in the carotenoid pathway (Kumagai *et al.,* 1995). More recently, other viruses, including *Potato virus* X, *Tomato golden mosaic* DNA virus, and TRV have also been developed into VIGS vectors (Burch-Smith *et al.,* 2004). As part of my work, I used TRV (Liu *et al.,* 2001a, b) and a fast-forward genetics approach to identify plant genes involved in COR mediated chlorosis.

TRV is a plant virus whose genome consists of two positive-sense single stranded RNAs that are encapsidated separately into rod shaped particles (MacFarlane, 1999).

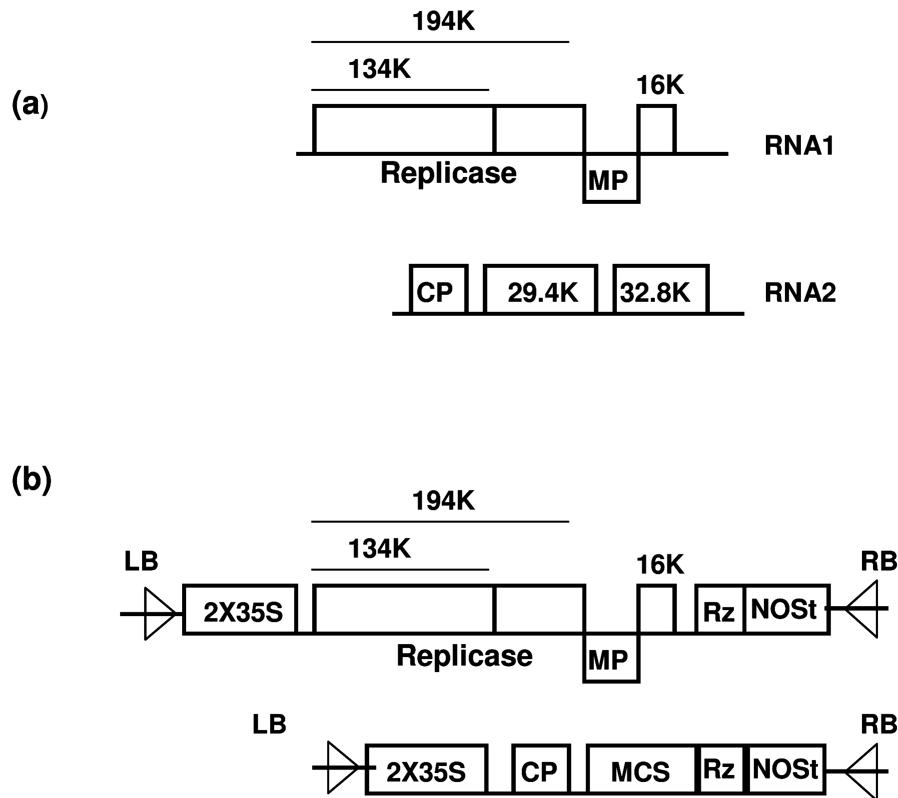

Figure 4: The *Tobacco rattle virus* (TRV) genome and the VIGS vector. A) TRV has a bipartite RNA genome known as RNA1 and RNA2. RNA1 encodes for a replicase that is composed of two enzymes; a 134 kilodalton (kDa or K) helicase and a 194 K RNA-dependent RNA polymerase. RNA1 also contains genes that encode for a movement protein (MP) and a 16 K cysteine-rich protein. TRV coat protein (CP) is encoded by RNA2 along with two other non-structural 29.4 K and 32.8 K proteins. B) In order to develop TRV as a VIGS vector, both RNA1 and RNA2 were inserted in a recombinant *Agrobacterium tumefaciens* T-DNA immediately downstream of the CaMV 35S dual promoter (2X35S) to generate a new recombinant T-DNA vector construct. In addition, sequences encoding a self-cleaving ribozyme (Rz) was also added immediately upstream of the nopaline-synthase termination sequence (NOSt) of the T-DNA. Furthermore, in RNA2, the two non-structural genes were replaced with a multiple cloning site (MCS) to enable insertion of sequences that are targeted for silencing. LB and RB indicates the left border and the right border, respectively, of the T-DNA vector. The resulting recombinant RNA1 and RNA2 are now called the pTRV1 and pTRV2. These two constructs are transformed separately into *A. tumefaciens*. Figure adapted from Liu *et al.,* 2002a.

RNA1 encodes for a replicase proteins that consists of a 134 kDa helicase and a 194 kDa RNA-dependent RNA polymerase (Figure 4a). RNA1 also encodes a 29 kDa movement protein and a cysteine-rich 16 kDa protein (MacFarlane, 1999). RNA2

encodes the coat protein and two non-structural proteins (MacFarlane, 1999; Liu *et al.,* 2002c).

To develop TRV as a VIGS vector, Liu and associates (2002b) generated Agrobacterium binary constructs containing cDNA clones of RNA1 and RNA2, individually within the T-DNA (Figure 4b). The cDNAs corresponding to RNA1 and RNA2 were inserted immediately downstream of the double CaMV 35S promoter. In addition, a self-cleaving ribozyme was added to the 3' end. In the construct containing RNA2 of TRV, the two non-structural genes were replaced with a multiple cloning site (MCS), which was used as the insertion site for cDNA clones chosen for silencing experiments. The T-DNA vector constructs that resulted were designated pTRV1 and pTRV2, and these were transformed into *Agrobacterium tumefaciens.* Approximately equal amounts of *A. tumefaciens* transformants carrying pTRV1 and pTRV2 were mixed together and then infiltrated into plants to achieve silencing of the target genes.

Application of VIGS to study COR function

VIGS has been widely applied to understand gene functions in several plant species including Arabidopsis (Burch-Smith *et al*., 2006; Lin *et al*., 2007), tomato (Liu *et al*., 2002a; Ryu *et al*., 2004; Fu *et al*., 2005) and *N. benthamiana* (Liu *et al*., 2002c; Anand *et al*., 2007b; Hirano *et al*., 2007). VIGS has also been used to probe gene function on a whole genome scale by using it as a screening tool to perform fast-forward genetics (Lu *et al*., 2003; Anand *et al*., 2007b).

Leaf tissues of tomato, collard and turnip treated with purified COR or infected with COR-producing strains of *P. syringae* show chlorosis (Gnamanickam *et al*., 1982;

Uppalapati *et al*., 2005, 2007; Elizabeth and Bender, 2007). Furthermore, COR also induces chlorosis on *Nicotiana benthamiana* (Figure 5a), thus provided a host plant where VIGS could be used to identify plant genes associated with COR and/or *Pst* DC3000-induced chlorosis.

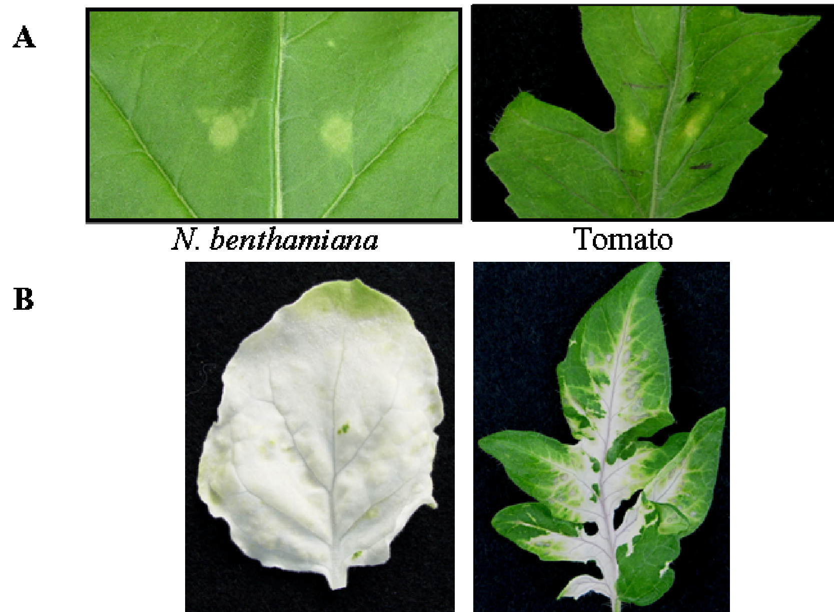

Figure 5. A): Leaves of *Nicotiana benthamiana* and tomato show chlorosis in response to COR. Photos were taken five days after COR application. B): Leaves showing the phenotype resulting from the silencing of *phytoene desaturase* (*PDS*) in *N. benthamiana* and tomato using VIGS. Notice that unlike in *N. benthamiana*, the photo-bleaching in tomato is not as pronounced and uniform throughout the leaf indicating inefficient silencing of *PDS* gene. Pictures were taken four weeks after silencing.

As part of my dissertation research, *N. benthamiana* was chosen for VIGS instead of tomato because similar to earlier observations (Ekengren *et al*., 2003; Ryu *et al.,* 2004; Uppalapati *et al*., 2007), efforts to silence *PDS* using VIGS in tomato resulted in non-uniform silencing, which was evident from the inconsistent photobleaching phenotype (Figure 5b). However, in *N. benthamiana*, the silencing of *PDS* resulted in a pronounced and uniform photobleaching (Figure 5b). Thus, we decided that *N. benthamiana* would be

a better host to assess altered chlorotic phenotypes in a high-throughput fashion. Moreover, a cDNA library of *N. benthamiana* is already available that contains ~4000 clones. Thus, the cDNA clones of *N. benthamiana* lines that have been silenced using VIGS could be readily screened to find lines displaying an altered phenotype in response to COR. The identification of silenced genes could then be used in sequence analysis to identify orthologs in tomato and Arabidopsis. Orthologous genes could then be investigated in these biologically relevant hosts in pathogencity and genetic studies, with the goal of identifying genes involved in COR and *Pst* DC3000-induced chlorosis and their contribution to disease.

OBJECTIVES

I. Identification of plant genes involved in the perception of coronatine, a phytotoxin produced by *Pst* DC3000, using the following approaches:

A. Screening of *N. benthamiana* silenced lines for altered chlorosis in response to COR (Figure 6): The silenced lines were obtained by: (**A**) Germinating and raising *N. benthamiana* on seed beds for 3 weeks; (**B**) Transplanting each seedling into individual six inch pots; and (**C**) performing VIGS based fast-forward genetics using a cDNA library (~4000 *N. benthamiana* cDNA clones); these were cloned into a VIGS vector based on tobacco rattle virus (TRV). (**D**) Pronounced silencing was generally achieved in three weeks. (**E**) Purified COR was applied onto expanded leaves, which were then examined for altered chlorosis.

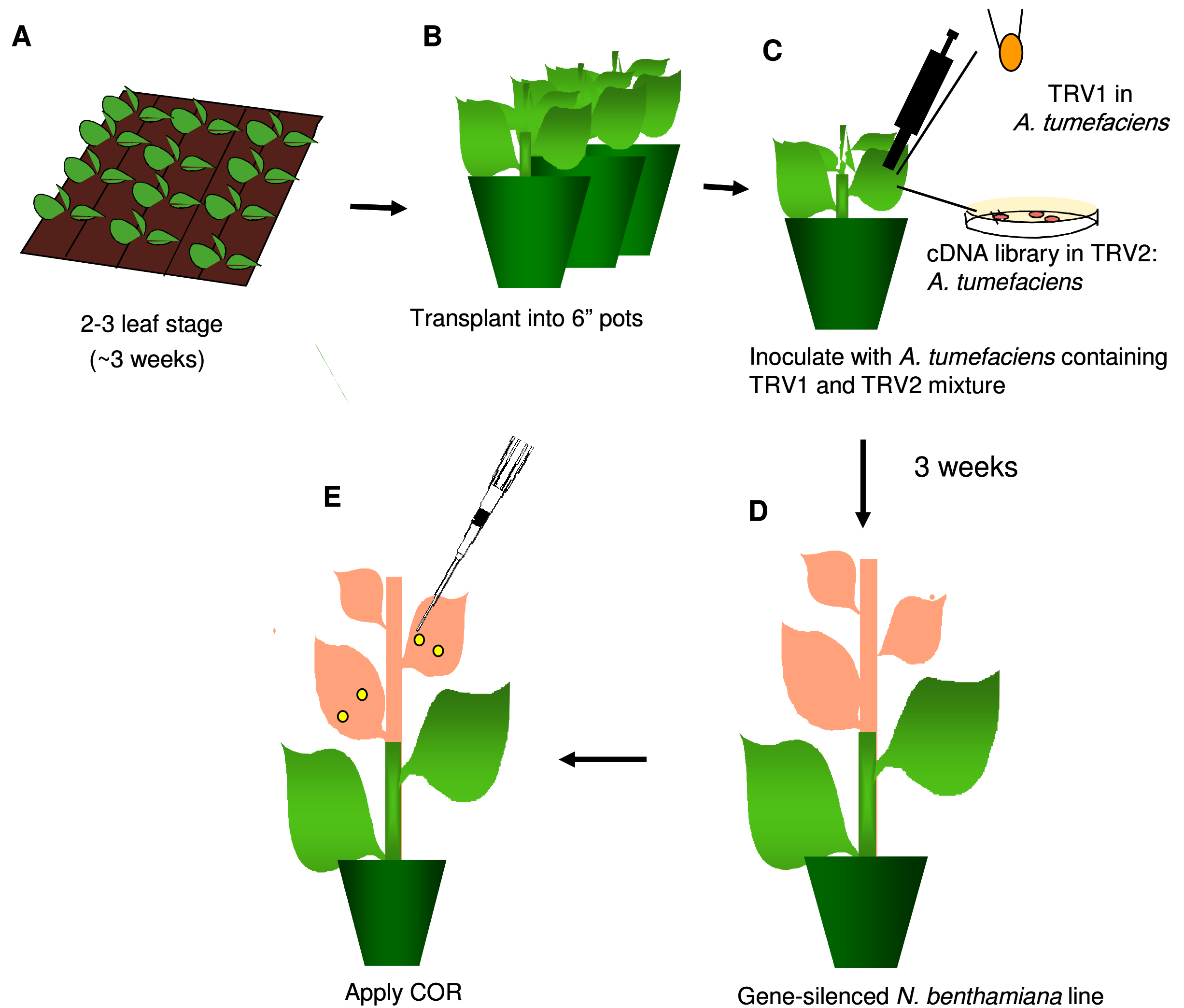

Figure 6. Silencing of *N. benthamiana* genes. *N. benthamiana* seedlings were raised for 3 weeks (**A**) and then transplanted into pots (**B**). A mixture of TRV1 and TRV2, which contain the *N. benthamiana* cDNA library, were Agro-inoculated into the lower leaves (**C**), and silencing was achieved by 3 weeks post Agro-inoculation (**D**). COR was applied onto silenced leaves, and phenotypes were observed after 3-4 days.

B. Assessment of altered response to COR on tomatoes silenced using tomato sequences homologous to selected *N. benthamiana* cDNAs. Briefly, the inserts in the *N. benthamiana* cDNA clones selected in part **A** (see above) were sequenced, and the sequence information was used to perform a BLAST search to look for orthologs in tomato. The cDNAs of tomato orthologous sequences were then transformed into the TRV2 VIGS vector and used to

silence tomato plants. The silenced tomato lines were then assessed for phenotypic response to COR to compare with the corresponding *N. benthamiana* lines. The response to COR-producing *Pst* DC3000 was also assessed on silenced lines to study the overall contribution of COR during disease development.

C. Use of Arabidopsis to further understand the involvement of the tomato ortholog (as obtained in part **B** above) in COR/*Pst* DC3000-mediated chlorosis. Since Arabidopsis is also a host of *Pst* DC3000, Arabidopsis T-DNA knock-out mutants corresponding to the *N. benthamiana* and tomato silenced lines showing an altered chlorosis phenotype were obtained and assessed for response to COR and *Pst* DC3000. Furthermore, a gene expression analysis was performed to better understand the involvement of the identified gene in COR and *Pst* DC3000-mediated chlorosis.

II. Develop a high through-put assay for assessing the pathogenicity of *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) on tomato.

A. Define parameters for assay, including:

i. Methods for seedling incubation (treatment and handling of seeds, temperature of incubation, choice of support).

ii. Age of seedlings, inoculation method, quantitative and qualitative variables.

B. Validation of seedling assay with known mutants.

C. Expression of known marker genes in the seedling assay.

D. Comparison of incompatible and compatible interactions (*Pst* DC3000 on Rio Grande (*PtoR*) and the near isogenic line (*PtoS*) in the seedling assay.

E. Screening of a *Pst* DC3000 mutant library for virulence on tomato seedlings

CHAPTER III

The *Thylakoid Formation1* gene is required for coronatine-induced chlorosis in response to *Pseudomonas syringae* pv. *tomato* DC3000 infection

SUMMARY

Pseudomonas syringae pv. *tomato* DC3000 (*Pst* DC3000), which causes disease in tomato and Arabidopsis, produces coronatine (COR), a non-host specific phytotoxin. COR, which functions as a methyl jasmonate (MeJA) mimic, is required for full virulence of *Pst* DC3000 and for the induction of chlorosis in host plants. Previous genetic screens based on insensitivity to COR and/or MeJA identified several potential targets for COR and MeJA. Despite these observations, the mechanisms involved in COR-mediated chlorosis remain unclear. In this study, we utilized *Nicotiana benthamiana* and viral-induced gene silencing to reduce the expression of over 4,000 cDNA clones mined from a *N. benthamiana* cDNA library. The silenced lines of *N. benthamiana* were then screened for altered response to purified COR. Using this forward genetic approach, several genes were identified with a potential role in COR-induced chlorosis. These were designated as altered COR-induced chlorosis (*ALC*) genes. One of the identified genes, *ALC1*, produced a hypersensitive/necrosis-like phenotype instead of chlorosis when silenced. To understand the involvement of *ALC1* during the *Pst* DC3000-host interaction, we used the nucleotide sequence of *ALC1* and identified its ortholog in Arabidopsis (*Thylakoid Formation1, THF1*) and tomato

(*SlALC1*). In pathogenicity assays performed on an Arabidopsis *thf1* mutant and *SlALC1*-silenced on tomato plants, *Pst* DC3000 induced necrotic lesions but chlorosis was absent, indicating that these genes are required for *Pst* DC3000-mediated chlorosis in Arabidopsis and tomato. Furthermore, genetic studies suggest that *THF1* regulates senescence during pathogenesis in Arabidopsis.

INTRODUCTION

In nature, plants come in contact with numerous microbes most of which are not capable of causing disease. This is mainly due to basal resistance mechanisms exhibited by plants against potential pathogens, which include physical barriers and preformed and inducible antimicrobial compounds (Dangl and Jones, 2001; Dixon *et al*., 2002; Nurnberger *et al*., 2004). In addition, plants also recognize surface derived molecules known as pathogen-associated molecular patterns (e.g. lipopolysaccharides, flagellin and harpin), which trigger the induction of defense responses including oxidative burst, apoptosis-like cell death and production of phytoalexins (Nurnberger *et al*., 2004; Hann and Rathjen, 2007). Another type of defense mechanism is mediated by disease resistance (*R*) genes and is induced by effector proteins secreted by pathogens. This results in a hypersensitive response (HR), which is characterized by necrotic cell death and may be followed by localized reinforcement of plant cell walls (Abramovitch *et al*., 2003; Hauck *et al*., 2003). Plant defense mechanisms, in general, involve a complex network of three genetically distinct signaling pathways, known as the salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) pathways (Kunkel and Brooks, 2002; Glazebrook, 2005).

Pathogens, in turn, have co-evolved by developing mechanisms that suppress plant defense pathways.

Several strains of *Pseudomonas syringae* produce phytotoxins. In plants, these phytotoxins generally induce chlorosis (e.g. coronatine, phaseleotoxin, and tabtoxin) (Gnanamanickam, 1982; Mitchell, 1976; Levi, 1986) or necrosis (e.g. syringomycin and syringopeptin) (Paynter and Alconero, 1979; Iacobellis *et al*., 1992). Some toxins do not induce visible symptoms, but instead influence metabolic processes in the host that are manifested at the biochemical level (Bender *et al*., 1999). In many cases, the toxins produced by *P. syringae* are not required for pathogenicity; they act as virulence factors and contribute to increased disease severity by facilitating bacterial movement *in planta* (Patil *et al*., 1974), lesion size (Bender *et al*., 1987; Xu and Gross, 1988) and pathogen multiplication (Bender *et al*., 1987; Feys *et al*., 1994; Mittal and Davis, 1995). Most phytotoxins produced by *P. syringae* are considered non-host specific because they cause disease symptoms on plant species not infected by the toxin-producing pathogen (Bender *et al*., 1999).

Coronatine (COR), a phytotoxin produced by *P. syringae* pv. *tomato* (*Pst* DC3000), is induced in the presence of the plant host metabolites such as malic, citric, shikimic, and quinic acids, which are present in leaf extracts and apoplastic fluids of tomato (Li *et al*., 1998). COR contributes to the virulence of *Pst* DC3000 in Arabidopsis, tomato, collard and turnip (Brooks *et al.,* 2004; Uppalapati *et al.,* 2005; Elizabeth and Bender, 2007). The structural component of COR consists of the polyketide coronafacic acid (CFA) and the cyclicized isoleucine derivative, coronamic acid (CMA) (Parry *et al*., 1994). It has been shown that COR has structural and functional resemblance to 12-oxo-phytodienoic acid

(12-OPDA), methyl jasmonate (MeJA), and related derivatives known as the jasmonates (Feys *et al.,* 1994; Weiler *et al.,* 1994). MeJA is a plant growth hormone that plays a key role in plant defense response to biotic and abiotic stress (Howe *et al*., 1996; McConn *et al*., 1997; Vijayan *et al*., 1998; Truman *et al*., 2007).

During a compatible interaction with a host, *Pst* DC3000 infection results in the activation of the JA signaling pathway (Zhao *et al*., 2003; Laurie-Berry *et al*., 2006). This results in suppression of the SA pathway because of mutual antagonism with the JA pathway (Kloek *et al*., 2001; Kunkel and Brooks, 2002). The suppression of the SA pathway during the *Pst* DC3000-host interaction has been proposed to be caused by COR, which acts as a molecular mimic of JA (Feys *et al.,* 1994; Bender *et al.,* 1999; Staswick and Tiryaki, 2004).

Pst DC3000 causes disease on several plant species including tomato and Arabidopsis. A typical symptom on tomato is bacterial speck that includes necrosis surrounded by a chlorotic halo (Mittal and Davis, 1995; Zhao *et al*., 2003). In Arabidopsis, the infected area exhibits water-soaked lesions accompanied by diffuse chlorosis (Mittal and Davis, 1995; Brooks *et al*., 2004). *Pst* DC3000 infection also causes chlorosis in other plants belonging to Brassicaceae family such as collard and turnip (Elizabeth and Bender, 2007). In addition to chlorosis, *Pst* DC3000-infected collard plants exhibit water-soaked lesions and anthocyanin, suggesting that *Pst* DC3000 elicits unique responses in different plants. Studies have shown that tomato plants inoculated with a COR-defective mutant of *Pst* DC3000 did not develop typical chlorotic symptoms; furthermore COR contributed to pathogen fitness and disease development in SA-independent manner (Uppalapati *et al.,* 2007). Unlike tomato, purified COR does not

elicit chlorosis on Arabidopsis leaves (Mach *et al.,* 2001). However, in Arabidopsis, COR is required for full disease symptom development and pathogen fitness in a SA-dependent manner (Kloek *et al.,* 2001; Brooks *et al.,* 2005). These results suggest that COR functions as an important virulence factor in tomato and Arabidopsis, although it functions differently in these hosts.

Despite our present understanding of COR function, it is not clear how chlorosis impacts or benefits pathogen virulence. Furthermore, the identity of host molecular targets for COR and the downstream signaling cascades that ensue are not well understood. Based on similarities between COR and JA in terms of structure and function (Feys *et al*., 1994; Uppalapati *et al*., 2005), it seems likely that COR and JA interact with at least one common host receptor. Thus, in addition to furthering our understanding of disease development, studies aimed at understanding the molecular mechanism of COR may provide information on JA-mediated plant defense. Therefore, in an effort to identify plant proteins that are the molecular targets of COR, we used virus-induced gene silencing (VIGS) as a fast-forward genetics tool to screen a *Nicotiana benthamiana* cDNA library for altered chlorosis in response to COR.

VIGS is a mechanism adopted by plants to defend against viral attack via RNA-mediated viral gene suppression (Baulcombe, 1999). VIGS reduces the expression of the targeted host gene when a recombinant viral genome, inserted with a partial sequence from the host target gene, is introduced into the plant (Baulcombe, 1999; Liu *et al*., 2002b). VIGS has been widely used to understand gene function in several plant species including Arabidopsis (Burch-Smith *et al*., 2006; Lin *et al*., 2007), tomato (Liu *et al*., 2002a; Ryu *et al*., 2004; Fu *et al*., 2005) and *N. benthamiana* (Liu *et al*., 2002c; Anand *et*

al., 2007a; Hirano *et al*., 2007). Furthermore, VIGS has been utilized as a screening tool to perform fast-forward genetics (Lu *et al*., 2003; Anand *et al*., 2007b).

In this study, tobacco rattle virus (TRV) was used as a VIGS vector (Liu *et al.,* 2001b, c), and a fast-forward genetics approach was implemented to identify potential molecular targets of COR. Using this strategy, we identified a *N. benthamiana* cDNA clone that displayed an unexpected hypersensitive/necrosis-like phenotype when silenced rather than the typical chlorotic phenotype observed in response to COR. The cDNA clone identified using this approach had homology to an Arabidopsis gene that encodes a light-regulated protein located in the chloroplast, which was previously named *Thylakoid Formation1* (Wang *et al.,* 2004). The pathogenicity assays performed in this study indicate that *Thf1* regulates necrosis and chlorosis in response to *Pst* DC3000. Furthermore, gene expression studies using senescence-related JA marker genes (e.g. *LOX2*, *PDF1.2*, *CORI1* and *SAG12*) indicate that *Thf1* may also regulate senescence during pathogenesis.

MATERIALS AND METHODS

Plant material and bacterial cultures

Seeds of *N. benthamiana* were germinated and maintained in the greenhouse facility of the Noble Foundation, Ardmore, OK, as described previously (Senthil-Kumar *et al*., 2006). Seeds of tomato (*Solanum lycopersicum*) cv. Glamour were obtained from Stokes Seeds, Inc. (Buffalo, NY, U.S.A.). Seeds of the Arabidopsis *thf1* T-DNA mutant, its complement, and overexpression lines were kindly provided by Dr. Ken Korth, University of Arkansas. Agar and broth cultures of *Pst* DC3000, and *P. syringae* pvs.

glycinea, *maculicola* and *tabaci* were grown on King's B medium (King *et al.,* 1954). *Agrobacterium tumefaciens* and *Escherchia coli* cultures were grown on Luria Bertani (LB) medium (1% yeast extract, 0.5% Bacto-tryptone, 1% NaCl). When required, growth media were supplemented with antibiotics at the following concentrations: rifampicin (50 mg/L), kanamycin (50 mg/L), ampicillin (100 mg/L) and gentamicin (20 mg/L). For pathogen infection assays on silenced-tomato lines, plants were inoculated with a bacterial suspension as described (Uppalapati *et al.,* 2007). Bacterial suspensions (optical density at 600 nm [OD_{600}] =0.1) were prepared in distilled water containing 0.0025% Silwet L-77 (OSi Specialities Inc., Danbury, CT, USA), and sprayed on plants using a Paasche VL airbrush (Paasche Airbrush Co. Chicago, IL, USA) to runoff. The spray-inoculated plants were then incubated in growth chambers at 90-100% relative humidity (RH) for the first 24 h followed by 70% RH until the end of the experimental period. In Arabidopsis infection experiments, the leaves of 4-week old plants were either infiltrated (OD_{600}=0.2) with bacteria using a needleless syringe, or the plants were inverted and dipped into the culture suspension (OD_{600}=0.002). The plants were then placed in trays and covered with transparent lids and incubated in growth chambers for the rest of the experimental period. Although the bacterial concentrations were determined routinely based on OD, the initial number of colony forming units (CFU) was determined by plating homogenized leaf samples harvested on the day of inoculation (0 dpi).

Screening of a *N. benthamiana* cDNA library based on COR responsiveness

A library of *N benthamiana* consisting of approximately 4000 cDNA clones (del Pozo *et al.*, 2004) was used to screen for plant genes involved in COR-mediated chlorosis.

Briefly, the cDNA library was cloned into a GATEWAY-ready VIGS vector based on TRV (Liu *et al*., 2002b, c). TRV RNA1 (TRV1) and TRV RNA2 (TRV2) were integrated into a modified T-DNA plasmid and maintained separately in *A. tumefaciens* strain GV2260 (Liu *et al*., 2002b). TRV1 contains the viral RNA-dependent RNA polymerase (RdRp) and the viral movement protein, whereas TRV2 contains the viral coat protein gene and the insertion site for the target cDNA clone. Approximately equal proportions (OD_{600}=0.1) of *A. tumefaciens* containing TRV1 and TRV2 were mixed together in a buffer containing 10 mM MES, 10 mM $MgCl_2$ and 100 µM acetosyringone. The culture mixture was then infiltrated into two leaflets of three-week old *N. benthamiana* seedlings with a 1 ml needleless syringe. About three weeks post inoculation, 2 µl of COR (2 nmol) was placed on either side of the midrib of two fully expanded leaves per plant. *N. benthamiana* inoculated with *TRV::GFP* was used as a control, and altered phenotypes in response to COR were recorded 5-7 days post application.

Cloning of a full length *Nb28C12* gene

A tobacco (*N. tabacum*) full length cDNA sequence of *Nb28C12* (226 bp) was obtained from the J. C. Venter Institute's plant genome database (Accession no. TC10126; www.tigr.org). Based on the tobacco sequence, primers for PCR were designed (forward: 5' CAA CTC CAT TCT CTA AAG CAA C 3'; reverse: 5' GTC AAT GAG GTC CAA GCA GG 3'); these spanned approximately 70 bp upstream and 40 bp downstream of the putative coding region. The 1 kb PCR product thus obtained was then cloned into the pGEMT EASY vector (Promega, Madison WI) and transformed into *E. coli* JM109 competent cells. The identity of the insert was then confirmed by sequencing.

Construction of pTRV::*Sl28C12* and VIGS in tomato

The vectors pTRV1 and pTRV2 (Liu *et al*. 2002b) were kindly provided by Dr. Dinesh-Kumar, Yale University, U.S.A. An antisense *Sl28C12* sequence consisting of a 324 bp fragment, (TIGR accession no. TC162724, currently http://compbio.dfci.harvard.edu/tgi/ accession no. 178313) was PCR-amplified from tomato (cv. Glamour) by reverse transcriptase-PCR (RT-PCR) using primers *Sl28C12*attB1: 5'- ggg gac aag ttt gta caa aaa agc agg ct TTC CAC CTC TCG CTT TGT CG -3' and *Sl28C12*attB2: 5'- ggg gac cac ttt gta caa gaa agc tgg gt GCA TCA GCT CTG TAT TGC TC -3' (the small letters indicate the GATEWAY adapters). The amplified fragments were then introduced into GATEWAY-ready pTRV2 (Liu *et al*. 2002b). The construct pTRV2-*Sl28C12* was then introduced into *A. tumefaciens* strain GV2260 by electroporation. TRV::*SlPDS* (*PDS* encodes *p*hytoene *des*aturase) was used as a positive control for VIGS and has been described previously (Ryu *et al*. 2004). The inserts and PCR fragments were verified by sequencing.

For gene silencing in tomato, a mixture of *A. tumefaciens* containing TRV1 and TRV2-*Sl28C12* was made as described for *N. benthamiana* and agitated at room temperature for 3-4 h. To prepare tomato seedlings for transformation with *A. tumefaciens*, an infiltration method for tomato seedlings (Ekengren *et al*., 2003) was used with slight modifications. Two-week old tomato seedlings with fully-expanded cotyledons were removed from pots, completely submerged in the *A. tumefaciens* culture mixture, and then vacuum-infiltrated for 2 min. The seedlings were then transplanted into Professional Blend potting mixture (Sun Gro, Bellevue, WA). To improve the silencing efficiency, the remaining culture solution was dispensed around the seedlings using the

Agrodrench method (Ryu *et al*., 2004). Inoculated, potted seedlings were then maintained in growth chambers for 10-14 days, with a 12 h photoperiod at 22°C (day) and 18°C (night). Then the plants were moved to greenhouse and maintained at 14 h (day, 25°C) and 22°C (night) for 10-14 days.

Generation of tomato *Sl28C12* transgenic RNAi lines

In order to generate a tomato *Sl28C12* RNAi line, the *Sl28C12* fragment described above was introduced into plasmid vector pDONR 207 (Invitrogen, Carlsbad, CA, USA), and the resulting clone was then transformed into *E. coli* TOP10 competent cells (Invitrogen, Carlsbad, CA, USA). The plasmid was then isolated and the fragment was then introduced into a GATEWAY-ready binary RNAi vector pK7GWIWG2(I) (Karimi *et al*., 2002) to generate the *Sl28C12* RNAi construct. This construct was transformed into *E. coli* DH5α competent cells, isolated and then transformed into *A. tumefaciens* strain GV2260 by electroporation. For the transformation of tomato plants, a tomato tissue culture method developed by Frary and Van Eck (2005) was followed with slight modifications (e.g. tobacco feeder cell layers were not used). Cotyledons of 7-8 day-old tomato seedlings were dissected and maintained on KCMS (KC Biological MS medium; Frary and Van Eck, 2005) for 24 h. Tomato cotyledons were then co-cultivated with *A. tumefaciens* cultures carrying the RNAi construct and maintained in darkness for 48 h. For all subsequent steps, the transformation protocol described by Frary and Van Eck (2005) was followed. The presence of the construct in the transgenic line *3-2* was confirmed in PCR experiments using primer combinations of attB2 with the 35S

promoter (P35S) or the 35S terminator (T35S) that flanks the insertion sequence (Karimi *et al*., 2002).

Table 1. Primers used for qRT-PCR

Primers (F/R)	Primer sequences	Fragment size	Source
AtEF1αF AtEF1αR	CGGAGCTCAATTCTCGGAATT AGGAAGCTCGAGTGCCAAGTAC	68	Li Kang
AtLOX2F AtLOX2R	CATTTCCGCTACACCATGGA CCACCTCCGTTGACAAGACTTT	62	This study
AtThf1F AtThf1R	TTCGCGTTCCACTTCGAAA AGGCACATCGGCGGTAAC	61	This study
AtSAG12F AtSAG12R	GTGTCTACGCGGATGTGAAG CAGCAAACTGATTTACCGCA	129	(Huynh le et al., 2005)
AtActin2F AtActin2R	TCGTTGCACCACCTGAAAGGAA TGGAATGTGCTGAGGGAAGCAA	73	Li Kang
AtCORI1F AtCORI1R	GCTTCGCATGGTTACATTCTTG TCCCGGCGGCAATAATTT	57	This study
AtPDF1.2F AtPDF1.2R	CATCATGGCTAAGTTTGCTTCC GCATGTCATAAAGTTACTCATAGAGTG	328	This study
Nb/SlActinF Nb/SlActinR	TGGTGCTGAGAGATTCCGCT TGGTTTCATGAATGCCAGCAG	83	This study
Nb/SlThf1F Nb/SlThf1R	GGAGGGCTACCCCAGTGAA TCCTTTAGCGCCTCTACATATGC	68	This study

RNA isolation and reverse transcription-PCR (RT-PCR) analysis

Total RNA was isolated from leaves of *N. benthamiana*, tomato and Arabidopsis plants using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). RNA samples were

treated with RNAse free DNAse (Promega, Madison, WI, USA), followed by phenol-choloroform-isoamyl alcohol (Sigma, St. Louis, MO, USA) to remove the DNase. The first strand cDNA was synthesized using oligo (dT) 15 primer and Omniscript RT kit (Qiagen, Valencia, CA, USA). For quantitative analysis of transcripts, primer pairs were designed using the Primer Express software (Applied Biosystems Inc., Foster City, CA) to amplify the target sequences (Table 1). qRT-PCR was performed with ABI HT7900 machine using SYBR Green method (Applied Biosystems Inc., Foster City, CA) to analyze the quantitative expression of the samples. PCR efficiency was determined using linear regression software LinRegPCR (Ramakers *et al*., 2003). In order to normalize the data, parallel reactions were run using the elongation factor-alpha (EF1α) primers as the endogenous control for Arabidopsis and actin primers as the endogenous controls for *N. benthamiana* and tomato (Table 1). For the analysis of the data, the transcript levels were relatively quantified as described previously (Pfaffl, 2001).

RESULTS

Purified coronatine produces chlorosis on *Nicotiana benthamiana* leaves

Tomato leaf tissues treated with purified COR or infected with COR-producing strains of *P. syringae* show chlorosis (Gnamanickam *et al*., 1982; Uppalapati *et al*., 2005, 2007). To identify novel genes involved in COR perception and COR signaling leading to chlorosis, we explored the utility of a fast-forward genetic screens using VIGS and *N. benthamiana* (Ryu *et al*., 2004; Burch-Smith *et al*., 2004; Anand *et al.,* 2007b). Unlike tomato, the efficiency of VIGS is quite uniform in *N. benthamiana* and thus this plant offers a better system for large-scale functional characterization of genes (Lu *et al*., 2003;

Anand *et al*., 2007b). Thus we initially investigated whether exogenous COR would induce a chlorotic response in *N. benthamiana*. When purified COR was applied to *N. benthamiana* leaves at different concentrations (0.002 – 2.0 nmol in 2 µl aliquots), a visible chlorotic zone was observed in a dose-dependent manner (Figure 7a). Although COR produced visible chlorosis at concentrations as low as 0.002 nmol, the chlorotic phenotype was more defined and reproducible at 0.2 nmol (Figure 7b). Based on these results we concluded that a VIGS-based approach in *N. benthamiana* was suitable for screening silenced plants for an altered chlorosis phenotype upon COR application.

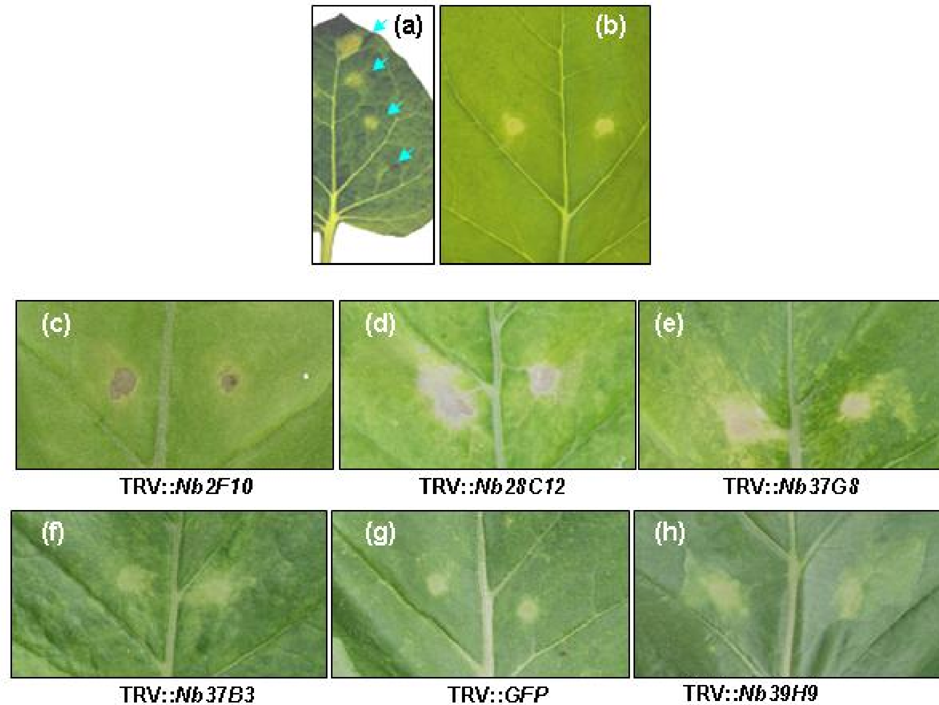

Figure 7. Coronatine (COR) induces visible chlorosis on *N. benthamiana* leaves**.** (a) Purified COR applied to *N. benthamiana* leaves in 2 µl aliquots (arrows) at different concentrations (0.002, 0.02, 0.2, 2 nmol, from lower to upper parts of the leaf); a visible chlorotic zone was observed 4 dpi. (b) Chlorotic phenotype induced by 0.2 nmol COR (quantity per inoculation site) on *N. benthamiana*. (c-h). Response of silenced lines of *N. benthamiana* leaves to 2 nmol COR. COR was applied three weeks post Agro-inoculation to silenced lines of *N. benthamiana*. In response to COR, leaves of silenced lines showed necrosis (c) or a necrosis-like phenotype (d and e). Some lines exhibited an enhanced chlorosis (f and h). Photos were taken 7 days after COR application.

VIGS identifies five *N. benthamiana* genes potentially involved in COR-mediated chlorosis

To identify the plant genes involved in COR-mediated chlorosis, we used a normalized *N. benthamiana* cDNA library cloned in pTRV2 (del Pozo *et al.,* 2004). Approximately 4,000 cDNA clones were inoculated in duplicates to silence their corresponding genes in *N. benthamiana* (Anand *et al*., 2007b). COR (2 nmol) was applied to the leaves of silenced plants, and the phenotypes were recorded 5-7 days after COR application.

During the initial screening, approximately 100 silenced plant lines (representing 100 cDNA clones) manifested an atypical response when treated with COR. After secondary and tertiary screenings, we identified five non-redundant cDNA clones that when silenced, resulted in plant lines that responded atypically to exogenous COR (Figure 7c-f, h). The application of COR to wild-type (Figure 7b) or TRV::*GFP* (vector control, Figure 7g) *N. benthamiana* plants resulted in a defined chlorotic halo. The silenced lines resulting from the five different cDNA clones exhibited either hypersensitive (HR)-like necrosis (Figure 7c-e) or increased chlorosis (Figure 7f, h) in response to COR. For example, *Nb28C12*-silenced plants exhibited a diffuse HR-like necrosis that extended beyond the area inoculated with COR (Figure 7d). Plants silenced with cDNA clone *Nb2F10* displayed a well-defined necrosis (Figure 7c), whereas *Nb37G8* (Figure 7e) silenced lines displayed a necrotic phenotype surrounded by chlorosis. *Nb37B3*- and *Nb39H9*-silenced plants displayed an enhanced, expanded chlorotic phenotype in response to COR (Figure 7f, h).

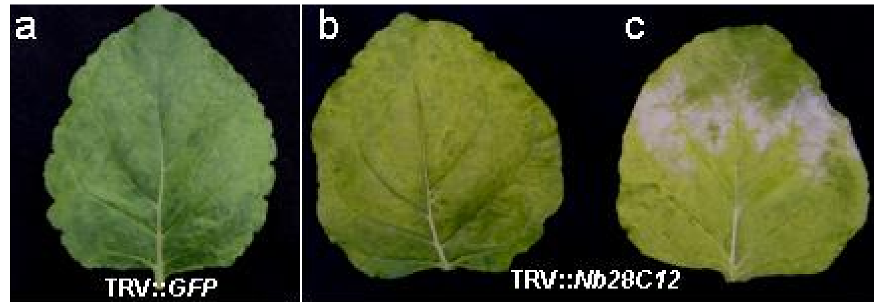

Figure 8. Phenotype of wild-type and *Nb28C12*-silenced *N. benthamiana*. (a) Leaves of *N. benthamiana* (vector control, TRV::GFP) and (b, c) plants silenced with *TRV::Nb28C12*. Notice that the silenced leaf on panel (c) has a variegated phenotype. Photos were taken five weeks after Agro-inoculation.

***ALC1* is an ortholog of the gene encoding Thylakoid Formation 1 protein**

Since the silencing of *Nb28C12* produced the most dramatic phenotype in response to COR application, this line was selected for further study. The phenotype of *N. benthamiana* plants silenced with TRV::*Nb28C12* was similar to control plants (TRV::*GFP*) up to four weeks post-silencing. However, after the fifth week, leaves of *Nb28C12*-silenced plants turned slightly pale green in color (Figure 8). At six weeks post-inoculation, portions of some leaves showed a variegated grey coloration (Figure 8c). To confirm the suppression of *Nb28C12* mRNA in silenced plants, quantitative real-time RT-PCR (qRT-PCR) was performed. The relative expression ratio of *Nb28C12* gene in the silenced line was 0.023, indicating the transcript level was approximately six-fold lower than in the control plant (Figure 9).

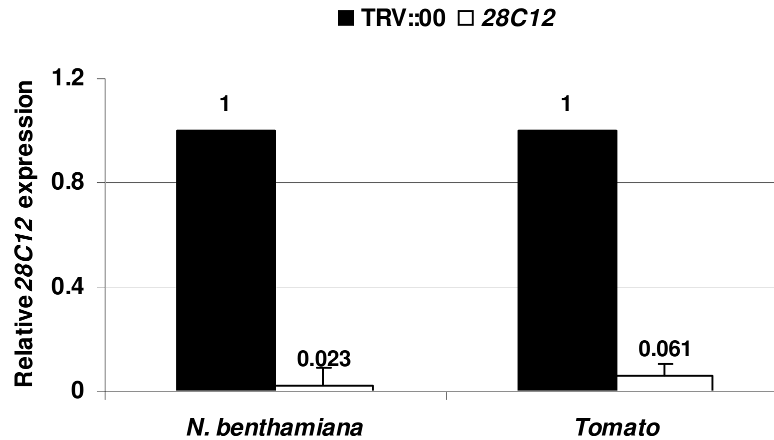

Figure 9. Real time qRT-PCR analysis comparing the transcript levels of 28C12 in *N. benthamiana* (left) and tomato leaves (right) silenced with *TRV::28C12* and *TRV::Sl28C12,* respectively. Expression levels in silenced lines are shown relative to the control (*TRV::GFP*). *N. benthamiana* and tomato samples were collected three weeks and four weeks post Agro-inoculation, respectively.

To further characterize the cDNA clone, the *28C12* insert in vector pTRV2 was amplified by PCR using vector-specific primers and sequenced. We termed this gene as altered COR-induced chlorosis 1 (*ALC1*). The sequence information was then analyzed to predict gene function. A BLASTn search against the TIGR database using *ALC1* sequence revealed 77% identity to an Arabidopsis gene *THF1* (Genbank ID AY899908); 92% identity to a potato gene that encodes a light-regulated chloroplast localized protein (*Solanum tuberosum THF1*, Genbank ID AY342161); 81% identity to a rice (*Oryza sativa*) gene encoding inositol phosphatase-like protein (Genbank ID AY224446); and 79% identity to a wheat (*Triticum aestivum*) gene encoding Ptr Tox A binding protein (Genbank ID AY377991). To facilitate a more comprehensive comparative analysis of *ALC1*, we designed a primer pair to clone the full length *ALC1* gene based on tobacco (*N. tabacum,* TC10126, Figure 10) and tomato (TC178313, Figure 10) orthologous sequences present in the TIGR database (www.tigr.org). The cloned gene was then

sequenced and results were translated using the "Translate tool" program (http://www.expasy.ch/tools/dna.html). The translated sequence was then aligned with orthologous plant genes using ClustalW (http://www.ebi.ac.uk/clustalw/). As shown in Figure 11a, *N. benthamiana* shows strong sequence identity with the tobacco (*N. tabacum*) ortholog with an alignment score of 96 followed by tomato (score of 89), potato (89), rice (68), Arabidopsis (67) and wheat (66). *N. benthamiana* also displays a higher degree of evolutionary relatedness with the tobacco ortholog when compared to other plant orthologs that were analyzed (Figure 11b).

```
Tob 11    TTTCTCCATTCTCCACAGAAAAA-TACAAATGGAAAACAACTCCATTCTCTAAAGCAAC   68
          |||||||||| | |||||||||| |  |||||||| ||||||| |||||||||||||||
Tom 32    TTTCTCCATTTTTCACAGAAAAAATTGAAATGGAATACAACTCTATTCTCTAAAGCAAC   90

Tob 116   ATGGCGGCAGTTACTTCGGTATCGTTCTCGGCGATTTCTCAATCCGCCGAAAGGAAATCA  175
          |||||||| |||||||| ||||| |||||||||||| ||||||| || ||||||||||| 
Tom 134   ATGGCGGCTGTTACTTCAGTATCTTTCTCGGCGATTACTCAATCGGCGGAAAGGAAATCC  193

Tob 176   TCCGTTTCTCCGTCTCGTTATGTTGATACATTTAGGTTCCGTTCCAACGTCTCCTTTGAT  235
          ||||||||| ||||||||| | |||||||||||||||||||||||||| |||||||||||
Tom 194   TCCGTTTCTTCGTCTCGTTCTATTGATACATTTAGGTTCCGTTCCAACTTCTCCTTTGAT  253

Tob 236   TGTTTCAATGTTCGATCTTCAAATT/---/TGAGGCTGTCACAAAATGCTTGGGAGATTA  990
          |   |||||||||||||||| ||||     |||| |||| ||||||||||||||||| ||
Tom 254   TCCGTCAATGTTCGATCTTCCAATT/---/TGAGACTGTGACAAAATGCTTGGGAGACTA  996

Tob 991   CCAATATGCTGGGAGGTAAGGCAAAGATCATTTTGTCCTCCTGCTTGGACCTCATTGACT  1050
          ||||||||||||||||||||| |   |||||||||| ||||||| |||||||||||||||
Tom 997   CCAATATGCTGGGAGGTAAGGGAGTCATCATTTTGTACTCCTGCCTGGACCTCATTGACT  1056

Tob 1051  TGTTTCACTA/---/ATTTGTAGATAA  1242
          |||| |||||      |||||||||||
Tom 1057  TGTTCCACTA/---/GTTTGTAGATAA  1248
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Figure 10. Pairwise alignment of full length tobacco (*N. tabacum* TC10126) and tomato (TC178313) orthologous sequences of *28C12*(*ALC1*) using BLAST (bl2seq) program from NCBI. For design of primer pairs to generate a full length *ALC1* sequence, consensus sequences upstream and downstream of the coding region were chosen (sequences with yellow background). The coding region is highlighted with green background. The symbol /---/ indicates the continuity of the sequences. Sequence source: TIGR database (www.tigr.org).

(a)

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N.benthamiana   --MAAVTSVSFSAIAQSAERKSSVSPSRSVDTFRFRSNVSIDCFNVRSSNSSFSSNPSTS
Tobacco         --MAAVTSVSFSAISQSAERKSSVSPSRYVDTFRFRSNVSFDCFNVRSSNSSFSSNSSTS
Tomato          --MAAVTSVSFSAITQSAERKSSVSSSRSIDTFRFRSNFSFDSVNVRSSN-------STS
Potato          --MAAVTSVSFSAITQSAERKSSVSSSRSIDTFRFRSNFSFDSVNVRSSN-------STS
Arabidopsis     MAATAISSLSFPALGQSDKISNFASSRPLASAIRICTKFSRLSLNSR----------STS
Rice            --MAAISSLPFAALRRAADCR----PSTAAAAGAGAGAVVLSVRPR----------RGS
Wheat           --MAAISSLPFAALRRAADWR----PSTATAAVSV-SGGVMLNARAR----------RGS

N.benthamiana   RFVVHCMS---TDLPTVAETKMNFLKAYKRPIPTVYNTVLQELIVQQHLIKYKKSYRYDP
Tobacco         RFVVHCMS---TDLPTVAETKMNFLKAYKRPIPTVYNTVLQELIVLQHLIKYKKSYRYDP
Tomato          RFVVHCTSSSAADLPTVADTKMKFLTAYKRPIPTVYNTVLQELIVQQHLTRYKKSYQYDP
Potato          RFVVHCTSSSAADLPTVADTKLKFLTAYKRPIPTVYNTVLQELIVQQHLTRYKKSYQYDP
Arabidopsis     KSLIHCMSNVTADVPPVSETKSKFLKAYKRPIPSIYNTVLQELIVQQHLMRYKKTYRYDP
Rice            RSVVRCVATAGDVPPTVAETKMNFLKSYKRPILSIYSTVLQELLVQQHLMRYKTTYQYDA
Wheat           RSVVRCVATAGDIPPTVADTKMNFLKSYKRPIPSIYSTVLQELLVQQHLMRYKSTYQYDP

N.benthamiana   VFALGFVTVYDQLMEGYPSEEDRDAIFKAYIEALNEDPVQYRADAQKFEEWARTQNANTL
Tobacco         VFALGFVTVYDQLMEGYPSEEDRDAIFKAYIEALNEDPVQYRADAQKFEEWARTQNANTL
Tomato          VFALGFVTVYDQLMEGYPSEEDRNAIFKAYVEALKEDPEQYRADAQKLEEWARTQNANTL
Potato          VFALGFVTVYDQLMEGYPSEEDRNAIFKAYIEALKEDPEQYRADAQKLEEWARTQNANTL
Arabidopsis     VFALGFVTVYDQLMEGYPSDQDRDAIFKAYIEALNEDPKQYRIDAQKMEEWARSQTSASL
Rice            VFALGFVTVYDQLMEGYPSNEDRDAIFKAYITALNEDPEQYRADAQKMEEWARSQNGNSL
Wheat           VFALGFVTVYDQLMEGYPSTEDRDAIFKSYVTALNEDPEQYRADAQRMEEWARSQNGNLL

N.benthamiana   VDFSSRDGEVENILKDIAQRAGTKDSFCYSRLFAVGLFRLLELANVTDPTILEKLCASLN
Tobacco         VDFSSRDGEVENILKDIAQRAGTKNSFCYSRLFAVGLFRLLELANVTDPTILEKLCASLN
Tomato          VDFSSKDGEIENIFKDIAQRAGTKDGFCYSRLFAVGLFRLLELANVTDPTILEKLCAALN
Potato          VDFSSKEGEIENIFKDIAQRAGTKDGFCYSRLFAVGLFRLLELANVTDPTILEKLCAALN
Arabidopsis     VDFSSKEGDIEAVLKDIAGRAGSKEGFSYSRFFAVGLFRLLELASATDPTVLDKLCASLN
Rice            VEFSSKDGEIEAILKDISERAQGKGSFSYSRFFAVGLFRLLELANATEPTILDKLCAALN
Wheat           VEFSSRDGEIESILKDISERAQGKGNFSYSRFFAVGLFRLLELSNATEPTVLDKLCAALN

N.benthamiana   INKKSVDRDLDVYRNLLSKLVQAKELLKEYVEREKKKRGERE-SQKANEAVTKCLGDYQY
Tobacco         TDKKSVDRDLDVYRNLLSKLVQAKELLKEYVEREKKKRGERE-SQKASEAVTKCLGDYQY
Tomato          VNKKSVDRDLDVYRNLLSKLVQAKELLKEYVEREKKKRGERE-TQKANETVTKCLGDYQY
Potato          VNKKSVDRDLDVYRNLLSKLVQAKELLKEYVEREKKKRGERE-TQKANETVTKCLGDYQY
Arabidopsis     INKKSVDRDLDVYRNLLSKLVQANELLKEYVEREKKKQEERAQSQKANETISKCLGDTLY
Rice            INKRSVDRDLDVYRNILSKLVQAKELLKEYVEREKKKREERSETPKSNEAVTKFDGSLNS
Wheat           INKKSVDRDLDVYRNLLSKLVQAKELLKEYIEREKKKREERLETPKPNEAVAKFDGSAYP

N.benthamiana   AGR-------   Alignment scores
Tobacco         AGR-------      96
Tomato          AGR-------      89
Potato          AGR-------      89
Arabidopsis     NPSFLVERKS      67
Rice            MRH-------      68
Wheat           LKH-------      66
```

(b)

N. benthamiana
Tobacco
Tomato
Potato
Arabidopsis
Rice
Wheat

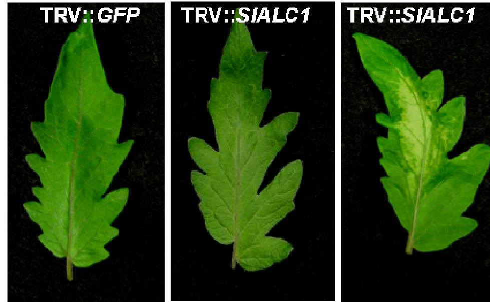
Figure 11. Comparative analysis of the translated sequence of *ALC1* from *N. benthamiana* (Genbank EU106046) with orthologs in tobacco (*N. tabacum,* TIGR: TC10126), tomato (*S. lycopersicon,* TIGR: TC162724), potato (*S. tuberosum,* Genbank ID AY342161), Arabidopsis (*THF1*, Genbank AY899908), rice (*Oryza sativa,* inositol phosphatase-like protein; Genbank AY224446) and wheat (*Triticum aestivum,* Ptr ToxA binding protein. Genbank AY377991). (a) A multiple sequence alignment program (ClustalW) was used to align the sequences. Identical amino acid residues are shaded in blue. Residues with conserved or semi-conserved substitutions are highlighted in green and yellow, respectively. (b) A phylogram of the sequences was generated using PHYLIP TREE and shows the evolutionary distance of all the homologs in panel (a) relative to *ALC1*.

ALC1-silenced tomatoes show necrosis in response to COR or _Pst_ DC3000

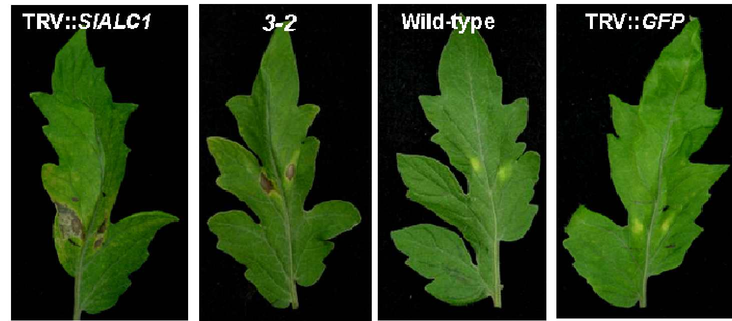
To understand the potential role of *Nb28C12* in host-pathogen interaction, we decided to use tomato for further study. Tomato, unlike *N. benthamiana*, is infected by *Pst* DC3000. Furthermore, purified COR induces chlorosis when applied to tomato leaves (Palmer and Bender, 1995; Zhao *et al*., 2003; Uppalapati *et al*., 2005). A tomato ortholog of *ALC1* was identified using a BLASTn search against a tomato database (www.tigr.org, TC162724). Using primers specific to the tomato sequence, we cloned the tomato ortholog of *ALC1* (see Experimental Procedures), which we refer to as *SlALC1*. A fragment of *SlALC1* was subcloned into pTRV2 and used for VIGS in tomato. qRT-PCR analysis of silenced tomatoes revealed that the relative expression ratio of *SlALC1* transcripts was 0.061 (about four-fold lower) in the silenced plants when compared to mock-inoculated (TRV::*GFP*) plants (Figure 9). Although the majority of leaves in *SlALC1*-silenced tomatoes did not exhibit an obvious phenotype (Figure 12a, middle panel), some of the older leaves showed variegated coloration on the leaf surface (Figure 12a, right panel). When purified COR (2 nmol) was exogenously applied, the silenced line showed a necrotic phenotype with little or no chlorosis (Figure 10b, left panel).

To study the influence of *SlALC1* on the virulence of *Pst* DC3000 in tomato*,* *SlALC1* silenced and control (TRV::*GFP*) tomato plants were spray-inoculated with *Pst* DC3000 (10^8CFU/ml). Control (TRV::*GFP*) plants showed typical bacterial speck symptoms at 5 dpi, which consisted of necrotic lesions surrounded by chlorotic halos (Figure 12c, left panel). At 5 dpi, the leaves of *SlALC1*-silenced plants showed necrosis

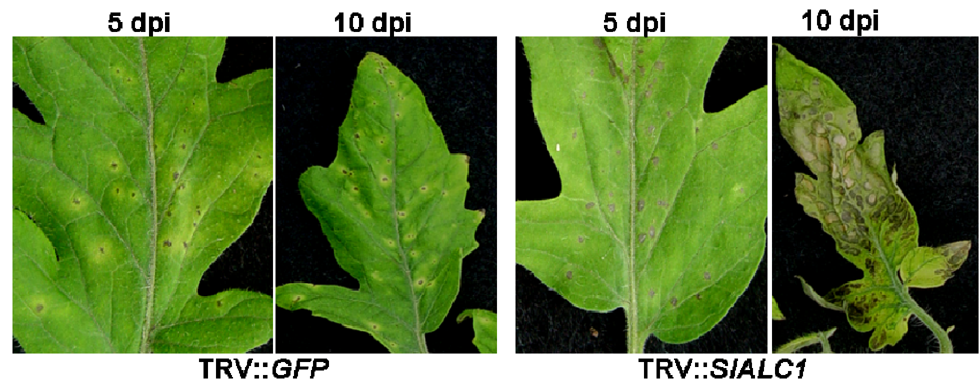
(a)



(b)



(c)



(d)

(e)

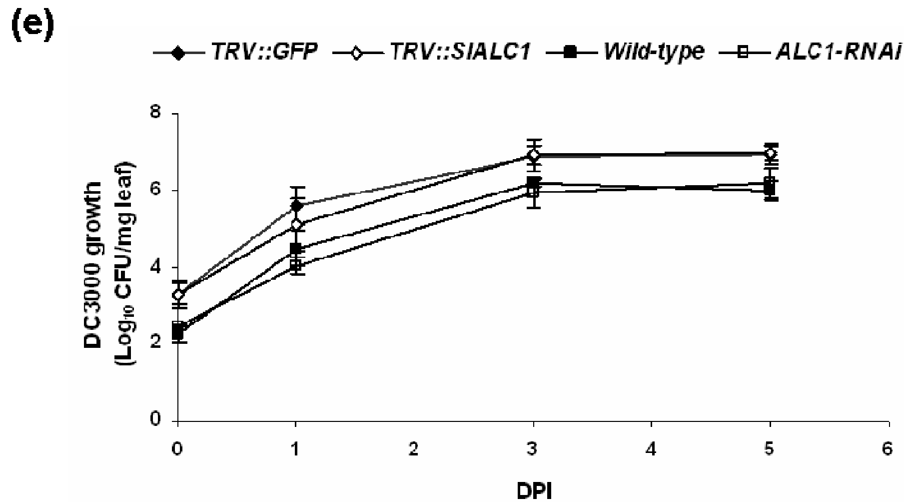


Figure 12. Phenotype of wild-type and silenced tomato lines. (a) Tomato leaves silenced with TRV::*GFP* (control, left) and *SlALC1* (middle and right panel) five weeks after agro-inoculation. The *SlALC1* silenced lines largely appeared similar to that of the wild-type plants (middle panel), however, a few leaves showed a variegated phenotype (right panel). (b) Phenotype of tomato lines inoculated with 2 nmol COR. Transiently (TRV::*SlALC1*) and stably silenced (*3-2*) tomato lines show necrosis in response to COR; whereas wild-type and vector control (e.g. TRV::*GFP*) lines show chlorosis. (c) Response of control and transiently silenced tomato lines to *Pst* DC3000. (d) Response of wild-type and stably silenced tomato lines to *Pst* DC3000. (e) Population dynamics of *Pst* DC3000 on wild-type and silenced tomato lines. All experiments were repeated at least twice, and the data shown are representative of each experiment.

with little or no chlorosis (Figure 12c). At 10 dpi, the necrosis observed on the silenced plants was severe (Figure 12c, right panel).

As noted above, severe necrosis was observed in transiently (TRV::*SlALC1*) silenced plants in response to *Pst* DC3000 (Figure 12c). To determine if this could be explained by a higher amount of bacterial growth in silenced lines, the population of *Pst* DC3000 was monitored at 1, 3 and 5 dpi. Interestingly, the bacterial population on the silenced plants was not significantly different from that on the inoculated control (TRV::*GFP*) (Figure 12e). These results suggest that *SlALC1*-mediated chlorosis does not have a significant effect on growth of the bacteria in tomato plants.

Although we were fairly successful in transiently silencing the tomato *SlALC1* gene, a uniform and pronounced silencing, such as that observed in *N. benthamiana,* is often difficult to achieve in tomato (Ekengren *et al.,* 2003; Ryu *et al.*, 2004). It is therefore difficult to assess the extent of silencing especially if the silencing does not result in any appreciable phenotypic change in the silenced plants (Liu *et al*., 2002a; Ryu *et al*., 2004). Therefore, to achieve stable, uniform silencing and to confirm the necrosis phenotype induced by COR and *Pst* DC3000 on *SlALC1*-silenced tomato lines, we generated *SlALC1* RNAi lines. We assayed three independent transgenic RNAi lines and all responded similarly to COR application and *Pst* DC3000 infection. Here, we discuss the data for one of the transgenic, stably silenced lines, *3-2.* Results obtained from qRT-PCR indicated the transcript levels of *SlALC1* were five-fold less in RNAi line *3-2* when compared to wild-type tomato plants (data not shown). When COR (2 nmol) was applied to the leaves of the silenced line *3-2*, necrosis appeared five days later (Figure 12b, second panel from left). When line *3-2* was inoculated with *Pst* DC3000 (10^8 CFU/ml), leaves developed necrotic lesions without chlorosis (Figure 12d), further confirming that *SlALC1* is required for COR-induced chlorosis. Consistent with the results obtained with VIGS, there was no difference in the growth of *Pst* DC3000 in the RNAi line *3-2* and wild-type tomato (Figure 12e).

Arabidopsis *thf1* mutant displays necrosis without chlorosis upon *Pst* DC3000 inoculation

As mentioned above, *ALC1* is closely related to an Arabidopsis gene called *THF1* (Figure 11a). An Arabidopsis *thf1* mutant has been previously identified and shown to

have variegated leaves (Figure 13), which were shown to lack normal chloroplast development in the variegated regions (Wang *et al.,* 2004). Additionally, *THF1* expression in leaves was positively regulated by light (Wang *et al.,* 2004). Another study showed that *THF1* is expressed throughout Arabidopsis, including seedlings, hypocotyls, flowers and roots (Huang *et al*., 2006).

Figure 13. Phenotype of the Arabidopsis *thf1* mutant: The mutant *thf1* grew slower than the wild-type Col-0 and the foliage was variegated in coloration (inset). On maturity, the mutant line attained Col-0 size (data not shown).

We obtained the Arabidopsis *thf1* mutant and reconfirmed the mutation by ascertaining the insertion of T-DNA in *THF1* (data not shown). Unlike *N. benthamiana* and tomato, exogenous application COR on Arabidopsis leaves does not induce chlorosis. Instead, Arabidopsis seedlings respond to COR by displaying a strong purple hue indicative of anthocyanin accumulation (Bent *et al*., 1992; Laurie-Berry *et al*., 2006). To further characterize the *thf1* mutant, we germinated seeds of Arabidopsis Col-0, the *thf1* mutant line, the *thf1* line complemented with *THF1*, and a *THF1*-overexpressing line on half strength MS medium containing 2 nmol COR (Laurie-Berry *et al*., 2006). As expected, Col-0 seedlings showed anthocyanin accumulation within ten days after germination (Figure 14a). Strikingly, the *thf1* mutant showed hypersensitivity to COR by

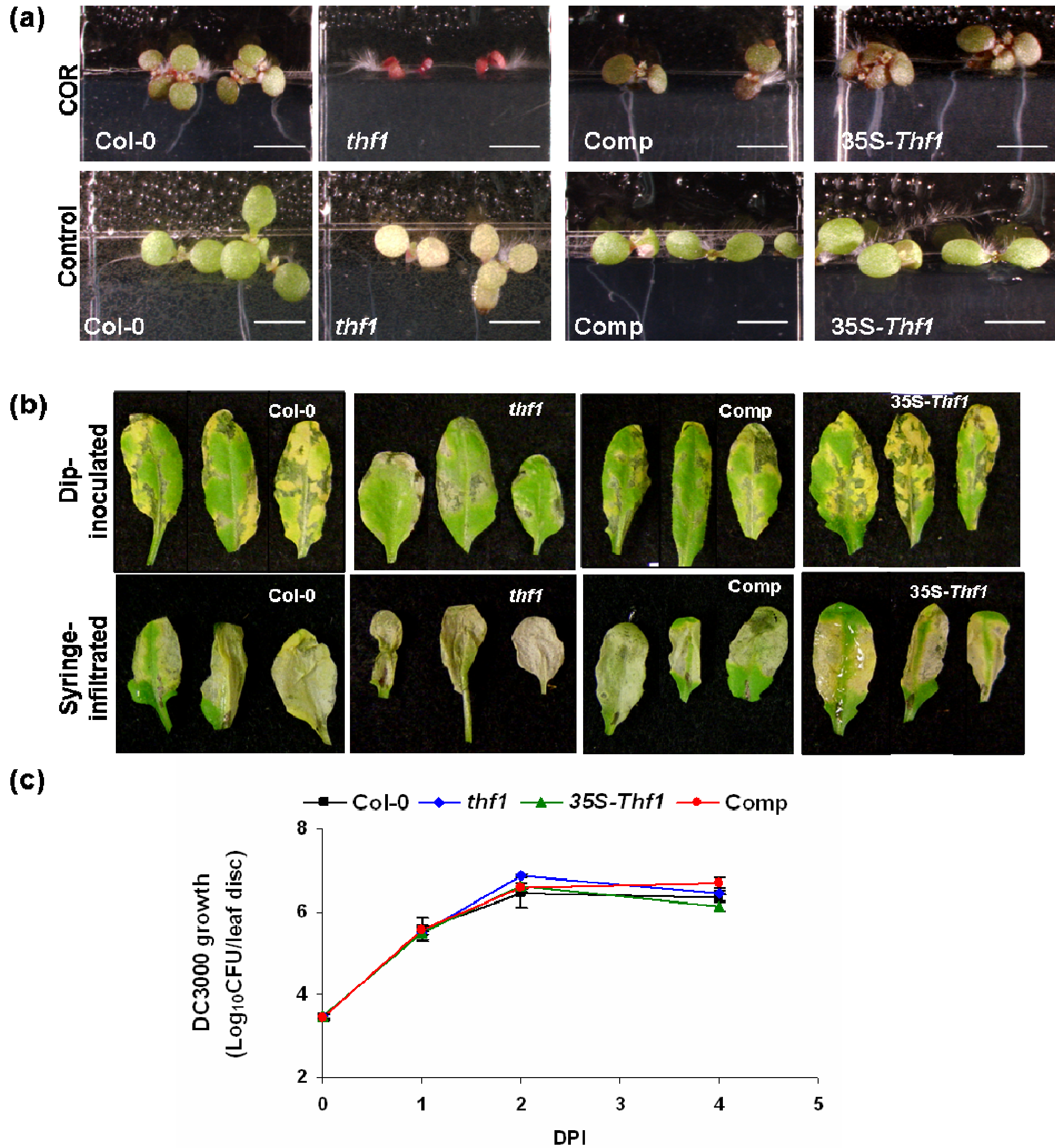
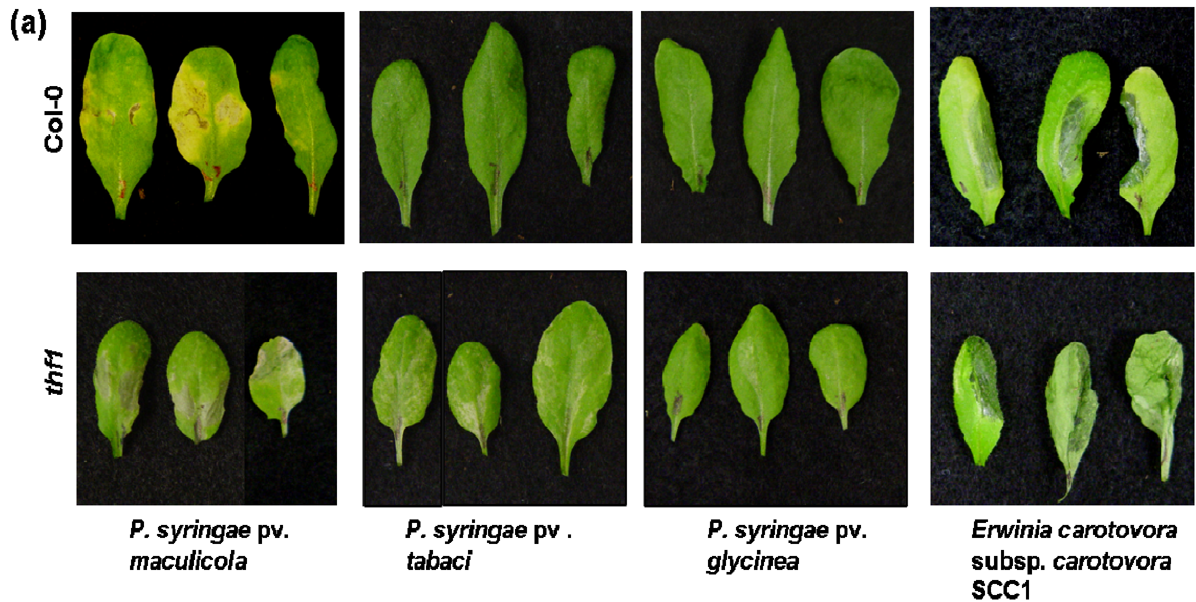


Figure 14. Arabidopsis *thf1* displays hypersensitivity in the presence of COR and produces necrosis with no chlorosis upon *Pst* DC3000 inoculation. (a) Arabidopsis wild-type Col-0, the *thf1* mutant line, the complemented line of *thf1* (Comp), and the *THF1* overexpression line (35S-*Thf1*) were germinated on half strength MS medium containing 2 nmol COR (upper panels). These lines were also germinated on half strength MS medium alone without COR (lower panels). Photos were taken of 10-day old seedlings, and each scale bar indicates 3 mm. (b) Foliage of four-week old Arabidopsis lines were either dipped in a *Pst* DC3000 culture suspension (10^8 CFU/ml; upper panel) or infiltrated with (10^6 CFU/ml, using a needle-less syringe, lower panel). Photos were taken 6 days post inoculation. (c) Arabidopsis leaves infiltrated with *Pst* DC3000 (b, lower panel) were homogenized in water and were then plated on KB medium, and the bacterial population was quantified. All experiments were conducted at least three times, and the data are representative of each experiment.

displaying a severe growth defect and more visible anthocyanin accumulation than Col-0 (Figure 14a, second panel from left). Both the *THF1* complemented and overexpressing lines showed phenotypes similar to the wild-type (Figure 14a).

To determine whether *THF1* has an effect on *Pst* DC3000-induced disease symptoms on Arabidopsis, we dip-inoculated (10^8 CFU/ml) and syringe-infiltrated (10^6 CFU/ml) the wild-type Col-0 and *thf1* mutant with *Pst* DC3000. As expected, Col-0 showed water-soaked necrotic lesions accompanied by chlorosis (Figure 14b). However, the *thf1* mutant plants exhibited necrotic lesions lacking chlorosis (Figure 14b). Complemented lines of the *thf1* mutant and *THF1*-overexpressing plants displayed disease symptoms similar to wild-type Col-0 after inoculation with *Pst* DC3000 (Figure 14b). These results clearly indicate that *THF1* is required for *Pst* DC3000-induced chlorosis. Interestingly, when the growth of *Pst* DC3000 was monitored at 0, 1, 2 and 4 dpi, no significant differences in bacterial growth were observed between the wild-type Col-0, the *thf1* mutant, the complemented line of *thf1*, and the *THF1* overexpression line (Figure 14c). These results suggest that *Pst* DC3000-induced chlorosis does not contribute to the overall fitness of the pathogen, at least for the duration of time the bacterial growth was monitored.

Necrosis occurred much earlier on *Pst* DC3000-infected *thf1* leaves than on leaves of the wild-type Col-0. We therefore investigated whether the *thf1* mutant had a weaker defense response and was more susceptible to biotic and abiotic stress because of defects in thylakoid formation (Wang *et al.,* 2004). To investigate this, leaves of Col-0 and the *thf1* mutant were infiltrated with two pathogens that do not infect Arabipdopsis, *P. syringae* pv. *tabaci* and *P. syringae* pv. *glycinea,* and growth and symptoms were



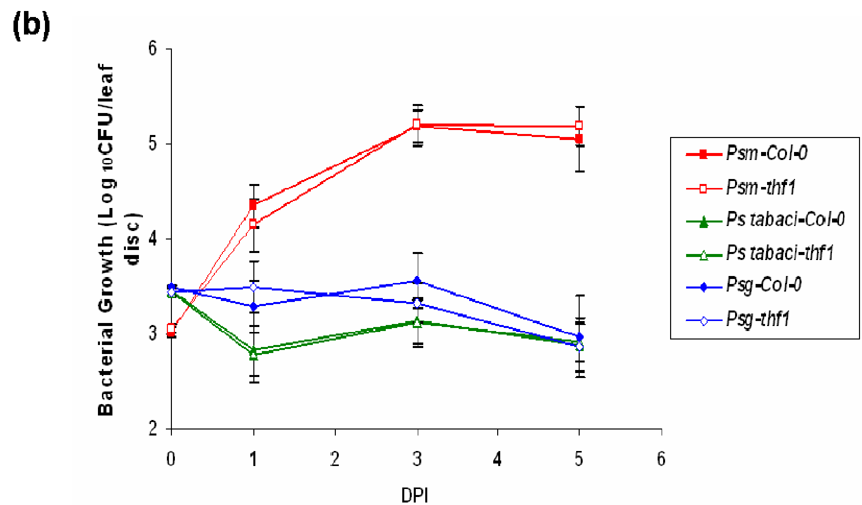


Figure 15. Response of Arabidopsis *thf1* mutant line to other pathogens (*P. syringae* pv. *maculicola* and *Erwinia carotovora* subsp. *carotovora*) and non-pathogens (*P. syringae* pvs. *tabaci* and *glycinea*): (a) Wild-type Arabidopsis Col-0 and the *thf1* mutant were syringe-infiltrated with pathogens (5 x 10^5 CFU/ml) and non-pathogens (10^6 CFU/ml), and photos were taken 5 dpi. (b) Growth of *P. syringae* pvs. *maculicola, tabaci and glycinea* on Co1-0 and the *thf1* mutant line 0, 1, 3 and 5 dpi. Inoculated samples were homogenized in water and plated on KB medium supplemented with antibiotics when necessary.

compared with *P. syringae* pv. *maculicola*, which is pathogenic to Arabidopsis (Dong *et al.,* 1991; Mishina and Zeier, 2006). As expected, the population of *P. syringae* pv. *maculicola* increased approximately 100-fold on both Col-0 and *thf1* leaves by 3 dpi;

however, neither *P. syringae* pv. *glycinea* or *tabaci* multiplied on Col-0 or *thf1* plants (Figure 15b) .

Arabidopsis Col-0 and the *thf1* mutant were also monitored for symptom development in response to inoculation with *P. syringae* pvs. *maculicola*, *glycinea* and *tabaci* and the soft rot pathogen *Erwinia carotovora* subsp. *carotovora*. *P. syringae* pv. *maculicola* induced chlorosis on Col-0 but not on *thf1* mutant line (Figure 15a). Neither Col-0 nor *thf1* plants developed visible symptoms in response to *P. syringae* pvs. *tabaci* or *glycinea* (Figure 15a). *E. carotovora* subsp. *carotovora* induced soft rot on both Col-0 and *thf1* with no apparent difference in phenotypic response between the wild-type and the mutant line. Infiltration of leaves with cell death inducing agents such as NaCl (500 mM) or H_2O_2 (3%) (Peart *et al.,* 2002; Kang *et al.,* 2004) caused similar cell death response on both Col-0 and *thf1* mutant line (data not shown). From the above experiments, only *P. syringae* pv. *maculicola* induced unique response on *thf1* when compared to Col-0. This response was similar to the one induced by *Pst* DC3000. The results indicate that the early death of the infected leaf tissues observed in the *thf1* mutant as a result of severe necrotic lesion is specific to *Pst* DC3000 and *P. syringae* pv. *maculicola*.

***Pst* DC3000-induced chlorosis is associated with senescence**

The results obtained in pathogenicity studies (Figure 14c and 15b) indicated that early death in the *thf1* mutant line was not due to elevated bacterial growth. Thus we speculated that the early death observed in leaves of the *thf1* mutant was somehow associated with pathogen-induced chlorosis. Since chlorosis is associated with

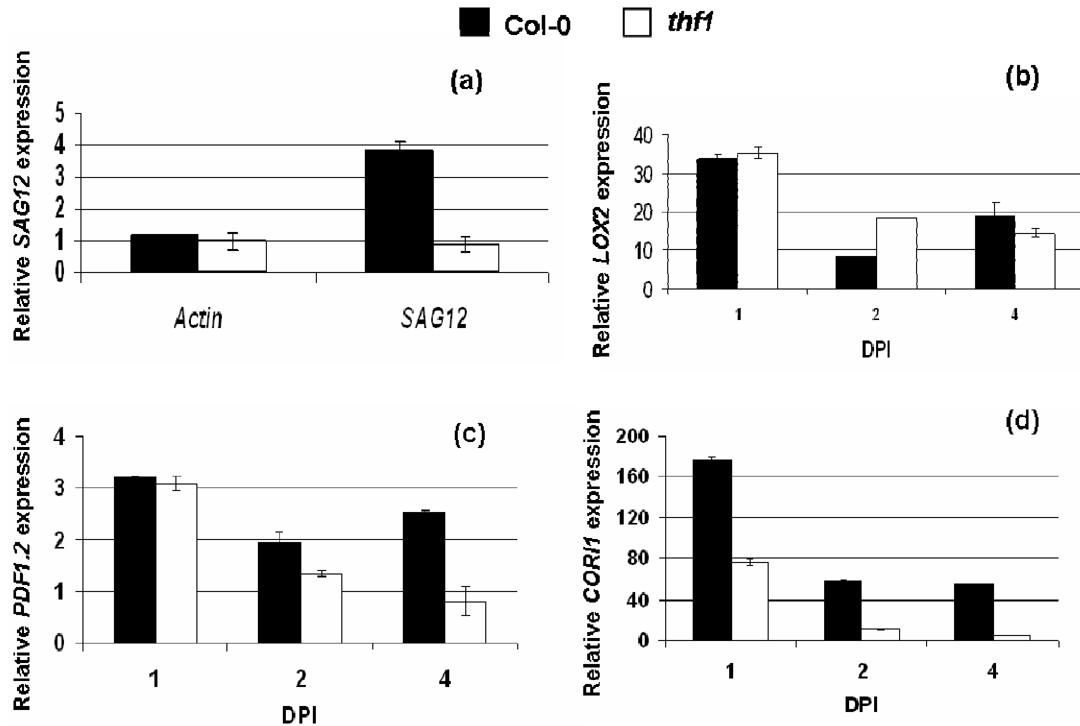


Figure 16. Arabidopsis *THF1* is required for the induction of senescence in response to *Pst* DC3000. For real time qRT-PCR, approximately 4-week old wild-type Col-0 and the *thf1* mutant line were syringe-infiltrated with *Pst* DC3000 (10^6 CFU/ml). Total RNA was extracted from the leaves of the infected plants collected 1, 2 and 4 dpi. Control samples (mock inoculation) were collected on the day of inoculation. Gene specific primers were used for amplification of the *SAG12, LOX2, PDF1.2* and *CORI1* transcripts. The transcript levels were normalized against the elongation factor EF1α that was used as endogenous control as described by Pfaffl (2001). (a) qRT-PCR showing the induction of the senescence-associated gene, *SAG12*, in the Col-0 and *thf1* mutant lines at 4 dpi. Actin, a house-keeping gene, was used to ascertain that the lack of expression of *SAG12* in the *thf1* mutant was not due to RNA degradation. (b-d) Expression of JA pathway marker genes (*LOX2* and *PDF1.2*) and (d) *CORI1* (encoding chlorophyllase) are activated in *Pst* DC3000-infected Col-0 and the *thf1* mutant. All experiments were repeated at least three times. The data shown here represent the average of three biological replicates and three technical replicates with the standard deviation values shown as the error bars.

senescence (Quirino *et al.*, 2000) and because JA (a functional analog of COR) induced premature senescence in Arabidopsis (He *et al.*, 2002), we investigated whether the senescence-associated gene *SAG12* was differentially expressed in the *thf1* mutant in response to *Pst* DC3000. *SAG12* encodes a cysteine protease that is expressed only in senescing tissues (Noh and Amasino, 1999a, b); furthermore, *SAG12* is activated during methyl jasmonate (MeJA)-induced senescence in Arabidopsis leaves (He *et al.*, 2002).

Using qRT-PCR, we analyzed the transcript level of *SAG12* four days after infiltration with *Pst* DC3000. Infected leaves of Col-0 expressed approximately four-fold higher levels of *SAG12* (Figure 16a) as compared to the *thf1* mutant line.

One possible explanation for the reduced level of *SAG12* transcripts (as shown in Figure 16a) was the possible degradation of RNA in the *Pst* DC3000-infected *thf1* mutant as a result of premature death. Thus we measured the transcript levels of the actin house-keeping gene. There was no difference in the actin transcript levels between Col-0 and *thf1* infected with *Pst* DC3000 4 dpi (Figure 16a). Additionally, we ran an agarose gel loaded with 2 µg aliquots of total RNA from all samples examined for qRT-PCR. Visual examination of the gel indicated that all of the RNA samples appeared intact (data not shown). The results clearly suggest that *Pst* DC3000-induced chlorosis is directly associated with senescence.

The JA pathway appears intact in *thf1* mutant plants after *Pst* DC3000 inoculation

By using a JA-insensitive Arabidopsis mutant, *coronatine insensitive1* (*coi1*), He *et al*. (2002) demonstrated that JA signaling pathway is required for the promotion of senescence in Arabidopsis. The study also indicated that senescing leaves show high level of *SAG12* accumulation. Thus, it remained possible that the absence of chlorosis and reduced levels of *SAG12* transcripts in the *Pst* DC3000-infected *thf1* mutant was due to disruption of the JA- dependent signaling pathway. Thus, we used qRT-PCR to analyze transcript levels of *Lipoxygenase2* (*LOX2*) and *Plant defensin1.2* (*PDF1.2*). Transcripts of *LOX2* and *PDF1.2* were induced in both Col-0 and *thf1* in response to *Pst*

DC3000. Although expression of both genes was lower in *thf1*, especially at 4 dpi, the JA pathway appears to be functional (Figure 16b, c) at the time points analyzed.

Chlorosis occurs due to the degradation of proteins in the chloroplast (Quirino *et al*., 2000), and the Arabidopsis *CORI1* gene (encoding chlorophyllase), is induced upon COR or MeJA application (Benedetti *et al.,* 1998), resulting in chlorophyll degradation (Benedetti and Arruda, 2002). We wanted to know if the lack of chlorosis in *thf1* could be due to repression of *CORI1* as a result of loss of *THF1* function. Thus, we analyzed *CORI1* transcript levels in *Pst* DC3000 inoculated Col-0 and *thf1* plants. Relative to Col-0, *CORI1* expression in *thf1* was lower; however, induction of *COR11* in the *thf1* mutant was ~75 fold lower compared to the basal expression level (Figure 16d). These results suggest that the JA dependent pathway is not affected in the *thf1* mutant.

DISCUSSION

COR produces visible chlorosis when applied to tomato, collard and turnip leaves (Uppalapati *et al.,* 2005; Elizabeth and Bender, 2007). However, in Arabidopsis COR does not produce chlorosis; instead it induces anthocyanin accumulation in seedlings (Bent *et al.,* 1992). Depending on the host and tissue, COR also induces hypertrophy in potato, inhibits root elongation, and stimulates ethylene production (Feys *et al.,* 1994; Bender *et al.,* 1999). COR contributes to the virulence of *Pst* DC3000 in Arabidopsis, tomato, collard and turnip (Brooks *et al*., 2004; Uppalapati *et al*., 2005; Elizabeth and Bender, 2007) and functions as a virulence factor in other *P. syringae* pathovars including pv. *atropurpurea, glycinea,* and *maculicola*, which infect ryegrass, soybean, and crucifers, and respectively (Bender *et al.,* 1999). More recently, COR was shown to

induce the opening of stomata to promote the entry of pathogen into the host (Melotto *et al.,* 2006).

Our observation that COR also induced chlorosis on *N. benthamiana* provided a perfect tool to identify plant genes, by application of VIGS, that play a role in COR and/or *Pst* DC3000 induced chlorosis. Here, we have clearly shown that the *N. benthamiana* gene *ALC1* and its orthologs, *SlALC1* in tomato and *THF1* in Arabidopsis, are required for the induction of chlorosis in response to COR and/or *Pst* DC3000. Furthermore, based on genetic studies we performed on the Arabidopsis mutant line *thf1*, we speculate that *Pst* DC3000-induced chlorosis is associated with premature senescence of the plant. Spray-inoculation of *Pst* DC3000 on *ALC1* silenced tomatoes induced necrotic lesions without chlorosis on the majority of the leaves instead of the typical bacterial speck symptom with a chlorotic halo (Mittal and Davis, 1995; Zhao *et al*., 2003). Furthermore, necrosis appears to progress beyond the infected areas (Figure 12c right panel) in a similar fashion as runaway cell death phenotype that has been reported earlier in Arabidopsis *lsd1* mutant (Jabs *et al.,* 1996). Arabidopsis mutants that die earlier than the wild-type in response to *P. syringae* infection include the *npr1* (Cao *et al.,* 1994) and *acd5* (Greenberg *et al.,* 2000). Interestingly, these mutants harbored more bacteria. In our study, evaluation of *Pst* DC3000 multiplication did not indicate a higher number of bacteria on *ALC1*-silenced tomato plants. This suggests that the early death induced by the pathogen is caused by reasons other than the overwhelming growth of the pathogen.

To determine the role of *ALC1* in the development of symptoms in response to COR or *Pst* DC3000, we chose Arabidopsis since it is genetically tractable and a host of *Pst* DC3000. The ortholog of *ALC1* in Arabidopsis, known as *THF1,* is a single-copy

gene with no closely related sequences in the Arabidopsis genome (Wang *et al.,* 2004). A Genbank database sequence query indicated that *THF1* orthologs are present almost exclusively in photosynthetic organisms. Expression of the Arabidopsis *THF1* gene is positively regulated by light, and the gene product is a chloroplast-localized protein. A T-DNA knockout mutant of *THF1* has been previously identified (Wang *et al*., 2004). The mutant line, *thf1,* is slightly stunted and has variegated leaves. The ultrastructure of chloroplasts in the *thf1* mutant shows shorter stacks of thylakoids in the green sector of the leaves and accumulation of membrane vesicles, but no thylakoids within the intact chloroplast membrane in the white sector of leaves (Wang *et al*., 2004). A recent study showed that *THF1* is ubiquitously expressed in Arabidopsis (Huang *et al.,* 2006). Unlike leaves, expression of *THF1* in roots is light-independent, THF1 interacts with GPA1*,* a component of the heterotrimeric G-protein complex, possibly through two transmembrane domains that span into the cytosol (Huang *et al*., 2006). Degradation of THF1 in presence of D-glucose and the epistatic relationship between the mutant lines, *thf1-1* and *gpa1-4*, which are hypersensitive to D-glucose, suggest that THF1 functions as the downstream partner of GPA1 in a G-protein-coupled sugar signaling pathway (Huang *et al.,* 2006).

Exogenous application of COR (2 nmol) on Arabidopsis leaves did not produce any chlorosis. However, consistent with earlier observations (Bent *et al.,* 1992; Feys *et al.,* 1994), our study showed that Arabidopsis seedlings grown on MS medium supplemented with COR accumulated anthocyanin. Interestingly, anthocyanin accumulation was significantly elevated in the *thf1* mutant as compared to the wild-type plants. Therefore, the *THF1* mutation has a positive effect on anthocyanin accumulation

in Arabidopsis. This contrasts with observations made in tomato where silencing of the homolog *SlALC1* negatively affected COR-induced chlorosis. These results are consistent with earlier an observation that COR induces different phenotypes in Arabidopsis and tomato (Mach *et al.,* 2001; Uppalapati *et al.,* 2005). It is possible that the induction of chlorosis in Arabidopsis seedlings is suppressed by anthocyanin accumulation. Furthermore, unlike Arabidopsis Col-0 where MeJA induces anthocyanin accumulation (Feys *et al.,* 1994), in the Arabidopsis *glabra1* (*gl1*) mutant, chlorosis is induced in response to MeJA (He *et al.,* 2002). *GL1* encodes a MYB-related transcription factor that positively regulates trichome initiation (Oppenheimer *et al.,* 1991). Another Arabidopsis gene required for trichome development, *TRANSPARENT TESTA GLABRA* (*TTG1*), is also required for anthocyanin production (Galway *et al.,* 1994). GL1 and TTG1 have been proposed to interact via GL3 to positively regulate trichome development (Schellmann and Hulskamp, 2005). Thus, it is plausible that *GL1* is required for anthocyanin accumulation and chlorosis is induced in the *gl1* mutant because the mutant cannot produce anthocyanin in the presence of MeJA. Thus, it is quite likely that the *gl1* mutant may display a chlorotic phenotype in response to COR.

Similar to *ALC1*-silenced tomato plants, inoculation of *thf1* with *Pst* DC3000 did not result in a typical chlorotic phenotype around the water-soaked lesion. Analysis of the *Pst* DC3000 population dynamics in the *thf1* mutant line indicated that there was no difference in the bacterial population dynamics between the wild-type Col-0 and the *thf1* mutant line. Interestingly, *Pst* DC3000-inoculated leaves of *ALC1*-silenced tomato and the Arabidopsis *thf1* mutant line died earlier than corresponding wild-type lines. Therefore, it is possible that *Pst* DC3000, a pathogen with hemi-biotrophic qualities, uses

ALC1/THF1 to gradually transition into a necrotrophic phase by reprogramming the plant to enter senescence. *P. syringae* pv. *maculicola* also induced symptoms on the *thf1* mutant similar to *Pst* DC3000, which is consistent with the fact that both are pathogenic on Arabidopsis and both produce coronatine (Dong *et al.,* 1991; Cuppels and Ainsworth, 1995). Furthermore*, P. syringae* pvs. *glycinea* and *tabaci*, which are not pathogenic to Arabidopsis, did not show any visible symptoms on Col-0 and *thf1*. Interestingly, *P. syringae* pv. *glycinea* produces coronatine (Cuppels and Ainsworth, 1995). However, its pathogenesis in Arabidopsis is largely dictated by the presence of the effector AvrB that interacts with the host *R* gene, *RPM1* (Ong and Innes, 2006). Mutation in either *avrB* or the host *R* gene leads to chlorosis (Nimchuk *et al.,* 2000; Ong and Innes, 2006).

Plant leaves undergo senescence at the terminal end of their developmental process. This is generally marked by chlorosis that represents the programmed degradation of the proteins in the chloroplast (Quirino *et al*., 2000). Senescence may also be induced by external factors such as environmental stress, nutrient supply and pathogen attack (Butt *et al*., 1998; Lim *et al*., 2003). In our study, we wanted to know whether *Pst* DC3000 induced chlorosis is associated with senescence. Several genes are up-regulated during senescence including *SAG12* (Quirino *et al.,* 2000; He *et al.,* 2002; Lim *et al.,* 2003). Our results showed that *SAG12* gene was up-regulated beginning 4-5 dpi in Col-0. However, this phenomenon was not observed in the *thf1* mutant, suggesting that *Pst* DC3000-triggered senescence is directly related to chlorosis.

Promotion of plant senescence by MeJA has been well-established (Ueda and Kato, 1980). Previously, MeJA induced senescence in the Arabidopsis *gl1* mutant, was represented by a chlorotic phenotype and *SAG12* accumulation, and was correlated with

the activation of JA-related marker genes such as *LOX* and *PDF1.2* (He *et al*., 2002). Additionally, the Arabidopsis mutant *coi1* in a Col-*gl1* did not show any chlorosis in response to MeJA, indicating that chlorosis is JA-mediated. To discover whether *SAG12* expression in the *Pst* DC3000-inoculated *thf1* mutant was lower because of defect in JA biosynthesis/perception; we looked at the expression levels of *LOX2* and *PDF1.2*. We observed elevated levels of *LOX2* and *PDF1.2* transcripts in both Col-0 and *thf1* that were inoculated with *Pst* DC3000, thus suggesting that the JA pathway is intact in the *thf1* mutant. We also looked at the expression level of the chlorophyllase gene, *CORI1* (also known as *AtCHL1*), that is inducible by COR or *Pst* DC3000 in Arabidopsis (Benedetti *et al*., 1998; Benedetti and Arruda, 2002; Laurie-Berry *et al*., 2006). Expression of *CORI1* was induced in both Col-0 and *thf1* upon inoculation with *Pst* DC3000. However, the induction of *COR11* was significantly higher in Col-0 when compared to *thf1*. Therefore, it appears that *THF1* is required for efficient chlorophyll degradation during *Pst* DC3000-induced chlorosis in Arabidopsis.

The photosynthetic electron transport system, a major source for reactive oxygen species (ROS), is present in the chloroplast (Foyer et al., 1994; Foyer and Noctor, 2003). The rapid accumulation of ROS in plant cells due to biotic and abiotic stress can exceed the capacity of the antioxidants to detoxify them. Therefore, efficient and tightly regulated degradation of chlorophyll is important in order to avoid the cellular damage caused by ROS (Matile *et al.,* 1999; Takamiya *et al.,* 2000). We speculate that in COR-treated or *Pst* DC3000-inoculated *ALC1* silenced tomatoes and in the *Pst* DC3000-inoculated Arabidopsis *thf1* mutant, the necrosis/HR-like cell death phenotype may appear because the effect of ROS supersedes the detoxifying capacity of antioxidants.

Moreover, a latter part of chlorophyll degradation takes place in the vacuole (Takamiya *et al.,* 2000). Thus we speculate that THF1, which is localized on the chloroplast membrane and stromules (Wang *et al.,* 2004), is somehow involved in the transport of the chlorophyll catabolic intermediates across the membrane for efficient and tightly-regulated degradation. Furthermore, SAG12 is located in the senescence-associated vacuoles that develop in the cytoplasm of the senescing leaves with intact chloroplasts (Otegui *et al.,* 2005) Thus, there may be a direct or indirect interaction between THF1 and SAG12. THF1 may play a signaling role during the transport of chloroplast proteins to the vacuoles containing proteases such as SAG12. The likelihood that THF1 plays a role in signaling stems from the fact that THF1 is an interacting partner in the G-protein mediated D-glucose signaling mechanism (Huang *et al.,* 2006). THF1 orthologs in other plants such as wheat ToxABP1 (Manning *et al.,* 2007) and rice inositol phosphatase-like protein that interacts with chilling-inducible protein (Cooper *et al.,* 2003) are potentially part of the signaling cascade during stress responses. Additionally, the inability to induce *SAG12* activation in the *thf1* mutant may suggest that SAG12 functions downstream of THF1.

Despite our current findings about the role of *ALC1/THF1* in pathogen-induced senescence, understanding its exact mechanism warrants further investigation. Current evidence suggests that *THF1* may have multiple functions (Huang *et al*., 2006). In leaves, *THF1* gene expression is light-dependent (Wang *et al*., 2004) and plays a role in the biogenesis of photosystem II; thus, the *thf1* mutant is sensitive to high light intensity (Keren *et al*., 2005). However, in Arabidopsis roots, the *THF1* expression is light independent and the gene product directly interacts with GPA1, the Gα part of the

heterotrimeric G-protein and is involved in the sugar signaling mechanism (Huang *et al*., 2006). Regulation of *Pst* DC3000 induced chlorosis may therefore be another unique function of *THF1*. Our results raise several questions. Does COR interact directly with ALC1/THF1 on the chloroplast membrane, or does COR induce chlorosis through a ALC1/THF1-mediated nuclear signal located downstream of other signaling molecules? Interestingly, a chloroplast protein in wheat, ToxABP1 (an ortholog of THF1; Figure 11), directly interacts with *Pyrenophora tritici-repentis* protein ToxA (Manning *et al*., 2007). ToxA is a determinant of virulence in *P. tritici-repentis*, a pathogen that causes the tan spot of wheat. Similar to our observation that *THF1* expression was not induced in the presence of pathogen, there was no difference between the expression level of ToxABP1 in ToxA sensitive and insensitive wheat (Manning *et al.,* 2007). Therefore (Uppalapati *et al.,* 2005; Thines *et al.,* 2007), it is possible that COR may also interact directly with ALC1/THF1 to induce senescence.

CHAPTER IV

Development of a high-throughput screening method for *Pseudomonas syringae* pv. *tomato* DC3000 pathogenicity using tomato seedlings

SUMMARY

Pseudomonas syringae pv. *tomato* DC3000 (*Pst* DC3000) is a plant pathogen that causes bacterial speck of tomato, an economically important disease. The interaction of tomato and Arabidopsis with *Pst* DC3000 are popular systems for analysis of pathogenesis and virulence. In the current study, we show that *Pst* DC3000 is a pathogen of tomato seedlings, an aspect of pathogen biology that has not been previously investigated. This finding resulted in the development of a virulence assay on tomato seedlings that has several advantages over labor-intensive foliar assays, including a shorter growth and incubation period, ease of inoculation and handling, and rapid generation of larger sample sizes per experiment. The utility of this assay was investigated by exploring the virulence functions of the Type III secretion system (TTSS) and coronatine (COR) on tomato seedlings. The current study also addresses the application of the seedling assay for the primary screening of *Pst* DC3000 virulence mutants. The seedling assay using defined *Pst* DC3000 mutants agreed well with results obtained by foliar inoculation of tomato plants. Furthermore, preliminary results using transcriptional fusions to the GUS reporter gene and tomato seedlings yielded gene

expression results that are consistent with those obtained in foliar inoculation of 3-4 week old tomato plants.

INTRODUCTION

Many pathogenic variants (pathovars or pv.) of *Pseudomonas syringae* cause diseases in plants and induce a wide variety of symptoms including blights, galls and leaf spots (Alfano and Collmer, 1996; Hirano and Upper, 2000). Bacterial speck disease caused by *Pst* is an economically important disease of tomato, and the pathogen causes necrotic lesions on the leaves, stems, and fruits of tomatoes (Goode and Sasser, 1980). Foliar lesions are generally surrounded by a yellow chlorotic halo due to production of the phytotoxin coronatine (COR) (Peñaloza-Vázquez, *et al.,* 2000). On tomato, fruit lesions are slightly raised and small, varying in size from tiny flecks to visible lesions approximately 3 mm in diameter (Bashan, 1980; Goode and Sasser, 1980) Fruit infection can lead to infestation of tomato seed, and *Pst* can survive on seeds, especially inside cavities present on the seed surface (Devash *et al*., 1980). Seedlings from infested seeds either develop visible disease symptoms or remain symptomless; in the latter case, asymptomatic plants grown under high relative humidity contain massive populations of the pathogen, thus serving as a reservoir for future infections (McCarter *et al*., 1983). Although several reports have shown that *Pst* is seed-borne (Bashan *et al*., 1982) and infects tomato seedlings (Gitaitis *et al*., 1992), these aspects of disease etiology and pathogen biology are not well understood.

Factors affecting the pathogenicity and virulence of *P. syringae*

The ability of *P. syringae* to cause disease in a host-specific manner led to investigations focusing on the molecular basis of host specificity (Lindgren *et al.*, 1986; Staskawicz *et al.*, 1984). Genetic studies using transposon mutants identified multiple genes that are induced during pathogenesis and are required for pathogenicity (Staskawicz *et al.,* 1984; Lindgren *et al.,* 1986; Collmer et al., 2000) or contribute to disease severity (e.g., virulence factors) (Mittal and Davis, 1995: Kloek *et al.,* 2001). In *Pst* DC3000, the type III secretion system (TTSS) determines pathogenicity (He, 1998; Galan and Collmer, 1999; Hutcheson *et al.,* 2001), and COR is an important component of *Pst* DC3000 virulence (Bender *et al.,* 1999). The TTSS and COR also play a major role in symptom production (Brooks *et al.,* 2004; Jin *et al.,* 2003). Other factors that contribute to the virulence of *Pst* DC3000 are exopolysaccharides (Yu *et al.,* 1999; Keith *et al.,* 2003) and a number of regulatory proteins including GacA, GacS, HrpL, CorR, and CorS (Hrabak and Willis, 1992; Rich *et al.,* 1994; Fouts *et al.,* 2002; Chatterjee *et al.,* 2003; Jin *et al.,* 2003; Sreedharan *et al.,* 2006)

The TTSS is a protein secretion complex used by many Gram-negative plant pathogenic bacteria to promote pathogenesis (Arnold *et al.,* 2003). Phylogenetic analyses of the sequences of the TTSS constituents indicate that the system is an evolutionarily divergent form of the bacterial flagellar apparatus (Nguyen *et al.,* 2000). Some of the genes encoding for the TTSS system are highly conserved and are hence called *hrc* genes (for *h*ypersensitive *r*esponse and *c*onserved) (Bogdanove *et al.,* 1996). Other genes encoding the TTSS that are not rigidly conserved among phytopathogenic bacteria are designated *hrp* (for *h*ypersensitive *r*esponse and *p*athogenicity) (He, 1998). The *hrp/hrc*

gene cluster (Figure 17) is required for the elicitation of the hypersensitive response (HR) on non-host plants and for pathogenicity on susceptible hosts (Badel *et al.,* 2003; Alfano and Collmer, 2004; Mudgett, 2005). In *P. syringae*, the *hrp/hrc* genes encode the structural and regulatory proteins associated with the TTSS. Functions for some of the *hrp/hrc* genes have been described based on sequence similarity with the bacterial flagellar assembly genes and the TTSS genes of animal pathogens (Jin *et. al.,* 2003; Cornelis, 2006), although the *P. syringae hrpA* gene has been biochemically shown to encode a pilus that elongates at the distal end by the addition of HrpA pilin subunits (He et al., 1993; Li *et al.,* 2002) and acts as a conduit for effector protein delivery into the host cell (Jin *et al.,* 2001).

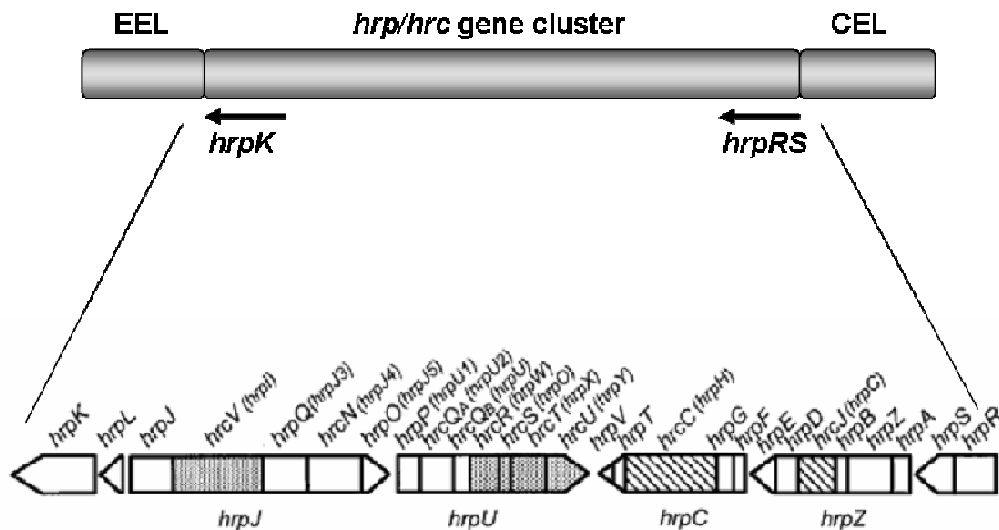


Figure 17. The *hrp/hrc* pathogenicity island in *P syringae* genome. The gene cluster is flanked by several effector genes encoded by the exchangable effector locus (EEL) and the conserved effector locus (CEL). While the genes in the EEL vary between strains of *P. syringae*, the CEL-encoded effectors are conserved and are required for the pathogenicity. The *hrp/hrc* gene cluster consists of *hrp* and *hrc* genes that encode for structural and regulatory proteins of TTSS. (Figure adapted from Charkowski *et al.,* 1997 and Arnold, 2003).

A number of regulatory genes impact virulence in *P. syringae* (Tang *et al.,* 2006). The *hrpL* gene is required for the expression of several transcripts in the *hrp* gene cluster

(Xiao *et al.,* 1994). HrpL recognizes a consensus sequence (GGAACC-N15/16-CCACNNA) on the promoters of the *hrp* operons known as the "hrp box" thus activating their transcription (Figure 18) (Fouts *et al.,* 2002). Expression of *hrpL* is regulated by *rpoN*, which encodes σ^{54} (Chatterjee *et al.,* 2002). The induction of *hrpL* induction also requires *hrpR and hrpS*, which are response regulator members of the two-component regulatory system (Xiao *et al.,* 1994). *hrpR* and *hrpS* are expressed as a single operon and then they interact to form a stable heterodimeric complex that positively regulates the σ^{54}-dependent *hrpL* promoter (Hutcheson *et al.,* 2001). Both *hrpRS* and *rpoN* are transcriptionally regulated by another two-component regulatory system, e.g. *gacA* and *gacS*, where GacA is a response regulator and GacS is the sensor kinase (Chatterjee *et al.,* 2003; Tang *et al.,* 2006). GacS presumably senses environmental signals, is autophosphorylated at a conserved histidine residue, and phosphorylates GacA, which then activates the transcription of target genes (Rich *et al.,* 1994). GacA acts as a central regulator that controls an assortment of transcriptional and post-transcriptional factors (Chatterjee *et al.,* 2003).

Experimental evidence combined with bioinformatic analyses indicates that *Pst* DC3000 produces over 40 effector proteins (Buell *et al.,* 2003; Lindeberg *et al.,* 2006). A large number of these proteins (including AvrPto) function to compromise plant defense by suppressing the HR (Nomura *et al.,* 2005). Transgenically expressed AvrPto repressed defense-related callose deposition in Arabidopsis cell walls and allowed substantial multiplication of the *Pst* DC3000 *hrcC* mutant (Hauck *et al.,* 2003). HrcC is a putative outer membrane protein that forms part of the basal structure of the TTSS needle-complex (He *et al.,* 2004). HrpZ, HrpW, and HrpJ are three harpins (Hrp proteins) in *P.*

syringae that are known to translocate through the TTSS, and all three proteins have a role in compromising host defense (He *et al.,* 1993; Charkowski *et al.,* 1998; Lee *et al.,* 2001; Fu *et al.,* 2006).

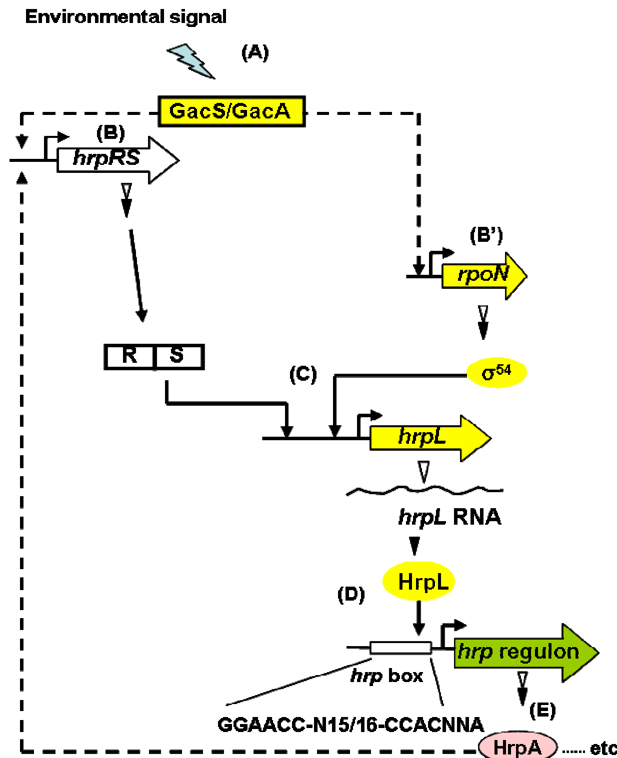


Figure 18. A simplified model showing regulation of the *P. syringae* type III secretion system (TTSS). (A) GacS is autophosphorylated in response to environmental signals. This leads to the phosphorylation of the response regulator GacA. The phosphorylated GacA then activates the transcription of *hrpRS* (B) and *rpoN* (B'). (C) HrpRS associate with the *rpoN*-encoded product σ^{54} at the *hrpL* promoter to activate *hrpL* expression. (D) HrpL recognizes the hrp box and activates the transcription of *hrp* operons. HrpA acts upstream of *hrpRS* transcription to stimulate TTSS activation. (Figure adapted from Tang *et al.,* 2006).

In the *Pst* DC3000-host interaction, disease severity is greatly enhanced by the virulence factor COR (Brooks *et al*., 2004; Uppalapati *et al*., 2007; Elizabeth and Bender, 2007). COR consists of the two structural components that are the precursors and intermediates in the COR pathway; e.g. the polyketide CFA and the cyclized isoleucine derivative CMA (Parry, 1994). To study the contribution of CFA, CMA and COR in virulence, well-defined mutants of *Pst* DC3000 were generated (e.g. *cfa6* and *cmaA*) (Brooks *et al.,* 2004). The *cfa6* and *cmaA* mutants exhibited reduced virulence phenotypes in Arabidopsis, and these phenotypes were comparable to a double mutant (*cfa6 cmaA*), thus suggesting that the intact COR molecule is necessary for virulence (Brooks *et al.,* 2004). Similar results were observed in tomato and edible brassicas

infected with the same *Pst* DC3000 COR$^-$ mutants (Uppalapati *et al*., 2007; Elizabeth and Bender, 2007).

P. syringae infection assays

To determine the virulence and pathogenicity of *P. syringae*, several bioassays have been developed. These include the assessment of the hypersensitive reaction (HR) on tobacco leaves and inoculation of fruit, seed pods, leaves and seedlings. Pathogenesis assays for the characterization of *P. syringae* virulence mutants have included: (i) puncture inoculation of primary bean leaves and bean pods with a toothpick dipped in the bacterial culture (Anderson and Mills 1985; Somylai *et al.,* 1986); (ii) wounding of bean leaves with carborundum and applying inoculum with cotton swabs (Anderson and Mills 1985); and (iii) vacuum infiltration of bean leaves with bacterial inoculum (Lindgren *et al.,* 1986). Mutants were also screened on tobacco leaves for their ability to elicit an HR; nonpathogenic mutants that were unable to induce an HR on tobacco leaves were later referred to as *hrp* mutants (Lindgren *et al.,* 1986). In another study, several inoculation methods were used to identify virulence mutants of *P. syringae* pv. *syringae* on common bean (*Phaseolus vulgaris*). These included syringe-infiltration and spray-inoculation of the primary leaves and stab inoculation of bean pods. Using this approach, mutants of *P. syringae* pv. *syringae* were identified that were defective in virulence on bean leaves but were still able to elicit a HR on tobacco (Willis *et al*., 1990).

Seedlings of apple, pear and peach were used for the detection of pathogenicity and measurement of virulence in *P. syringae* pv. *syringae* on woody plants (Endert and Ritchie, 1984). Symptoms ranged from small scabby lesions (low virulence) to extensive

rotting of the entire hypocotyl and epicotyl (highly virulent). In another study utilizing snap bean seedlings, both seeds and primary leaves were inoculated to evaluate the role of the TTSS in the growth of *P. syringae* pv. *syringae* B728a in the field (Hirano *et al.,* 1999). The results of this study suggested that mutants defective in the TTSS showed greater virulence defects in foliar assays than when inoculated to seeds. In a more recent study, alfalfa seedlings were used as a model for identifying pathogenicity factors in *P. aeruginosa*, which causes cystic fibrosis (CF) (Silo-Suh *et al*., 2002). This study showed a good correlation between virulence in alfalfa seedlings and factors that contribute to the persistence of *P. aeruginosa* in CF.

Assays using *P. syringae* pv. *tomato* (*Pst*)

Foliar assays have been used to screen for host resistance to *P. syringae* pv. *tomato* (formerly *P. tomato*); for example, Pilowsky and Zutra (1982) screened for resistance to *Pst* by spray-inoculating the foliage of wild tomato seedlings. In another study Cuppels (1986) used cotton swabs to inoculate the foliage of 15-day-old tomato seedlings with the aim of isolating *Pst* mutants with impaired virulence. More recently, Brooks *et al.* (2004) screened a library of Tn*5* mutants of *Pst* DC3000 for impaired virulence on Arabidopsis using a dip inoculation method.

Bacterial speck disease of tomato is largely regarded as a foliar disease (Preston, 2000), and studies of the *Pst* DC3000-tomato interaction have primarily been conducted in the leaves of 3-4 week old tomato plants. Foliar assays generally require high relative humidity to enable pathogen entry and development of typical disease symptoms following dip or spray inoculation (Lund *et al*., 1998; Peñaloza-Vázquez *et al*., 2000;

Zhao *et al*., 2003; Uppalapati *et al*., 2007). Unfortunately, these assays require extensive growth chamber or greenhouse space and are labor-intensive. Thus the development of a reliable seedling assay for *Pst*-tomato interactions could reduce both the time and space needed for virulence assays.

In the present study, I describe the development of a simple, inexpensive seedling-based assay that expedites the analysis of *Pst* DC3000-tomato interactions. Furthermore, this assay was then used to screen a mutant library of *Pst* DC3000 to identify mutants that were defective in virulence on tomato.

MATERIALS AND METHODS

Bacterial isolates and inoculum preparation

The bacterial strains and plasmids used in this study are listed in Table 2. Bacterial cultures were maintained at -80ºC in 15% glycerol. Working cultures were maintained at 4ºC on mannitol-glutamate medium (Keane *et. al.,* 1970) or King's medium B (King *et. al.,* 1954) with antibiotic selection when needed. Antibiotic concentrations (in μg/ml) were as follows: rifampicin, 50; spectinomycin, 25; streptomycin, 25; kanamycin, 25; and chloramphenicol, 25. Prior to inoculation, bacterial strains were subcultured at 28ºC for 36-48 h and suspended in 1 ml sterile distilled H_2O. Cultures were then adjusted to the desired concentration with a spectrophotometer (Spectronic 20, Bausch & Lomb). The final volume used for dip and vacuum infiltration was 40 ml. To study the effect of inoculum concentration on symptom development, three different concentrations of *Pst* DC3000 inoculum were

used: OD_{600} = 0.01, 0.1 and 1.0, which are equivalent to 5 x 10^6 CFU/ml, 5 x 10^7 CFU/ml and 5 x 10^8 CFU/ml, respectively.

Table 2. Bacterial strains and plasmids used in this study.

Strain	**Relevant characteristics**	**Reference or source**
P. syringae pv. *tomato* DC3000		
Wild-type	COR^+	Alan Collmer
hrcC	Cm^R, *hrcC*::Tn*5Cm*	Yuan & He, 1996
hrpS	Cm^R, *hrpS*::Tn*5Cm*	Yuan & He, 1996
VJ202	Rif^R Sp^R; *hrpL*::	Zwiesler-Vollick *et al*., 2002
CUCPB5114	Cm^r; DC3000 derivative containing deletion in entire *hrp* gene cluster	Fouts *et al*., 2003
hrpZ	Rif^R; Km^R; has deletion in *hrpZ*	Alan Collmer
CUCPB5096	Rif^R Sp^R; contains Ω Sp^R insertion in *hrpW*	Charkowski *et al*., 1998
DC3000-*pnlA*	Rif^R Sp^R; contains a deletion in *pnlA*	Barbara Kunkel
DB2H10	Rif^R Km^R; contains mini-Tn*5* insertion in a 381 bp ORF with an unknown function; defective in COR and CFA	Barbara Kunkel
Complemented lines		
KP430	RifR SpR/SmR KmR; VJ202 containing *hrpL* in pVSP61	This study
DC3000-*hrpS*	Cm^R Amp^R; contains hrpR-V on pCPP2201	Preston *et al*., 1995
DC3000-*hrcC*	Cm^R Amp^R; contains hrpR-V on pCPP2201	Preston *et al*., 1995
Plasmids		
pYXJIR	Sm^R, *hrpL*::*uidA* promoter fusion in pRG970	Xiao *et al*., 1994
pCFLP3	Cm^R, *cfl::uidA* promoter fusion in pBBR1MCS	Penaloza-Vazquez & Bender, 1998)
pDCalgDP	Cm^R, *algD::uidA* promoter fusion in pBBR1MCS	Keith *et al*., 2003
pBBR.Gus	Cm^R, 6.6-kb promoter probe vector containing promoterless *uidA* in pBBR1MCS	Penaloza-Vazquez & Bender, 1998)

Propagation of tomato seedlings

Seeds of tomato (*Solanum lycopersicum*) cv. Glamour were obtained from L. L. Olds Seed Company (Madison, WI). Tomato cv. wild-type Rio Grande (*PtoR*) and a near isogenic line containing the *PtoS* gene were kindly provided by Dr. Kiran Mysore. Two different support matrices were used to assess the efficiency of seed germination and

seedling elongation. In one method, a stack of three paper towels was placed in a Petri dish (9 cm, stackable, Kord Valmark). The towels were soaked with 4 ml sterile distilled H_2O. Seeds were placed on the soaked towels and incubated at 22 or 28°C (see below). In the second method, seeds were placed on water agar (1% agar, 9 cm Petri dishes) and incubated at 22 or 28°C.

Inoculation techniques

Tomato seedlings were four days old at time of inoculation. Two inoculation methods (dip and vacuum infiltration) were used to evaluate their effect on subsequent disease development with respect to time, efficiency, and reproducibility. Dip inoculation involved immersing the seedlings in culture suspensions (OD_{600} = 0.1) for 5 min prior to placing them on fresh water agar. In the other technique, seedlings were vacuum-infiltrated using a Speedvac vacuum evaporator (Savant Inc.) at 635 mm Hg (25 in Hg) for 20 sec. Seedlings were removed when the pressure re-equilibrated to 0 mm Hg. Sterile water was used as a control for non-inoculated seedlings in both methods. In each experiment, 20 seedlings were evaluated, and each experiment was repeated at least twice.

Disease severity scale

For quantitative expression of disease symptoms, a scale of 0 to 3 was developed as follows: 0 = no necrosis; 1 = slight necrosis of hypocotyls and at junction of root and shoot; 2 = severe necrosis of hypocotyls (root remains healthy); and 3 = seedling totally necrotic (including root) (Figure 19). Although the lesions were scored based on the

intensity of necrosis, the overall virulence of each mutant was compared using *Pst* DC3000 as a positive control, which was assigned a disease severity score of 3.

Figure 19. Disease severity scale for quantitative expression of disease symptoms on tomato seedlings as a result of *Pst* DC3000 infection.

Histochemical detection of GUS activity

Inoculated tomato seedlings were sampled at 72 h post-inoculation (hpi) and vacuum-infiltrated with a substrate surfactant solution [5-bromo-4-chloro-3-indoyl β-D-glucuronide (X-gluc, 0.5 mg/ml, and Silwet L77 (Osi Specialties Inc., Danbury, CT; 0.2 µl/ml in 50 mM sodium phosphate buffer, pH 7.0]. Vacuum-infiltrated seedlings were incubated at 37°C overnight and fixed and destained in 80% ethanol at 37°C (Hugouvieux *et al.*, 1998). Samples from infiltrated seedlings were assayed for GUS activity as described previously (Keith *et al.*, 2003).

RESULTS

Effect of seedling support matrix and temperature

Two matrices were evaluated for their ability to support efficient growth of the seedlings during germination. One matrix consisted of a layer of three paper towels

placed in a Petri dish and saturated with sterile water, and the other matrix consisted of water agar (1% agar) (Figure 20). Although the seedlings grew well on the paper towel matrix, the roots attached to the towels, which made it difficult to remove them without injury. Furthermore, the paper towels required re-wetting after two days, which added to the labor involved in the assay. Finally, the percent germination of seeds was poor on paper towels, and the seedlings grew at disparate rates, thus making it difficult to obtain uniform results.

When seeds were incubated on water agar, germination and seedling elongation were more uniform in comparison with the paper towel support matrix. Although seedlings were incubated on water agar for 4-5 days prior to inoculation, the medium could support the seedling growth for up to 10 days. Since the agar medium was semi-solid, it was much easier to remove the seedlings for inoculation without damaging the roots.

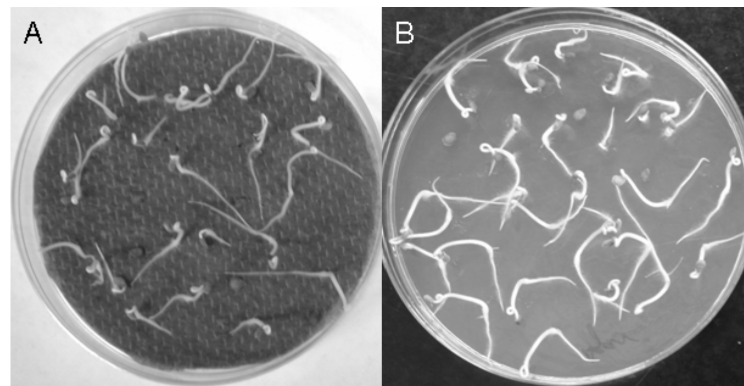


Figure 20. Evaluation of support matrices for the seedling assay. (**A**) Tomato seedling growth on water-soaked paper towels and (**B**) 1% water agar. Pictures were taken when seedlings were 4 days old.

The effect of temperature using both support matrices was evaluated by incubating seeds at 22 and 28°C. Seeds incubated at 28°C were approximately threefold longer than those incubated at 22°C (Figure 21). Therefore, in all subsequent experiments, seeds were incubated at 28°C on water agar.

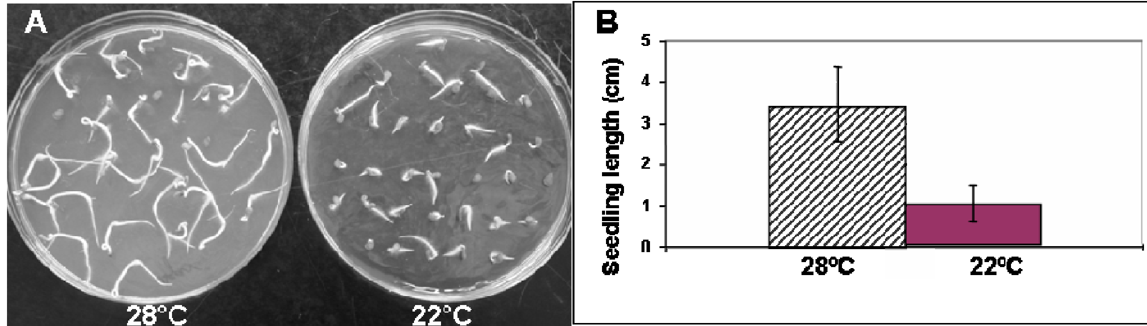


Figure 21. Growth rate of tomato seedlings on water agar incubated at 22 or 28°C. (**A**) Seedling growth at 4 days on 1% water agar. (**B**) Average seedling length obtained from 60 seedlings incubated at 22 or 28°C.

Effect of inoculation methods on symptom development on seedling

Tomato cv. 'Glamour' inoculated with *Pst* DC3000 showed similar disease symptoms when inoculated by dipping or vacuum infiltration. Dip-inoculated seedlings exhibited mild symptoms (Figure 22B) at 3 dpi and were completely necrotic at 6 dpi (data not shown). However, disease symptoms were observed in the vacuum-infiltrated seedlings two days earlier than those inoculated by dipping. Since most of the vacuum-infiltrated seedlings were completely necrotic at 3 dpi (Figure 22A), this time point was used to rate disease severity when screening the *Pst* DC3000 mutant library.

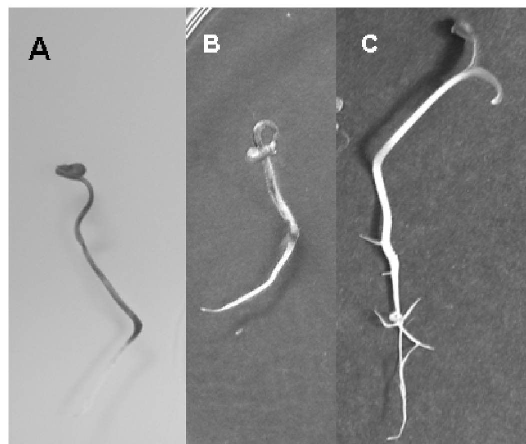


Figure 22. Symptoms on tomato seedlings at three days after (**A**) vacuum-infiltration with *Pst* DC3000; (**B**) dip-inoculation with *Pst* DC3000; and (**C**) H_2O-inoculated control using vacuum-infiltration.

Effect of inoculum concentration and age of seedling on symptom development

To study the effect of *Pst* DC3000 on symptom development, three different concentrations of inoculum were used: OD_{600}=0.01, 0.1 or 1.0. Seedlings vacuum-infiltrated with an OD_{600}=1.0 were severely necrotic within 24 h, and the seedlings died in less than 3 days (Figure 23). This concentration was considered too lethal for accurately studying disease progression. When an OD_{600}=0.01 was used, some necrosis was apparent at 3 dpi, which became more prominent after 5 days. Tomato seedlings inoculated with an OD_{600}=0.1 showed severe symptoms at 3 dpi. Thus for following disease progression and completing the assay in a high-throughput manner, an OD_{600}=0.1 was considerable most suitable.

The criteria used for choosing the age of seedlings for inoculation included: 1) the formation of both hypocotyl and root; and 2) a seedling root length of at least 1 cm. Seedlings that were 4 days old fulfilled these criteria and were used for subsequent experiments.

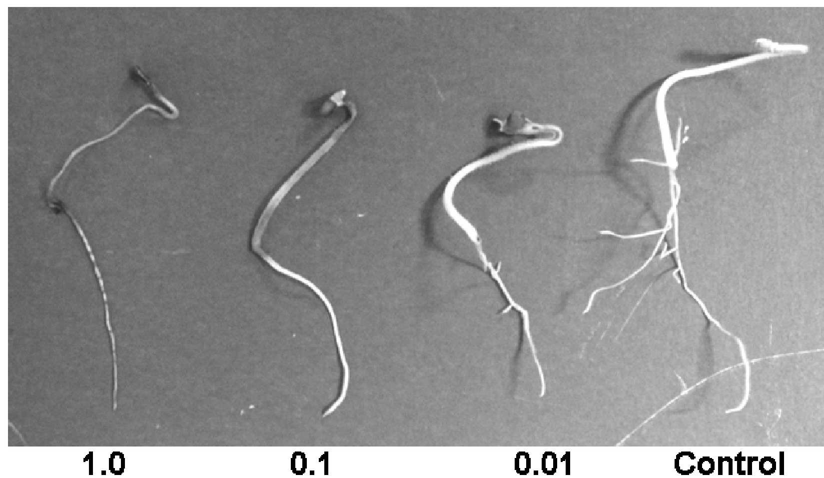


Figure 23. Disease symptoms on tomato seedlings vacuum-infiltrated with different concentrations of *Pst* DC3000 at 3 dpi. Numbers indicate bacterial cell concentration quantified at OD_{600}.

Evaluation of assay with known mutants

To study the host-pathogen interaction using the seedling assay, we initially used mutants of *Pst* DC3000 that had been studied using foliar assays. Several TTSS mutants, including *hrcC, hrpS, hrpL*, *hrpZ*, *hrpW*, and CUCPB5114 (deleted for the TTSS), and three COR-defective mutants (DB4G3, AK7E2 and DB4G3) were used. The disease severity scores and the characteristic phenotype in response to each mutant are given in Table 3.

When four-day old tomato seedlings were inoculated with *Pst* DC3000 mutants defective in COR production, the seedlings exhibited brown necrotic lesions 3 dpi and showed differences in root length relative to *Pst* DC3000 and the water-inoculated control. The root length of seedlings inoculated with DB4G3 (CFA^{-} CMA^{+} COR^{-}) and AK7E2 (CFA^{+} CMA^{-} COR^{-}) were comparable to *Pst* DC3000-inoculated seedlings. However, seedlings inoculated with DB29 (CFA^{-} CMA^{-} COR^{-}) had exhibited root lengths similar to the control (mock-inoculated with water) (Figure 24).

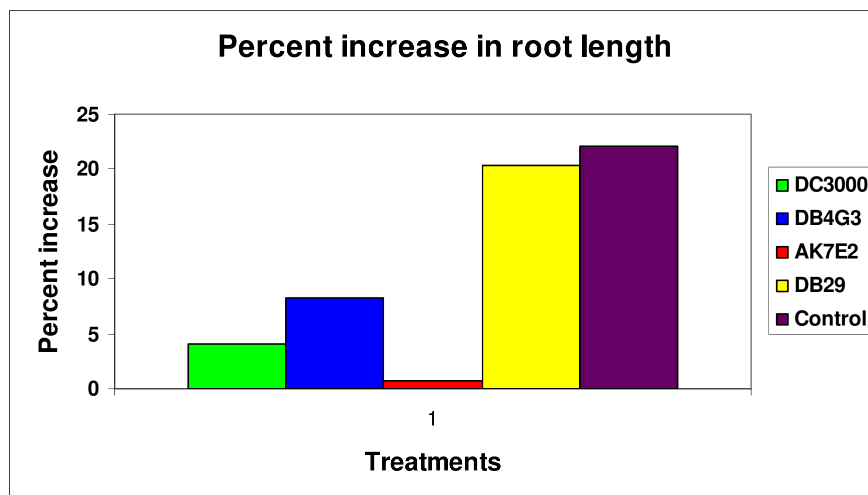


Figure 24. Graphical representation of the percent increase in root length of seedlings inoculated with *Pst* DC3000 (COR^{+}), DB4G3 (CFA^{-}), AK7E2 (CMA^{-}), DB29 (CFA^{-} CMA^{-}), and water (control); data represent increase in root length three days of inoculation.

Lesions on seedlings inoculated with different mutants were scored using the disease severity scale. Mock-inoculated seedlings did not show any lesions and received a score of 0. Ten of 20 seedlings inoculated with *Pst* DC3000 were completely necrotic (including the root) and were assigned a disease severity score of 3 (Figure 25). Similarly, seven of the 20 seedlings inoculated with AK7E2 received a disease severity score of 3. Seven, 14 and 13 seedlings (from a total of 20) inoculated with AK7E2, ChaDB4G3 and DB29, respectively, received a score of 2 and exhibited necrotic lesions on the hypocotyls (roots remained healthy). A few of the seedlings presented only a slight necrosis and were given a disease severity score of 1.

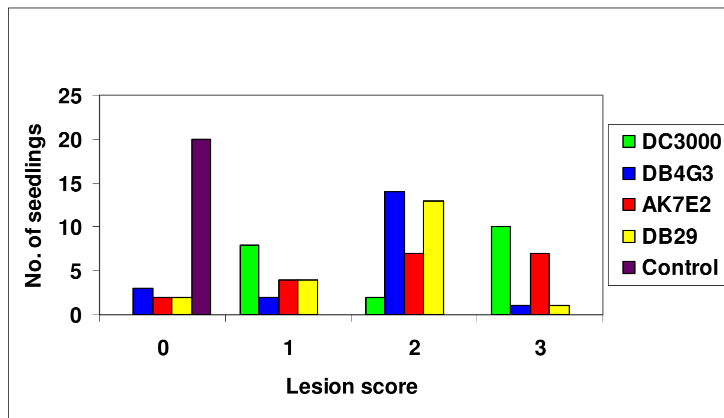


Figure 25. Seedlings inoculated with *Pst* DC3000 coronatine mutants and scored using the disease severity scale shown in Fig. 19.

Similarly, several the TTSS mutants of *Pst* DC3000 were inoculated onto tomato seedlings. For example, *hrpZ* encodes an effector protein known to traverse the Hrp pilus into the plant cell (Brown *et al*., 2001). In my work, the *hrpZ* mutant did not differ significantly from the wild-type *Pst* DC3000 in terms of disease severity (symptoms on tomato seedlings and number of lesions) (Figure 26A); this is consistent with results obtained in foliar inoculation of mature plants (Preston, 1997). Other mutants defective in the TTSS, including *hrpS*, *hrcC* (Figure 25A), *hrpL* (designated VJ202) and CUCPB5114 (deleted for the TTSS) showed little or no symptoms on tomato seedlings, which also

agrees with whole plant assays (Penaloza-Vazquez *et al.,* 2000; Preston, 1995; Zwiesler-Vollick *et al.,* 2002).

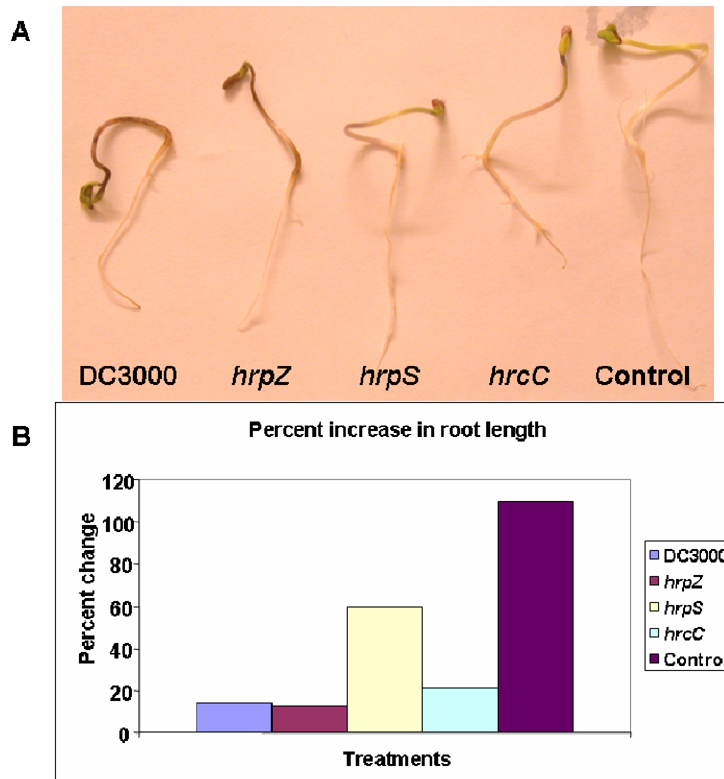


Figure 26. Genes encoding the *Pst* DC3000 type III secretion system are involved in the pathogenesis of tomato seedlings. (**A**) Phenotypes induced by *hrp/hrc* mutants of *Pst* DC3000 on tomato seedlings. *hrpZ*-inoculated seedlings were severely necrotic, whereas no necrotic lesions were seen on *hrpS*- and *hrcC*-inoculated seedlings. (**B**) Graphic representation of the percent increase in root length in seedlings inoculated with the wild-type *Pst* DC3000 and TTSS mutants (*hrpZ, hrpS* and *hrcC*); data represent the increase in root length 4 dpi.

When seedlings were scored for lesions in response to inoculation with *Pst* DC3000 and the TTSS mutants, four of 20 *hrpZ*-inoculated seedlings received a disease severity score of 3 and two were assigned a severity rating of 2; similar ratings were observed in response to tomato seedlings inoculated with *Pst* DC3000 (Figure 27). However, the *hrpS* and *hrcC*-inoculated seedlings received reduced disease severity scores relative to *Pst* DC3000 and the *hrpZ* mutant. For example, three of the *hrpS*-

inoculated seedlings were given a score of ‘2’, and the *hrcC*-inoculated seedlings did not develop lesions (score=0).

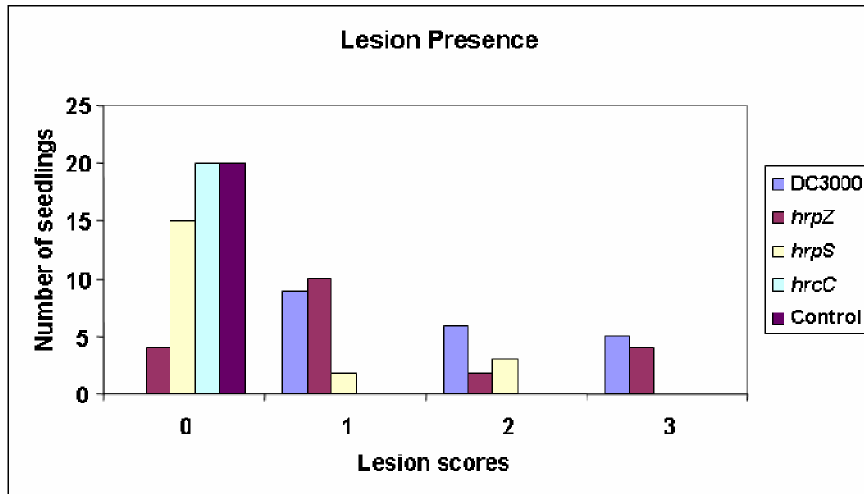


Figure 27. Seedlings inoculated with *Pst* DC3000 and selected TTSS mutants (*hrpZ*, *hrpS*, and *hrcC*) and scored using the following disease severity scale: 0, no necrosis; 1, slight necrosis of hypocotyls and at junction of root and shoot; 2, severe necrosis of hypocotyls (root remains healthy); and 3, seedling totally necrotic (including root).

Although the lesions were assigned based on the intensity of necrosis, the virulence of each mutant was compared using *Pst* DC3000 as a positive control, which received a disease severity score of 3. In Table 3, the disease severity of the mutants was scored by comparing virulence with that of *Pst* DC3000. Seedlings inoculated with the *hrpL* mutant (strain VJ202) showed minor lesions or were symptomless, with healthy root growth (Table 3). Additionally, *Pst* DC3000-*pnlA* and DB2H10, two previously uncharacterized mutants, induced mild symptoms on seedlings. *pnlA* (locus tag PSPTO4283) encodes for a protein that has a putative pectate lyase activity (Buell *et al.,* 2003); DB2H10 remains uncharacterized (B. N. Kunkel, pers. comm.).

Table 3: Symptoms and disease severity of *Pst* DC3000 mutants on tomato seedlings

Strain	Relevant characteristics	Disease Severity	Symptoms
P. syringae pv. *tomato*			
DC3000	COR^+	3	Complete necrosis of seedling, 'mushy' appearance, anthocyanin accumulation, stunted root growth.
DB4G3	COR^- Km^R; contains an insertion in *cfa6*	2	Severe necrosis of hypocotyl, root growth stunted
AK7E2	COR^- Sp^R; contains insertion in *cmaA*	2	Severe necrosis of hypocotyls and root, root growth stunted
DB29	COR^- Km^R Sp^R; *cmaA-cfa6* mutant of DC3000	1	Mild necrosis of hypocotyl, root growth stunted
CUCPB5114	Cm^r; DC3000 derivative containing deletion in entire *hrp* gene cluster	0	Stunted root
DC3000-*hrpZ*	Rif^R; Km^R; has deletion in *hrpZ*	2,3	Severe necrosis, Anthocyanin accumulation, severely stunted root
CUCPB5096	Rif^R Sp^R; contains Ω Sp^R insertion in *hrpW*	2,3	Severe necrosis, severely stunted root
DC3000-*pnlA*	Rif^R Sp^R; contains a deletion in *pnlA*	1	Mild necrosis, severely stunted root
DB2H10	Rif^R Km^R; contains mini-Tn*5* insertion in a 381 bp ORF of unknown function. COR^- CFA^-	1	Mild necrosis
AC811	Rif^R Km^R; contains mini-Tn5 insertion in *gacA*	1	Mild necrosis
DC3000-*gacS*	RifR KmR; contains a Tn5 insertion in *gacS*	1	Mild necrosis, stunted root
DC3000-hrpL	*Rif^R Sp^R;* hrpL*::Ω*	0,1	Mild to no necrosis
D3000-*hrcC*	*hrcC*::Tn*5Cm*	0	Mild anthocyanin accumulation, severely stunted root length
DC3000-*hrpS*	*hrpS*::Tn*5Cm*	0	Anthocyanin accumulation, severely stunted root
hrpL complement		2,3	Severe necrosis of seedling, stunted root growth
hrcC complement		3	Complete necrosis of seedling, 'mushy' appearance, anthocyanin accumulation, stunted root growth
hrpS complement		3	Complete necrosis of seedling, 'mushy' appearance, anthocyanin accumulation, stunted root growth

Overall, there was a good correlation between virulence on seedlings with results obtained with mature plants. To explore whether the seedling assay could be used in genetic complementation studies, representative mutants that were complemented for the genetic defect were analyzed for virulence in seedling assays. These experiments included the *hrcC, hrpL* and *hrpS* with the corresponding, complementing genes (Table 3). Seedlings were inoculated and disease severity was rated as described in the methods section. Three dpi, the original mutants showed few to no lesions, whereas the complemented mutants showed lesions and phenotypes comparable to that of *Pst* DC3000-inoculated tomato seedlings.

Validation of the assay using sensitive and resistant tomato

Tomato seedlings of cultivar Rio Grande that were either susceptible (*PtoS*) or resistant (*PtoR*) to *Pst* DC3000 were evaluated using the seedling assay. The *PtoR* gene confers resistance to strains of *Pst* that carry the avirulence gene *avrPto* (Ronald *et al*., 1992; Martin *et al*., 1993). Seedlings of *PtoS* and *PtoR* tomatoes were infiltrated with *Pst* DC3000 (OD_{600}=0.1) or water and evaluated 3 dpi. Seedlings of *PtoS* tomatoes inoculated with *Pst* DC3000 were severely necrotic and had well-defined lesions, whereas the *PtoR* tomatoes inoculated with *Pst* DC3000 were relatively healthy with minor lesion development (Figure 28A).

When seedlings were scored using the disease severity scale, none of the ten mock-inoculated seedlings of *PtoR* or *PtoS* lines showed any symptoms. However, of the ten *Pst* DC3000-inoculated *PtoS* seedlings, five received a disease severity score of 1, four received a rating of 2, and one was given a score of 3. For the *PtoR* line, five *Pst*

DC3000-inoculated seedlings received a score of 0 (no lesions) and the remaining five received a score of 1 (slight lesion development). In summary, the results indicate that *PtoR* seedlings were highly resistant to *Pst* DC3000, which agrees with results obtained with intact, mature plants (Martin *et. al.,* 1993).

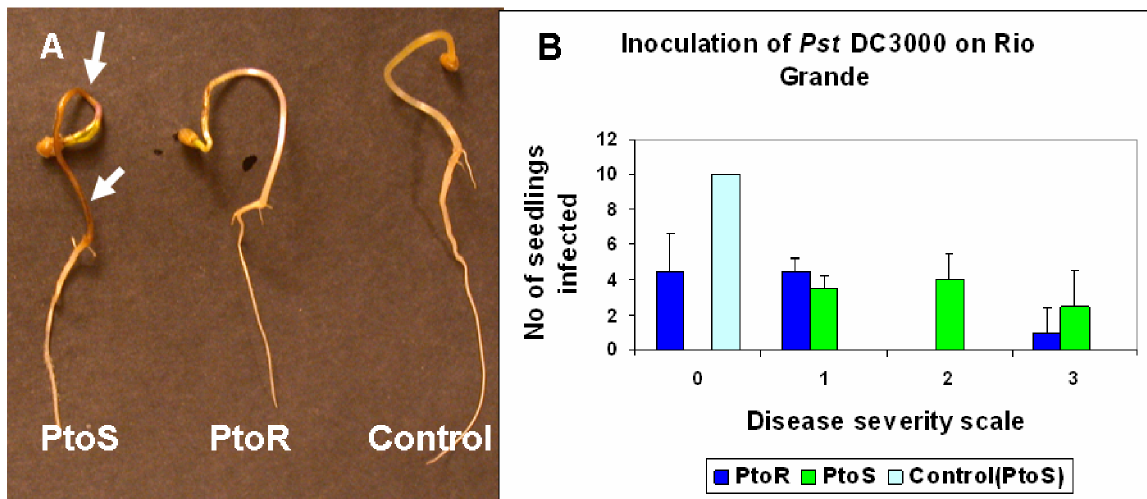


Figure 28. (A), Phenotype of susceptible (*PtoS*) and resistant (*PtoR*) Rio Grande seedlings at 4 days post inoculation with *Pst* DC3000. Arrows indicate necrotic regions. PtoS lines were inoculated with water as control lines. (B) Graphic representation of Rio Grande lines in response to *Pst* DC3000 inoculation. The experiment was performed twice with similar results.

Expression of GUS reporter activity in tomato seedlings

To determine whether *Pst* DC3000 virulence genes are expressed in infected seedlings, the transcriptional activity of several known virulence genes (e.g. representative of the TTSS, COR and the exopolysaccharide alginate) were studied. Tomato 'Glamour" seedlings were inoculated with three *Pst* DC3000 strains that carried promoters of the *hrpL* (representing the TTSS), *cfl* (COR pathway) and *algD* (alginate pathway). *algD* encodes GDP-mannose dehydrogenase and is the first gene to be transcribed in the alginate structural gene cluster of *P. syringae* (Penaloza-Vazquez et *al*., 1997).

These three promoters were previously fused to a promoterless glucuronidase (GUS or *uidA*) gene, and the constructs are described in Table 3 (see pYXJIR, pCFLP3, and pDCalgDP). Transcriptional activity (GUS gene expression) was evaluated in tomato seedlings 72 hpi. The *hrpL*::*uidA* fusion was highly expressed in tomato seedling roots (Figure 29A). Since *hrpL* encodes an alternate sigma factor that activates several transcripts in the TTSS (Collmer *et. al.,* 2002; Xiao *et. al.,* 1994; Jin *et. al.,* 2001), these results suggest that the TTSS is expressed in tomato seedling roots. The *cfl*::*uidA* fusion was also highly expressed in roots (Figure 29B). *cfl* is the first gene in an operon encoding the structural genes required for synthesis of CFA, the polyketide component of COR (Liyanage *et. al.,* 1995); and these results suggest that *cor* genes are highly expressed in tomato roots. However, the *algD*::*uidA* gene was not highly expressed in tomato seedling roots, suggesting that alginate is not expressed in this tissue (Figure 29C).

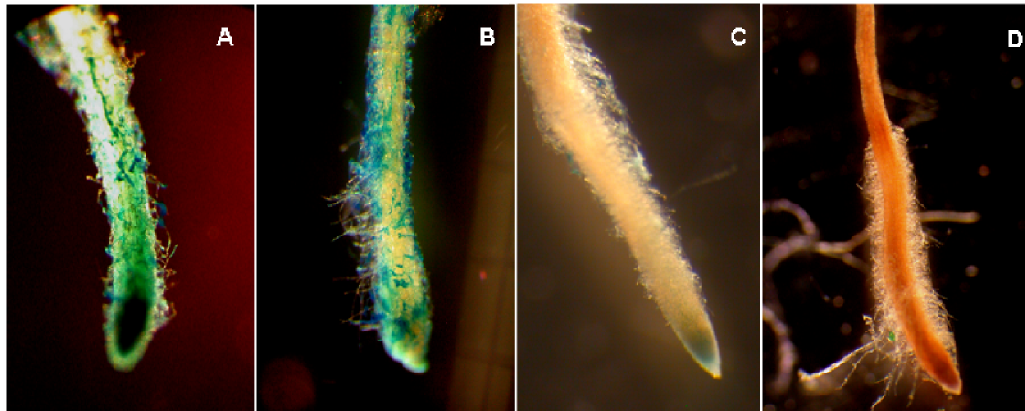


Figure 29. Virulence gene expression in the roots of *Pst* DC3000-infected tomato seedlings. The expression of several virulence genes was measured by monitoring the activity of transcriptional fusions to a promoterless glucuronidase gene (GUS; *uidA*). Reporter activity was monitored in tomato roots infected with *Pst* DC3000 carrying: (**A**) *hrpL::uidA* promoter fusion (indicating activity in the TTSS); (**B**) *cfl::uidA* (representing COR gene expression); (**C**) *algD::uidA*, (indicating alginate gene expression); and (**D**) control (promoterless *uidA*

Time course study of virulence gene expression

To understand the progression of virulence gene expression during seedling infection, transcriptional activity of *hrpL* and *cfl* were observed and visually quantified for up to 8 dpi. *Pst* DC3000 strains carrying *cfl*::*uidA* or *hrpL*::*uidA* transcriptional fusions were vacuum-infiltrated into tomato seedlings. *cfl* expression was visible beginning at 12 hpi; whereas, *hrpL* activity was not evident until 1-2 dpi (Figure 30). These results were consistent with studies performed on leaves of 4-week old tomato plants (Guzman-Hernandez *et al.,* unpublished).

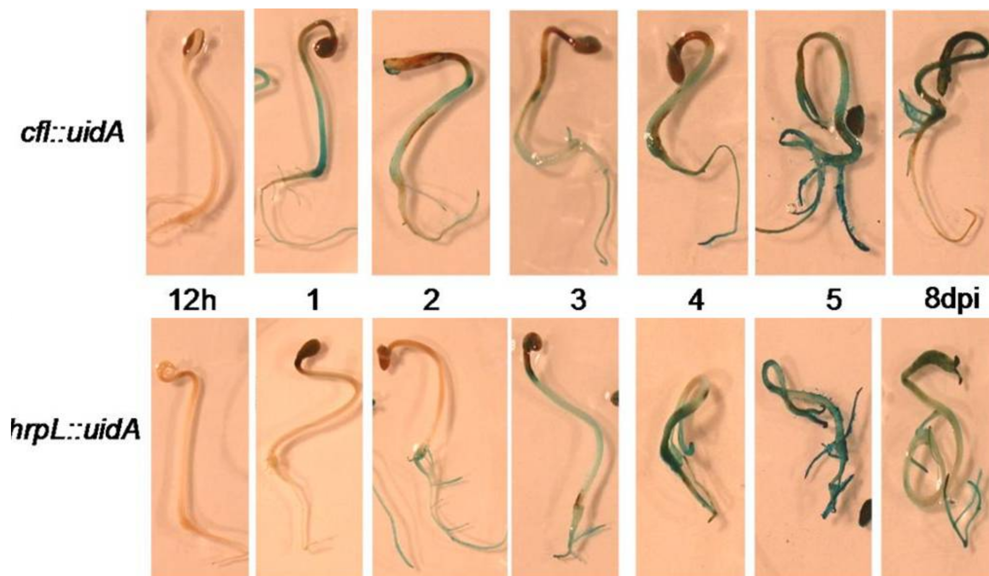


Figure 30. Dynamics of *Pst* DC3000 virulence gene expression in tomato seedlings. *Pst* DC3000 strains carrying either the *cfl* or *hrpL* promoter fused to the GUS (*uidA*) reporter gene were vacuum-infiltrated into 4-day old tomato seedlings. Each seedling shown is representative of five seedlings.

Application of the seedling assay to screen for *Pst* DC3000 virulence mutants

After the seedling assay was developed and validated using defined *Pst* DC3000 virulence mutants, I investigated whether the assay could be used to screen a library of uncharacterized *Pst* DC3000 mutants. A collection of approximately 1000 *Pst* DC3000

mutants (Kloek *et al*., 2000; Brooks *et al*., 2004), which was generated through insertional mutagenesis of transposon Tn*5*, was kindly provided by Dr. Barbara Kunkel (Washington University, St. Louis, MO). Tomato seedlings (4-5 days old) were submerged in bacterial culture suspensions (OD_{600}=0.1), and the inoculum was introduced by vacuum infiltration (see Methods). The inoculated seedlings were then incubated at 28°C on water agar. After 3-4 days, symptoms on the seedlings were observed and recorded, and twenty seedlings were used as biological replicates. The assay was performed in batches with each group consisting of not more than ten *Pst* DC3000 mutant strains excluding the wild-type *Pst* DC3000 and the control. The completion of each batch required approximately ten days. Approximately 200 mutants were screened using this approach, and I identified seven different mutants that consistently exhibited reduced disease symptoms on tomato seedlings relative to *Pst* DC3000 (Table 4). The phenotype of several mutants with reduced virulence is shown below (Figure 31).

Figure 31. *Pst* DC3000 mutants identified using the high-throughput seedling assay and shown to have a reduced virulence phenotype on tomato seedlings. Seedlings were vacuum-infiltrated with *Pst* DC3000 mutants and incubated on water agar. Pictures were taken 5 dpi.

Of the seven mutants mentioned above, a mutant designated as AKIIIA1 was previously shown to contain a transposon insertion in *dsbA* (Kloek *et al*., 2000). DsbA is a periplasmic enzyme involved in the direct oxidation of cysteine residues in proteins that localize to the periplasm (Bardwell *et al*., 1991). Furthermore, two other mutants, AKIIIA5 and AKIIIF1, were auxotrophic (Table 4) and did not grow on histidine as the nitrogen source.

The remaining four *Pst* DC3000 mutants were subsequently grown on minimal media supplemented with various carbon and nitrogen sources. Three of the mutants (AKIIA9, AKIIB12 and AKIID10) grew on par with *Pst* DC3000 (Table 4). However, one mutant (AKIID11) did could not utilize succinate or aspartate as a carbon source or glutamate, proline, L-alanine, or arginine as a nitrogen source.

Table 4. Growth characteristics of *Pst* DC3000 virulence mutants

Mutant	Characteristics	Reference
AKIIA9	Prototroph; reduced virulence on tomato seedlings	This study
AKIIB12	Prototroph; reduced virulence on tomato seedlings	This study
AKIID10	Prototroph; reduced virulence on tomato seedlings	This study
AKIID11	Auxotroph, no growth on several C and N sources; reduced virulence on tomato seedlings	This study
AKIIIA1	*dsb*A mutant; reduced virulence on tomato seedlings	Kloek *et al*., 2000
AKIIIA5	Auxotroph, no growth on histidine as sole N source; reduced virulence on tomato seedlings	Andrew Kloek
AKIIIF1	Auxotroph, no growth on histidine as N source; reduced virulence on tomato seedlings	Andrew Kloek

DISCUSSION

Foliar assays have been used to screen for host resistance on tomato in response to *P. syringae* pv. *tomato* (Pilowsky and Zutra, 1982) and to isolate *Pst* mutants with impaired virulence (Cuppels, 1986; Kloek *et al*., 2000). Tomato plants have also been used to conduct infection assays with *P. syringae* (Zhao *et al.,* 2003; Uppalapati *et al.,* 2007) and other pathogens including *Ralstonia solanacearum* (Gonzalez *et al.,* 2007), the oomycete *Phytopthora infestans* (Lee *et al*., 2006), and the fungal pathogen *Cladosporium fulvum* (van Kan *et al.,* 1991). Although these methods for assessing the virulence of *P. syringae* have been established, these are not rapid, high-throughput methods that facilitate the screening of large numbers of mutants. Thus the aim of this work was to develop a seedling assay that could be used in virulence screens and gene expression studies.

To establish the utility of the seedling assay as a screening tool for the identification of pathogenicity genes, the seedling assay results were compared with those obtained in foliar assays using well-characterized virulence mutants. For example, the well-defined TTSS mutants *hrcC, hrpS, hrpL*, *hrpZ*, *hrpW*, and CUCPB5114 showed similar disease phenotypes in the seedling and whole plant assays (Penaloza-Vazquez *et. al.,* 2000; Preston, 1997; Zwiesler-Vollick *et. al.,* 2002; Zhao *et. al.,* 2003). Similarly, results from the virulence assays using COR biosynthetic mutants were consistent between seedling (this study) and foliar assays (Uppalapati *et al*., unpublished).

The seedling assay also shows promise in identifying plant genes that confer pathogen resistance. This was shown using the resistant line of tomato cultivar Rio Grande that contains the *R* gene *PtoR.* PtoR confers resistance to *Pst* by physically

interacting with the *Pst* effector protein AvrPto (Tang *et al,* 1996), an event that triggers a signal transduction cascade leading to host defense. Inoculation of *Pst* DC3000 on *PtoR* seedlings resulted in limited necrotic lesions, suggesting that *Pto*-mediated resistance functions during the seedling stage of growth. Moreover, the results obtained with seedlings were similar to those obtained with mature plants (Martin *et. al.,* 1993).

During *Pst* DC3000 infection of plant hosts, the TTSS (Tang *et al*., 2006; Lindeberg *et al*., 2006) and the COR genes (Mittal and Davis, 1995; Brooks *et al*., 2004; Melotto *et al*., 2006; Uppalapati *et al*., 2007) are required for symptoms associated with bacterial speck disease. The results from our studies with tomato seedlings inoculated with *Pst* DC3000 carrying *hrpL*::*uidA* and *cfl*::*uidA* suggest that TTSS and COR genes are also expressed in tomato seedlings. Similarly, a qualitative assay that was performed using *Pst* DC3000 containing the *hrpL*::*uidA* and *cfl*::*uidA* constructs agreed with similar experiments performed on the foliar parts of tomato. Thus, the pathogen may use similar strategies to induce pathogenesis on both fully grown plants and seedlings.

The use of the seedling assay to screen a *Pst* DC3000 mutant library indicated that this approach could be used to identify virulence genes involved in bacterial speck disease. It may also be possible to use this approach to screen for virulence genes in other pathogens of tomato. Furthermore, some of the mutants that were isolated using the seedling assay were not identified using the whole plant, foliar assay (B.N. Kunkel, personal communication). Therefore, in addition to its utility as a rapid, high-throughput assay, the assay described above also has the potential to identify pathogen genes that are uniquely expressed during pathogenesis of seedlings.

CHAPTER V

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APPENDIX

Subcellular localization of ALC1

SUMMARY

The *N. benthamiana ALC1* described in Chapter III was predicted to be a homolog of Arabidopsis *THF1*. An earlier study showed that THF1 was located in the chloroplast. In this study, I used ChloroP, a program that predicts the chloroplast transit peptides and an ALC1-GFP fusion construct, to show that ALC1 is a nuclear-encoded protein that localizes to the chloroplast.

INTRODUCTION

In my earlier studies using fast-forward screening of the *N. benthamiana* cDNA library, I discovered several plant genes that are potentially involved in COR-mediated chlorosis (Chapter III). One of these genes, *ALC1*, resulted in a HR-like phenotype when silenced in *N. benthamiana*. The full-length *ALC1* gene was then sequenced, the putative amino acid coding region was predicted, and a BLAST search was performed. The search result indicated that the predicted ALC1 protein had homologs in several plants (Chapter III, Figure 11). One of the sequences that showed strong homology with ALC1 was *Arabidopsis* THF1 (alignment score=67). THF1 was previously shown to be a light-regulated, chloroplast-localized protein (Wang *et al*., 2004). My identification of *ALC1* as a gene involved in COR-mediated chlorosis in Arabidopsis was primarily based on the

strong homology between the two sequences. In the current study, I used transient expression of *ACL1* to determine if the protein localizes to the chloroplast in *N. benthamiana*, thus possibly functioning in a manner orthologous to THF1.

METHODS

Generation of an ALC1-GFP construct

To transiently express ALC1 in *N. benthamiana*, the GATEWAY-ready pMDC83 was used as a vector to generate a GFP fusion (Curtis and Grossniklaus, 2003). Full length *ALC1* sequence was amplified from *N. benthamiana* cDNA using the following gene specific primers: *ALC1*attB1: 5'- ggg gac aag ttt gta caa aaa agc agg ctt c ATG GCG GCA GTT ACT TCG-3'; and *ALC1*attB2: 5'- ggg gac cac ttt gta caa gaa agc tgg gtc CCT CCC AGC ATA TTG GT AAT CT-3' (small letters indicate the GATEWAY adapters). The amplified sequence was cloned into the donor vector pDONR 207 (Invitrogen, Carlsbad, CA, USA), and the resulting clone was then transformed into *E. coli* DH5α competent cells (Invitrogen, Carlsbad, CA, USA). The full length gene was further sub-cloned into pMDC83 (Figure 32a), and pMDC83-*ALC1* (Figure 32b) was then introduced into *A. tumefaciens* GV2260 by electroporation. To generate pMDC83 empty vector that can replicate in *A. tumefaciens* GV2260 without killing the host (Dao-Thi *et al.*, 2005), the vector was restriction digested with KpnI to remove the *ccdB* (controller of cell division or death) region (Figure 32c, d). The open ends were then ligated with T4 DNA ligase.

Microscopy

For visualization of GFP, a laser scanning confocal microscope (model TCS SP2 AOBS, Leica Microsystems Inc, Bannockburn, IL, USA) was used. GFP was excited at 488 nm by an argon laser, and emission was collected at 522 nm. The fluorescence of chlorophyll was obtained at 680 nm.

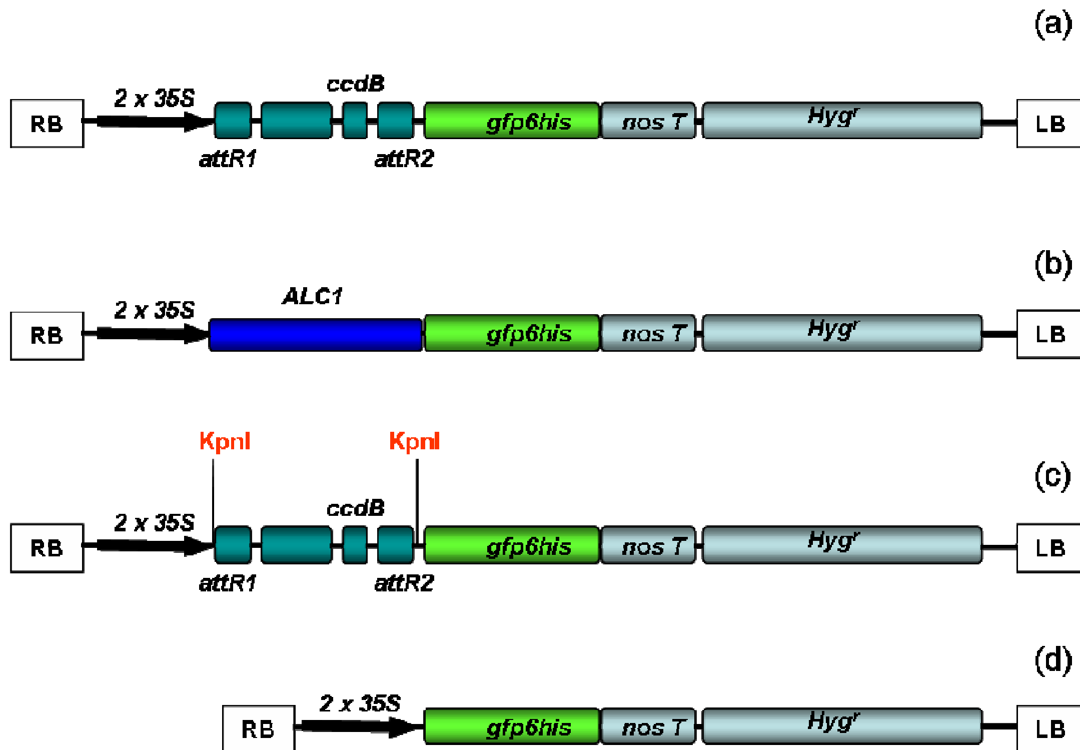

Figure 32. The GATEWAY-ready GFP vector pMDC83 was used for transient expression of ALC1. (**a**) pMDC83 carrying the GATEWAY recombination sites attR1 and attR2. The controller of cell division or death (*ccdB*), a lethal gene, is present as a negative selection marker. (**b**) The *ALC1-GFP* construct. (**c**) To generate an empty vector as a control, the *ccdB* region was removed by restriction digestion with *Kpn*I and, (**d**) the open ends were ligated with T4 DNA ligase.

RESULTS AND DISCUSSION

A previous study showed that *Arabidopsis* THF1, a homolog of *N. benthamiana* ALC1 is localized to the chloroplast. To determine if ALC1 is also localized to the

chloroplast, the predicted ALC1 amino acid sequence was first analyzed using ChloroP (http://www.cbs.dtu.dk/services/ChloroP/), a program that predicts the chloroplast transit peptide sequence (Emanuelsson *et al*., 1999). The results suggested that the protein has a chloroplast transit peptide (Figure 2), indicating that ALC1 is a nuclear-encoded, chloroplast-localized protein.

Table 5. Prediction of the chloroplast transit peptide.

Name	Length	Score	cTP	cTP-length
ALC1	295	0.597	Yes	70

The ChloroP program was used for predicting the chloroplast transit peptide (cTP) sequence. The first 70 of 295 amino acids residues in ALC1 were predicted to comprise a transit peptide.

To further confirm the localization of ALC1 to the chloroplast, the putative coding region of *ALC1* was cloned into a GFP fusion vector. *ALC1* was fused downstream of the CaMV 35S promoter and upstream of *gfp6his* in the GATEWAY-ready plasmid vector pMDC83 (Figure 32b). For transient expression of ALC1 in *N. benthamiana* leaves, the construct was transformed into *A. tumefaciens* GV2260. Six week old *N. benthamiana* plants were Agro-infiltrated with strain GV2260 (OD_{600}=1.0) carrying the pMDC83-*ALC1* construct. A slightly modified pMDC83 vector (Figure 32d) was transformed into *A. tumefaciens* GV2260 and infiltrated as a control. After 48 h, the infiltrated regions were observed under the confocal microscope. In control leaves, GFP was expressed in the cytoplasm (Figure 33a, b) whereas no GFP expression observed in the chloroplast (Figure 33b, c). Leaves infiltrated with the pMDC83-*ALC1* construct expressed GFP exclusively in the chloroplast (Figure 33d, e, f).

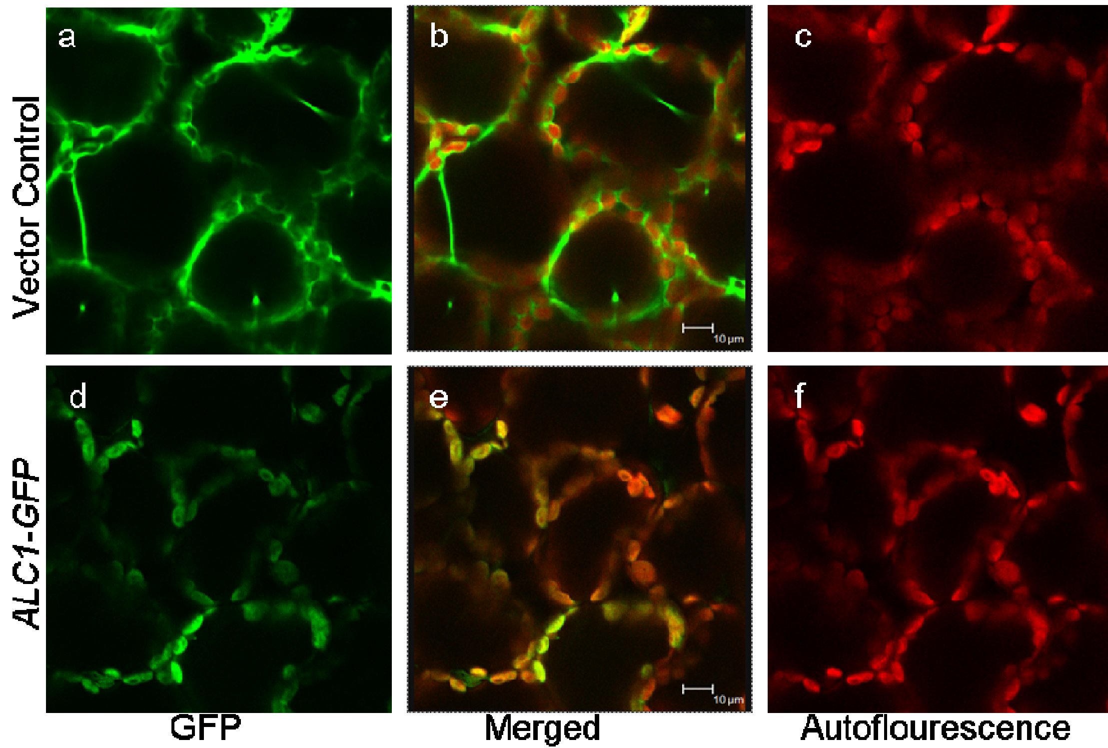

Figure 33. ALC1 is localized to the chloroplast. Six week old *N. benthamiana* leaves were inoculated with *A. tumefaciens* GV2260 carrying *GFP* (vector control, panels a-c) or an *ALC1-GFP* fusion construct (panels d-f). The Agro-inoculated leaves were incubated for 48 h and observed using a confocal microscope.

From the above results, I have concluded that ALC1 is a nuclear-encoded protein that is localized to the chloroplast. This would also indicate that ALC1 is an ortholog of Arabidopsis THF1.

VITA

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Thesis: APPLICATION OF A FAST-FORWARD GENETIC TOOL TO IDENTIFY PLANT GENES INVOLVED IN THE PERCEPTION OF CORONATINE, A PHYTOTOXIN PRODUCED BY *PSEUDOMONAS SYRINGAE* PV. *TOMATO* DC3000

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Major Field: Plant Pathology

Scope and Method of Study: The purpose of this study was to identify host factors that are involved during the interaction of the phytopathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) and host plants. The specific objectives of this project were to: (i) screen *Nicotiana benthamiana* lines silenced with a *N. benthamiana* cDNA library for altered chlorosis in response to the phytotoxin coronatine (COR); and (ii) further characterize the identified genes from tomato and *Arabidopsis*, which are hosts infected by *Pst* DC3000. Another purpose of this study was to (iii) develop a high-throughput tomato seedling assay that could be used for screening of *Pst* DC3000 virulence mutants.

Findings and Conclusions: Objective I. Several plant genes were identified with potential roles in COR-induced chlorosis. One of the genes, *ALC1*, was further characterized on tomato and Arabidopsis and found to be involved in COR/*Pst* DC3000-mediated chlorosis. Furthermore, genetic analysis suggested that the gene is involved in the regulation of *Pst* DC3000-induced senescence in Arabidopsis. Objective II. A high-throughput tomato seedling assay was developed and optimized. The assay was validated using well-characterized *Pst* DC3000 virulence mutants and compared with results previously obtained using mature tomato plants.

ADVISER'S APPROVAL: Dr. Carol L. Bender