

A *Pseudomonas syringae* pv. *tomato* DC3000 mutant lacking the type III effector HopQ1-1 is able to cause disease in the model plant *Nicotiana benthamiana*

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Summary

The model pathogen *Pseudomonas syringae* pv. *tomato* DC3000 causes bacterial speck in tomato and Arabidopsis, but *Nicotiana benthamiana*, an important model plant, is considered to be a non-host. Strain DC3000 injects approximately 28 effector proteins into plant cells via the type III secretion system (T3SS). These proteins were individually delivered into *N. benthamiana* leaf cells via T3SS-proficient *Pseudomonas fluorescens*, and eight, including HopQ1-1, showed some capacity to cause cell death in this test. Four gene clusters encoding 13 effectors were deleted from DC3000: cluster II (*hopH1*, *hopC1*), IV (*hopD1*, *hopQ1-1*, *hopR1*), IX (*hopAA1-2*, *hopV1*, *hopAO1*, *hopG1*), and native plasmid pDC3000A (*hopAM1-2*, *hopX1*, *hopO1-1*, *hopT1-1*). DC3000 mutants deleted for cluster IV or just *hopQ1-1* acquired the ability to grow to high levels and produce bacterial speck lesions in *N. benthamiana*. HopQ1-1 showed other hallmarks of an avirulence determinant in *N. benthamiana*: expression in the tobacco wildfire pathogen *P. syringae* pv. *tabaci* 11528 rendered this strain avirulent in *N. benthamiana*, and elicitation of the hypersensitive response in *N. benthamiana* by HopQ1-1 was dependent on SGT1. DC3000 polymutants involving other effector gene clusters in a *hopQ1-1*-deficient background revealed that clusters II and IX contributed to the severity of lesion symptoms in *N. benthamiana*, as well as in Arabidopsis and tomato. The results support the hypothesis that the host ranges of *P. syringae* pathovars are limited by the complex interactions of effector repertoires with plant anti-effector surveillance systems, and they demonstrate that *N. benthamiana* can be a useful model host for DC3000.

Keywords: Hrp system, hypersensitive response and pathogenicity, plant defense, host specificity, Avr proteins.

Introduction

Pseudomonas syringae pv. *tomato* (*Pto*) DC3000 is a pathogen of tomato and Arabidopsis noted for its large and well-characterized repertoire of type III effectors: proteins injected into plant cells by the type III secretion system (T3SS). Strains in *P. syringae* are divided into 50 or so pathovars based largely on host specificity (Hirano and Upper, 2000). For example, *Pto* causes bacterial speck

of tomato but is avirulent and elicits the defense-associated hypersensitive response (HR) in tobacco, whereas *P. syringae* pv. *tabaci* (*Pta*) causes wildfire disease in tobacco but is avirulent in tomato. What controls such host specificity is not understood, although type III effectors are generally suspected (Alfano and Collmer, 2004).

The T3SS is encoded by *hrp* and *hrc* genes that are required for HR elicitation and pathogenesis in plants (*hrc* genes encode T3SS components conserved with animal pathogens) (Cornelis, 2006). Effectors contribute to pathogenesis by defeating plant defenses and by controlling the cell death that is associated with the blight, spot, speck and canker symptoms that are characteristic of various *P. syringae* diseases (Abramovitch *et al.*, 2006; Alfano and Collmer, 2004; Grant *et al.*, 2006; Nomura *et al.*, 2005). As an example of the latter ability, *Pto* DC3000 *hopN1* mutants produce more bacterial speck lesions in host tomato whereas *hopM1* or *avrE1* mutants produce fewer lesions (Badel *et al.*, 2003, 2006; López-Solanilla *et al.*, 2004). However, none of these mutants is reduced in its ability to grow *in planta* or is completely abolished in its ability to produce disease symptoms. Mutations affecting individual effector genes in *Pto* DC3000 typically have phenotypes that are subtle, at best, apparently because of redundancy. Although the strong phenotype of T3SS pathway mutations points to the collective importance of effectors, the study of individual effector functions has been complicated by this apparent redundancy.

One approach to overcoming the problem of weak phenotypes is to express individual effector genes *in planta* using stable transformants, or through transient expression using viral vectors or *Agrobacterium tumefaciens*. These approaches maximize the chance of detecting weak phenotypes and provide important data for the functional profile of an effector repertoire. A related approach is to examine virulence-related phenotypes of a T3SS-proficient strain that is lacking multiple effectors. For example, the ability of certain *Pto* DC3000 effectors to suppress basal resistance has been observed by using a DC3000 mutant deleted for three effectors in the conserved effector locus, and by using *Pseudomonas fluorescens* expressing a cloned *P. syringae* T3SS (DebRoy *et al.*, 2004; Oh and Collmer, 2005).

A complementary approach to the problem of redundancy is to reduce redundancy by constructing polymutants in which multiple effector genes are deleted. This strategy has been used with Yop effectors in *Yersinia* and AvrBS3 family effectors in *Xanthomonas* (Castaneda *et al.*, 2005; Neyt and Cornelis, 1999; Yang *et al.*, 1996). *Pto* DC3000 is an ideal target for this approach. The DC3000 genome has been sequenced (Buell *et al.*, 2003), and the effector repertoire has been extensively characterized from the perspective of effector identification and deployment (Lindeberg *et al.*, 2006). DC3000 appears to deploy at least 28 effectors (plus several proteins that appear directed to the apoplast rather than the host cytoplasm) (Schechter *et al.*, 2006). Many of the effector genes are clustered in pathogenicity islands and islets on the chromosome or are present on pDC3000A, which is one of two native plasmids in DC3000. Deleting such clusters and pDC3000A provides an efficient way to reduce redundancy in the effector repertoire.

Polymutants can be used to ask fundamental questions about the role of effectors in controlling host specificity, growth *in planta* and symptom production by *P. syringae*. The issue of host specificity is particularly important. A given *P. syringae* strain is avirulent in most plant species it encounters, which are therefore considered to be non-hosts. Non-host resistance refers to the resistance of a plant species to a pathogen and contrasts with host resistance (race-specific resistance), which is possessed by a subset of genotypes within a host species and typically is effective against a subset of genotypes of the pathogen (Heath, 2000; Keen, 1990). Non-host resistance can be classified as type I (HR not elicited) or type II (HR elicited) (Mysore and Ryu, 2004). Type-II non-host resistance against *P. syringae* pathovars is prevalent, although the type-I non-host resistance of Arabidopsis against *Pph* and some other pathovars has attracted much interest (Davis *et al.*, 1991; Klement *et al.*, 1964; Mysore and Ryu, 2004). Type-II non-host and race-specific resistance against *P. syringae* often appear similar. When inoculated at a low level, *P. syringae* strains will grow well initially in plants with either type of resistance, but growth is sustained for several days and necrotic symptoms are produced only in susceptible species or cultivars. When inoculated at high levels into resistant plants, the rapid tissue collapse that is diagnostic of the HR is typically observed with both types of resistance. For example, the wild tobacco *Nicotiana benthamiana* is considered to be a non-host of *Pto* DC3000 and does not show symptoms when inoculated at a low level but responds with the HR at a high level (Mysore and Ryu, 2004).

Understanding the factors that prevent DC3000 from being virulent in *N. benthamiana* and other non-host plants is important for at least three reasons. Firstly, *N. benthamiana* complements Arabidopsis as a model in plant biology research, particularly in its amenability to rapid loss-of-function tests based on virus-induced gene silencing (VIGS). Thus, a disease model involving DC3000 and *N. benthamiana* would accelerate research. Secondly, the control of host specificity is related to fundamental questions about *P. syringae*-plant interactions. For example, do the virulence targets of effectors differ in plants, thus requiring specialized effectors for different plants in the host range of a given strain? Thirdly, disease resistance is important in crop defense, and non-host resistance is considered to be more durable in the field than race-specific resistance. Because the durability of resistance is determined by the genetics of the pathogen (Leach *et al.*, 2001; McDonald and Linde, 2002), a better understanding of the genetics of bacterial host specificity could have broad practical implications for the development of resistant crops.

In this report, we used T3SS-proficient *P. fluorescens* to test a panel of DC3000 effectors for their ability to cause cell death in *N. benthamiana*, and we constructed a series of polymutants that deleted 13 *Pto* DC3000 active effector

genes. Remarkably, we found that deleting a single effector gene that has avirulence activity enables DC3000 to cause disease in *N. benthamiana*. Additional polymutants were then constructed to compare contributions of the DC3000 effector repertoire to growth and lesion formation in *N. benthamiana*, *Arabidopsis* and tomato.

Results

Several Pto DC3000 effectors can elicit cell death in *N. benthamiana*

Plant cell death is associated with the avirulence activity of *P. syringae* effectors in resistant plants as well as with the formation of lesions in susceptible plants (Alfano and Collmer, 2004). Therefore, identifying the effectors that can cause cell death in a non-host or a host plant is a useful first step in cataloging potential functions of the effector repertoire. Our primary interest in this work was in identifying DC3000 effectors that may function as avirulence determinants in non-host *N. benthamiana*. The repertoire, listed in Table 1, comprises what is considered to be a complete set of Avr/Hop proteins that are expressed and translocated by DC3000 (Lindeberg *et al.*, 2006). In a previous study of

Table 1 Assay for ability of effectors to elicit cell death in *Nicotiana benthamiana* when delivered by the type III secretion system (T3SS) heterologously expressed by *Pseudomonas fluorescens* (pLN1965)^a

Effector	Plasmid	Cell death	Effector	Plasmid	Cell death
AvrE1	pLN2423	+	HopN1	pLN1324	-
AvrPto1	pLN1327 ^b	-	HopO1-1	pLN1622	-
AvrPtoB	pLN347 ^b	-	HopQ1-1	pLN348	+
HopA1	pLN1323	-	HopR1	pLN1154	-
HopB1	pLN271 ^c	-	HopT1-1	pLN256 ^b	+
HopC1	pLN50 ^c	-	HopU1	pLN223 ^c	-
HopD1	pLN167 ^c	-	HopV1	pLN517	+/-
HopE1	pLN162 ^c	-	HopX1	pCPP5068 ^b	+/-
HopF2	pLN1420	-	HopY1	pLN1528	-
HopG1	pLN460 ^c	-	HopAA1-1	pLN1326	+
HopH1	pLN150 ^c	-	HopAA1-2	pLN1419	-
HopI1	Not tested	-	HopAF1	pLN164 ^c	-
HopK1	pCPP5100 ^b	+	HopAM1 ^d	pCPP5063 ^b	-
HopM1	pLN1156	+	HopAO1	pLN130 ^e	-

^aLeaves were observed for confluent collapse of infiltrated areas 48 h after inoculation: + indicates that confluent necrosis was consistently observed; +/- indicates that necrosis was spotty or inconsistent; - indicates that necrosis was not observed. pLN1965 is a derivative of pLN18 (Jamir *et al.*, 2004) in which the $\Delta hopA1/shcA$ mutation is marked with Sp^R/Sm^R instead of Km^R. *P. fluorescens* (pLN1965) does not elicit cell death in *N. benthamiana*.

^b(Jamir *et al.*, 2004).

^c(Petnicki-Ocwieja *et al.*, 2002).

^dDC3000 carries two identical copies of *hopAM1* located on the chromosome (*hopAM1-1*) and pDC3000A (*hopAM1-2*).

^e(Espinosa *et al.*, 2003).

translocation using effectors with C-terminal Cya (*Bordetella pertussis* adenylate cyclase) fusions expressed from a *tac* vector promoter, we observed that two of nine translocated effectors tested (HopQ1-1 and HopK1) also elicited cell death in *N. benthamiana* (Schechter *et al.*, 2004). That study used *P. fluorescens* expressing cloned *P. syringae* T3SS genes to deliver individual effectors. Here we extended that analysis by using T3SS-proficient *P. fluorescens* and effectors expressed without Cya fusions expressed from the stronger *npt* promoter in pML123. *P. fluorescens* strains were infiltrated at 10⁸ colony forming units (CFU) ml⁻¹ into leaf tissue, and leaves were observed after 48 h for the development of visible necrosis. *P. fluorescens* expressing the T3SS without any effectors did not elicit necrosis, but eight of the effectors revealed some capacity to elicit necrosis in these assays (Table 1).

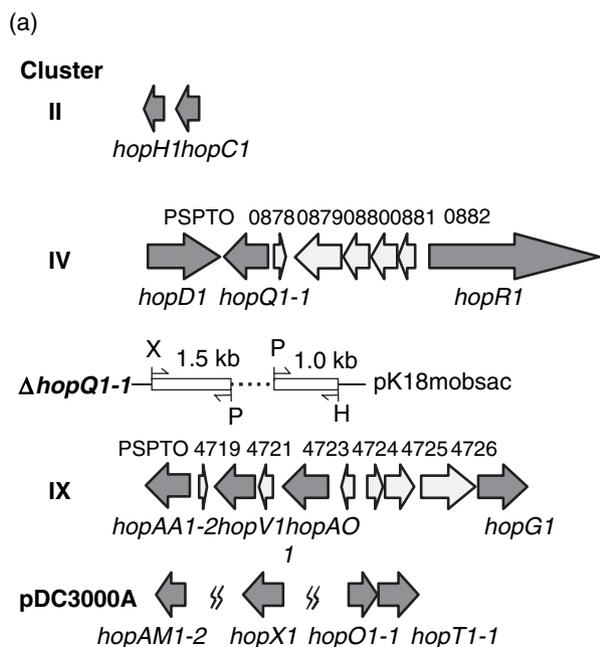
Pto DC3000 type III effector polymutants lacking various combinations of effector genes were constructed

More than half of the effector genes in DC3000 occur in ten clusters of two or more genes. These clusters are evident when the repertoire is ranked by PSPTO locus numbers (Schechter *et al.*, 2006) or visualized on the genome (<http://pseudomonas-syringae.org>), and they are presented in Table 2. We chose to delete four clusters containing a total of 13 active effector genes (Figure 1). These clusters encode four of the effectors with some capacity to elicit cell death in *N. benthamiana*. Cluster II contains *hopH1* and *hopC1*. Both effectors appear to be robustly produced in DC3000 (Chang *et al.*, 2005; Ferreira *et al.*, 2006), but their function is unknown. Cluster IV contains *hopD1*, *hopQ1-1* and *hopR1*. Cluster IX carries *hopAA1-2*, *hopV1*, *hopAO1* and *hopG1*. Weak phenotypes have been associated with individual *hopAA1-2* and *hopAO1* mutations (Badel *et al.*, 2002;

Table 2 Effector gene clusters in *Pseudomonas syringae* pv. *tomato* DC3000^a

Cluster	Effector genes
I	<i>hopU1</i> , <i>hopF2</i>
II	<i>hopH1</i> , <i>hopC1</i>
III	<i>hopAJ1</i> , (<i>hopAT1</i>)
IV	<i>hopD1</i> , <i>hopQ1-1</i> , <i>hopR1</i>
V	(<i>hopAG::ISPssy</i>), (<i>hopAH1</i>), (<i>hopAI1</i>)
VI	<i>hopN1</i> , <i>hopAA1-1</i> , <i>hopM1</i> , <i>avrE1</i>
VII	(<i>hopAH2-1</i>), (<i>hopAH2-2</i>)
VIII	<i>hopS2</i> , (<i>hopT2</i>), (<i>hopO1-3'</i>), <i>hopT1-2</i> , <i>hopO1-2</i> , (<i>hopS1::ISPssy</i>)
IX	<i>hopAA1-2</i> , <i>hopV1</i> , <i>hopAO1</i> , (<i>hopD::IS52</i>), (<i>hopH::ISPssy4</i>), <i>hopG1</i> , (<i>hopQ1-2</i>)
pDC3000A	<i>hopAM1-2</i> , <i>hopX1</i> , <i>hopO1-1</i> , <i>hopT1-1</i>

^aClusters are numbered in the order of their location on the DC3000 chromosome. Putative pseudogenes are indicated with parenthesis. Cluster VI is also known as the conserved effector locus.



(b)

Strain	Effector gene cluster(s) deleted			
	II	IV	IX	pDC3000A
CUCPB5439			Δ	
CUCPB5440		Δ		
CUCPB5445	Δ			
CUCPB5138				Δ
CUCPB5447		Δ	Δ	
CUCPB5448	Δ	Δ		
CUCPB5451	Δ	Δ	Δ	
CUCPB5452	Δ	Δ	Δ	Δ

Figure 1. Type III effector gene clusters that were deleted to produce *Pseudomonas syringae* pv. *tomato* DC3000 effector polymutants.

(a) The distribution of the 13 deleted effector genes in three chromosomal clusters and on plasmid pDC3000A. Arrows indicate the direction of transcription. PSPTO locus numbers are provided for all genes other than the *hop* (effector) genes. The construct used to delete *hopQ1-1* is aligned below cluster IV. The *hopQ1-1* mutant, CUCPB5460, was screened by PCR using primers P2299 and P2300 (H, *Hind*III; P, *Pst*I; X, *Xba*I).

(b) Strain designations and genotypes of effector polymutants.

Espinosa *et al.*, 2003). pDC3000A carries *hopAM1-2*, *hopX1*, *hopO1-1* and *hopT1-1*. Strain CUCPB5138 has been cured of pDC3000A and pDC3000B, the two plasmids present in the wild type, but it is not markedly reduced in virulence in *Arabidopsis* or tomato (Buell *et al.*, 2003). We deleted the chromosomal clusters, singly and in combination, using a system of which key components are a Gateway[®]-ready derivative of pRK415 and PCR entry clones that contain an FRT cassette ligated between sequences flanking the deleted target gene. The cassette-marked region can be rapidly

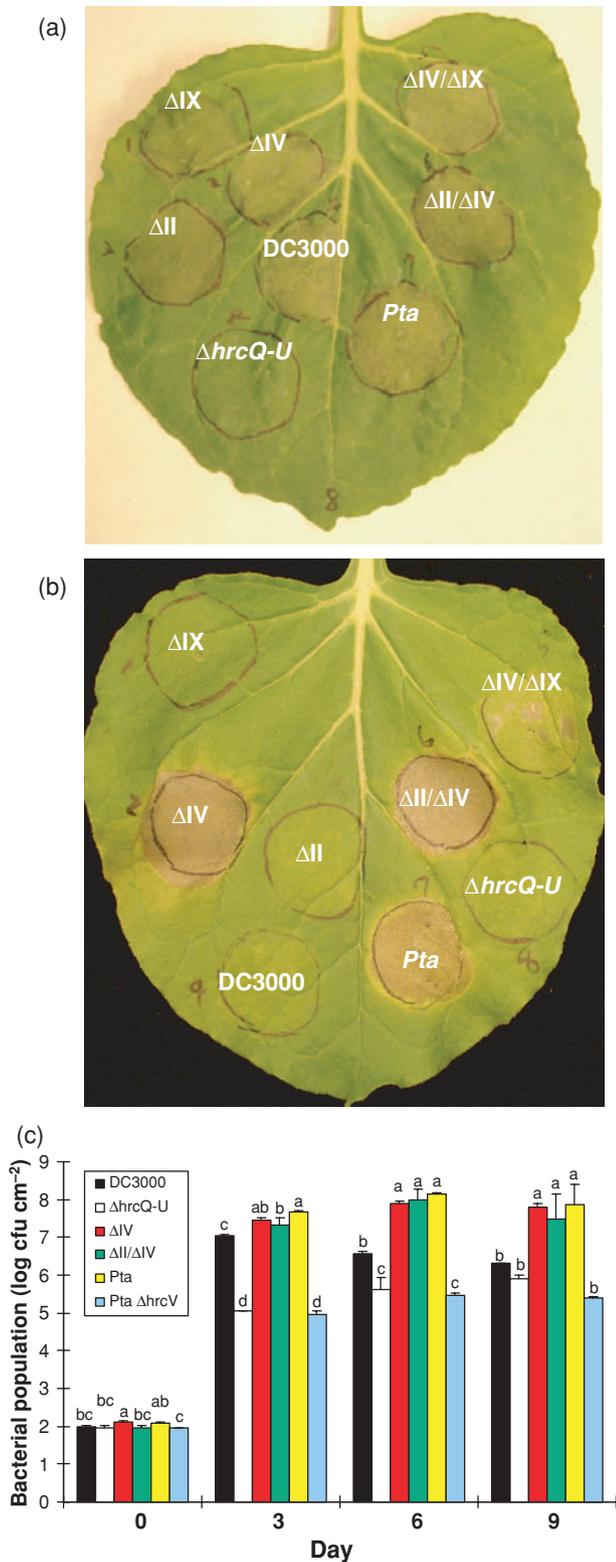
recombined into pRK415, transformed into *P. syringae*, and then introduced into the chromosome by homologous recombination. The cassette is then removed from the genome using the yeast Flp recombinase, which leaves the deletion marked only by FRT scars (Hoang *et al.*, 1998). All mutations were confirmed by PCR analysis using primers flanking the deletion, and polymutants with multiple deletions were simultaneously checked for the integrity of all of the deletions. Based on the predicted operon structure of genes associated with the T3SS effector system in DC3000 (Ferreira *et al.*, 2006), none of the deletions should have affected genes flanking those depicted in Figure 1a.

Pta DC3000 effector polymutants lacking cluster IV are able to cause disease in *N. benthamiana*

DC3000 and mutants lacking various combinations of the chromosomal effector gene clusters were inoculated into *N. benthamiana* leaves at 10^8 CFU ml⁻¹ using a blunt syringe. As expected, DC3000 elicited a rapid collapse of the infiltrated leaf tissue that is typical of the HR associated with type-II non-host resistance, but the T3SS-deficient Δ *hrcQ-U* mutant did not (Figure 2a). At this high level of inoculum, the compatible pathogen *Pta* 11528 also caused rapid cell death. In practice, low levels of inoculum are more useful in differentiating compatible and incompatible interactions, and we accordingly inoculated *N. benthamiana* leaves with DC3000 and effector mutants at 10^4 CFU ml⁻¹. Figure 2b shows symptoms on a representative leaf 10 days after inoculation. As expected, DC3000 did not produce visible necrosis, whereas *Pta* 11528 did. Surprisingly, the DC3000 mutant lacking effector gene cluster IV (hereafter referred to simply as Δ IV) caused extensive necrosis similar to that caused by *Pta*. In both cases, the necrosis developed after several days and spread beyond the area initially inoculated. This necrosis was not observed with the Δ IX and Δ II mutants. Interestingly, the necrosis induced by a Δ IV/ Δ IX mutant was markedly reduced in comparison with the Δ IV mutant.

We next analyzed the growth of DC3000 and the Δ IV mutant strains in *N. benthamiana* leaves following inoculation with a blunt syringe at 10^4 CFU ml⁻¹ (Figure 2c). DC3000 was able to grow in a T3SS-dependent manner early in the interaction, but then population levels declined. In contrast, the Δ IV mutant continued to grow for several days and attained population levels equivalent to that of *Pta*.

To further explore the ability of the DC3000 Δ IV mutant to cause disease in *N. benthamiana*, we inoculated whole plants by dipping them in 10^6 CFU ml⁻¹ of the test strains and observed symptoms after 8 days (Figure 3). Wild-type DC3000 caused no necrosis. In contrast, both the DC3000 Δ IV mutant and *Pta* 11528 caused extensive necrosis typical of *P. syringae* blight disease. The Δ IV/ Δ IX mutant also caused blight lesions, but these were much reduced. Surprisingly, the Δ II/ Δ IV mutant caused speck symptoms similar to those



caused by wild-type DC3000 in tomato. To further explore the virulence of the ΔIV mutant and its derivatives, we dipped *N. benthamiana* leaves in inoculum containing

Figure 2. *Pseudomonas syringae* pv. *tomato* DC3000 effector polymutants from which effector gene cluster IV was deleted cause necrotic symptoms and grow as well as *P. syringae* pv. *tabaci* in *Nicotiana benthamiana* leaves when inoculated at low levels.

(a) *N. benthamiana* leaf was infiltrated with the indicated strains at 10^8 CFU mg⁻¹ using a blunt syringe and photographed 1 day after inoculation.

(b) *N. benthamiana* leaf was infiltrated with the indicated strains at 10^4 CFU mg⁻¹ using a blunt syringe and photographed 10 days after inoculation.

(c) Bacterial growth in *N. benthamiana*. Bacteria were infiltrated at 10^4 CFU ml⁻¹ and populations were measured from three 0.6-cm-diameter leaf discs at 0, 3, 6 and 9 days after inoculation. Error bars indicate the standard deviation of populations measured from three leaf discs from each of two plants. Means with the same letter were not significantly different at the 5% confidence level based on Duncan's multiple range test. The experiment was repeated three times with similar results.

10^5 CFU ml⁻¹ and observed the symptoms 8 days later (Figure 3). At this lower level of inoculum, the ΔIV strain caused both blight and speck symptoms, whereas the $\Delta II/\Delta IV$ and $\Delta IV/\Delta IX$ mutants caused only speck lesions. In summary, these observations demonstrate that *Pto* DC3000 is capable of causing disease in *N. benthamiana* if the IV cluster is deleted, and that the character of the symptoms is altered by the repertoire of remaining effectors.

hopQ1-1 acts as an avirulence determinant in *N. benthamiana* for *Pto* DC3000 and *Pta* 11528

Our observations suggested that the cluster IV effector HopQ1-1 could be an avirulence determinant in the interactions of DC3000 with *N. benthamiana* (Table 1) (Schechter *et al.*, 2004). We accordingly deleted *hopQ1-1* singly from the DC3000 genome and inoculated *N. benthamiana* leaves with mutant and control strains at 10^4 CFU ml⁻¹. A representative leaf was photographed 8 days after inoculation and shows that the $\Delta hopQ1-1$ mutant produced necrosis that was even more extensive than that triggered by the ΔIV mutant at this time point, and equivalent to that of *Pta* (Figure 4a). Furthermore, the $\Delta hopQ1-1$ mutant grew in *N. benthamiana* leaves to the same population levels achieved by *Pta* (Figure 5), and expression of *hopQ1-1* *in trans* restored avirulence in *N. benthamiana* to the $\Delta hopQ1-1$ mutant (Figure S1).

To further test the notion that HopQ1-1 was acting as an avirulence determinant, we transformed *Pta* 11528 with pCPP3033, which expresses HopQ1-1-Cya (Schechter *et al.*, 2004) and inoculated *N. benthamiana* with wild-type and transformed *Pta* at both 10^4 and 10^5 CFU ml⁻¹ (Figure 4b). Wild-type *Pta* caused extensive necrosis at both levels of inoculum that was visible 8 days after inoculation. In contrast, the strain expressing HopQ1-1 failed to cause any symptoms at 10^4 CFU ml⁻¹ and only faint chlorosis at 10^5 CFU ml⁻¹. These observations suggest that HopQ1-1 can act as an avirulence determinant in *Pta*, and that this effector is the sole avirulence determinant responsible for the failure of DC3000 to cause disease in *N. benthamiana*.

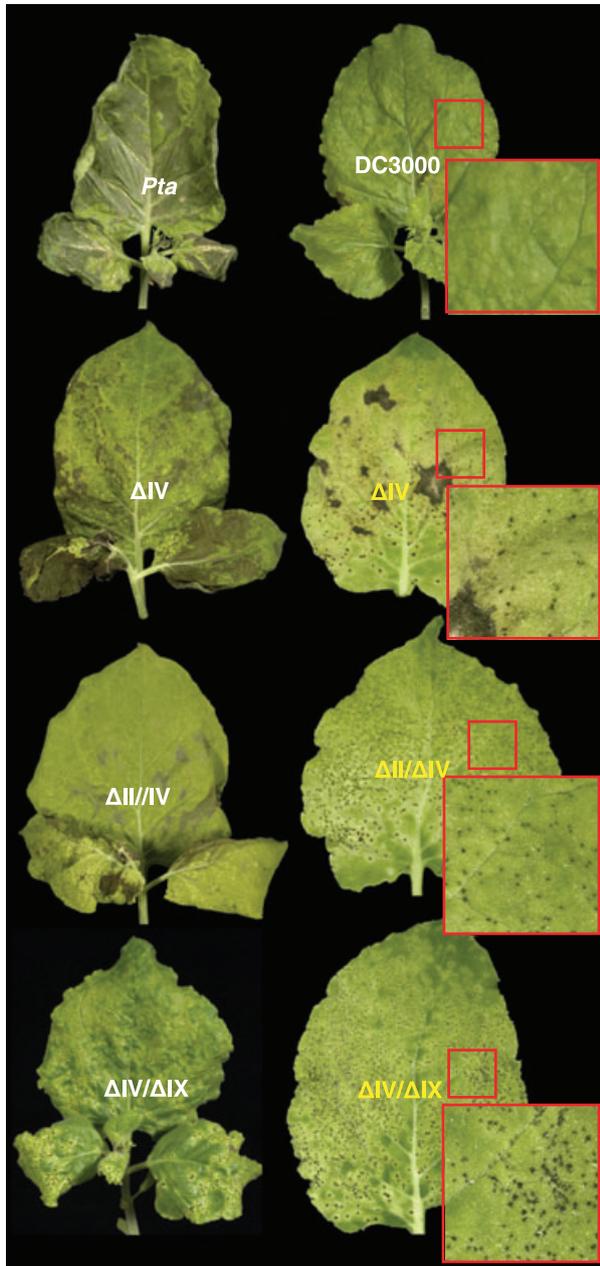


Figure 3. *Pseudomonas syringae* pv. *tomato* DC3000 mutants lacking effector gene cluster IV can cause speck-like disease lesions when dip-inoculated. Plants were dip-inoculated with the indicated strains and photographed 8 days after inoculation. Leaves labeled with white lettering were inoculated at 10^6 CFU ml $^{-1}$ and those labeled in yellow (the three directly beneath DC3000) were inoculated at 10^5 CFU ml $^{-1}$. Insets show details of symptoms in the marked leaf areas.

Silencing SGT1 in *N. benthamiana* blocks elicitation of cell death by HopQ1-1

Many resistance (R) proteins require the ubiquitin ligase-associated protein SGT1 to elicit the HR in response to

the presence or activity of cognate effectors (Peart *et al.*, 2002). Thus, SGT1 dependency is another indicator that cell killing by an effector represents avirulence activity mediated by R-protein detection. To determine the SGT1 dependency of the cell killing elicited by HopQ1-1 when delivered by *P. fluorescens* (pLN18), we used VIGS to block SGT1 production in *N. benthamiana*. Plants were inoculated with *A. tumefaciens* carrying tobacco rattle virus vector pTRV1 and pTRV2 \pm SGT1 (Peart *et al.*, 2002), and then 4 weeks later were inoculated with *P. fluorescens* carrying pCPP3303 (*hopQ1-1-cya*) and either pLN18 (T3SS $^+$) or pCPP3297 (T3SS $^-$) at 10^9 CFU ml $^{-1}$ (Schechter *et al.*, 2004). HopQ1-1 delivered by the T3SS elicited cell death in leaves that were untreated or infected with TRV::00, but not in leaves infected with TRV::SGT1 (Figure S2). As expected, no cell death was elicited in leaves inoculated with T3SS-deficient *P. fluorescens*. It is important to note that the Figure S2 photographs were taken 48 h after bacterial inoculations, and that no symptoms were visible at 24 h. Thus, the cell death elicited by *hopQ1-1* in *N. benthamiana* develops relatively slowly.

N. benthamiana is resistant to *P. syringae* pv. *phaseolicola* 1448A and *P. syringae* pv. *syringae* 61

N. benthamiana has recently been reported to be susceptible to wild-type *P. syringae* pv. *syringae* (*Psy*) B728a (Vinatzer *et al.*, 2006). Here, we tested *Psy* B728a as a positive control along with two other model strains: *P. syringae* pv. *phaseolicola* (*Pph*) 1448A and *Psy* 61. B728a and 1448A cause brown spot of bean and halo blight of bean, respectively, and both strains have been sequenced (Feil *et al.*, 2005; Joardar *et al.*, 2005). DC3000, B728a and 1448A represent each of the three major clades within the *P. syringae* pathovars (Sarkar and Guttman, 2004; Sawada *et al.*, 1999), and their effector repertoires have been extensively characterized (Chang *et al.*, 2005; Lindeberg *et al.*, 2006; Vencato *et al.*, 2006; Vinatzer *et al.*, 2006). Strain 61, which is pathogenic on bean, is the source of cosmid pHIR11, which carries an *hrp/hrc* cluster that has been extensively studied because it functions in non-pathogens, such as *P. fluorescens* and *Escherichia coli* (Huang *et al.*, 1988). Derivatives of pHIR11 are used in this work. At the low inoculum level of 10^4 CFU ml $^{-1}$ only *Pta* 11528 and *Psy* B728a produced disease lesions, and these developed markedly faster with B728a (Figure S3). At the high inoculum level of 10^8 CFU ml $^{-1}$ both *Pph* 1448a and *Psy* 61 produced HR-like confluent cell death. Thus, *N. benthamiana* displays type-II non-host resistance against *Pph* 1448a and *Psy* 61, and does not appear to be broadly susceptible to diverse *P. syringae* strains.

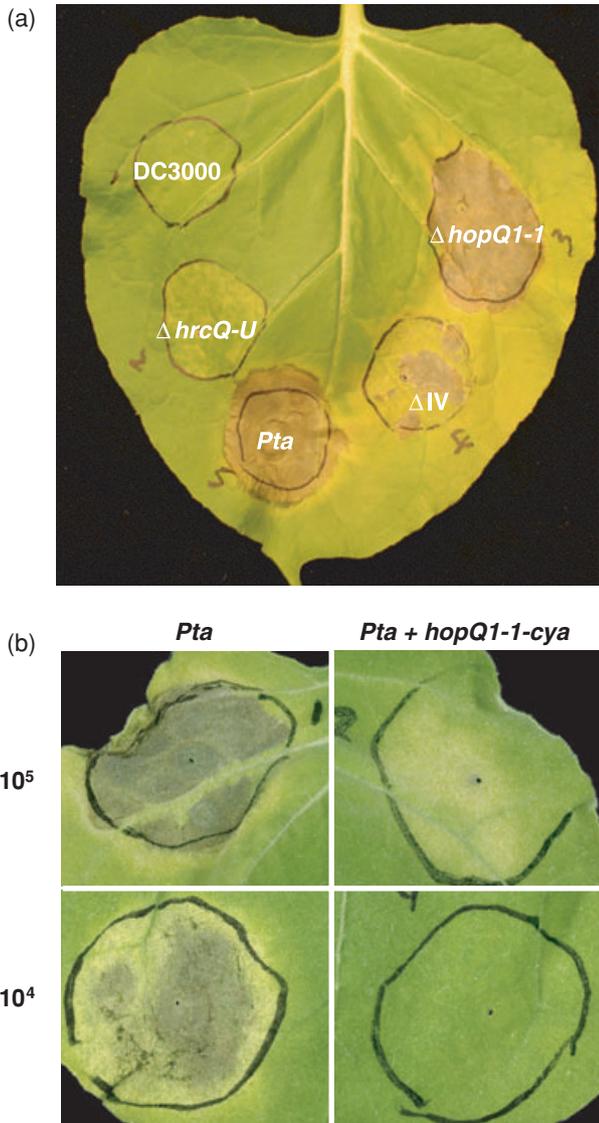


Figure 4. Evidence that HopQ1-1 is an avirulence determinant in *Nicotiana benthamiana* based on deletion of *hopQ1-1* from *Pseudomonas syringae* pv. *tomato* DC3000 and heterologous expression of *hopQ1-1* in *P. syringae* pv. *tabaci* (*Pta*) 11528.

(a) DC3000 $\Delta hopQ1-1$ mutant causes lesions in *N. benthamiana*. The leaf was infiltrated with the indicated strains at 10^4 CFU mg^{-1} using a blunt syringe and photographed 8 days after inoculation.

(b) *Pta* 11528 carrying pCPP3033 expressing HopQ1-1-Cya from a *tac* vector promoter is avirulent in *N. benthamiana*. Leaves were infiltrated with the indicated strains at 10^4 and 10^5 CFU ml^{-1} and photographed 8 days later.

A Pto DC3000 mutant lacking 13 effectors is strongly reduced in lesion formation, but not in growth, in N. benthamiana leaves

To further test the role of the effector repertoire in the ability of DC3000 strains lacking *hopQ1-1* to cause disease and grow to high levels in *N. benthamiana*, we deleted addi-

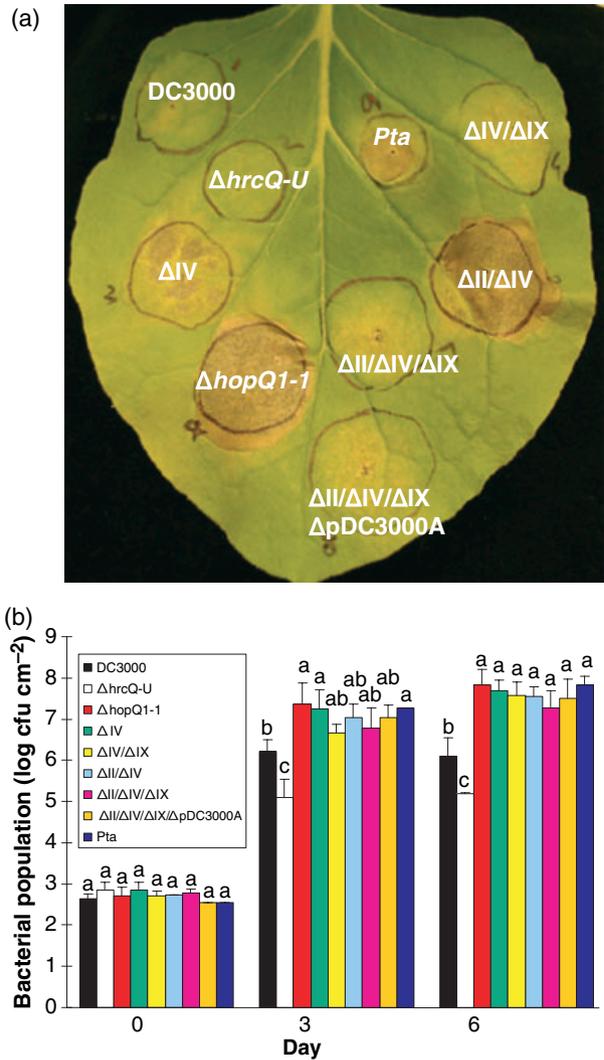


Figure 5. Deletion of effector gene cluster IX from *Pseudomonas syringae* pv. *tomato* DC3000 mutants lacking *hopQ1-1* results in a strong reduction in lesion symptoms without a commensurate effect on growth in syringe-infiltrated *Nicotiana benthamiana* leaves.

(a) *N. benthamiana* leaf was infiltrated with indicated strains at 10^4 CFU mg^{-1} using a blunt syringe and photographed 8 days after inoculation. *P. syringae* pv. *tabaci* (*Pta*) 11528 was used as a positive control.

(b) Bacterial growth in *N. benthamiana*. Bacteria were infiltrated at 10^4 CFU ml^{-1} and populations were measured from three 0.6-cm-diameter leaf discs at 0, 3 and 6 days after inoculation. Error bars indicate the standard deviation of populations measured from three leaf discs from each of two plants. Means with the same letter were not significantly different at the 5% confidence level based on Duncan's multiple range test. The experiment was repeated three times with similar results.

tional effector gene clusters from the strains already lacking cluster IV (Figure 1). These polymutants were inoculated into *N. benthamiana* leaves at 10^4 CFU ml^{-1} and examined for symptoms and growth 8 days later. The cluster IX deletion strongly reduced lesion formation, and this effect was not reversed by additional deletions (Figure 5a). Interestingly, deleting all four of the effector clusters addressed in

this work did not reduce the ability of the DC3000 derivatives lacking *hopQ1-1* to grow as well as *Pta* in *N. benthamiana* (Figure 5b).

Pto DC3000 effector cluster deletions reduce lesion formation and growth in Arabidopsis

To determine the contributions of the four tested effector gene clusters to the ability of DC3000 to cause disease lesions and grow in Arabidopsis, we inoculated Col-0 plants by vacuum infiltration with 10^5 CFU ml⁻¹ of each strain. Examination of the inoculated plants 6 days later revealed a striking reduction in lesions caused by the ΔIX mutant and the polymutants (Figure 6a). The mutant lacking all four of the test clusters produced virtually no lesions. Examination of bacterial population levels in Arabidopsis leaves after 4 days revealed that all strains containing the ΔII mutation were significantly reduced relative to wild-type DC3000 (Figure 6b). Plasmid pCPP5657, carrying *hopC1* and *hopH1*, restored wild-type growth in Arabidopsis to the ΔII mutant (Figure S4). The $\Delta II/\Delta IV/\Delta IX/\Delta pDC3000A$ polymutant was significantly reduced relative to the ΔII mutant but still showed significantly more growth than the $\Delta hrcQ-U$ mutant. It is noteworthy that the ΔIX mutant reduced lesion formation without a commensurate effect on growth *in planta*. In contrast, the similarly strong reduction in symptoms caused by the $\Delta II/\Delta IV$ mutant was accompanied by substantial growth reduction. Finally, the mutant lacking all four clusters was almost equivalent to the $\Delta hrcQ-U$ mutant in its reduced ability to cause symptoms in DC3000.

Deletion of multiple effector gene clusters reduces the virulence of Pto DC3000 in tomato

We similarly tested the contributions of the four deleted clusters of effector genes to the ability of DC3000 to cause bacterial speck symptoms and to grow in tomato (*Solanum lycopersicum* cv. Moneymaker). We first inoculated tomato plants by dipping them in the test strains at 10^6 CFU ml⁻¹, and observed symptoms 5 days after inoculation (Figure 7a). Wild-type DC3000 caused typical symptoms of bacterial speck, whereas the $\Delta hrcQ-U$ mutant produced no symptoms. The ΔIV mutant produced symptoms that were not substantially different from those produced by DC3000; however, the ΔII and ΔIX mutants produced substantially fewer bacterial speck lesions. The $\Delta II/\Delta IV/\Delta IX$ and $\Delta II/\Delta IV/\Delta IX/\Delta pDC3000A$ polymutants produced even fewer lesions. Figure 7a shows leaves that are representative of several independent experiments.

We also examined symptom development and bacterial growth in tomato leaves following inoculation with a blunt syringe at 10^4 CFU ml⁻¹. The extent of lesion formation was documented 5 days after inoculation by treating the leaves with Carnoy's solution, which clears leaf tissue and enhan-

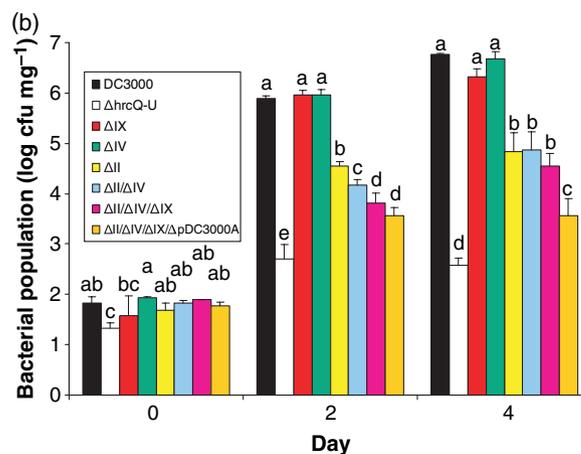
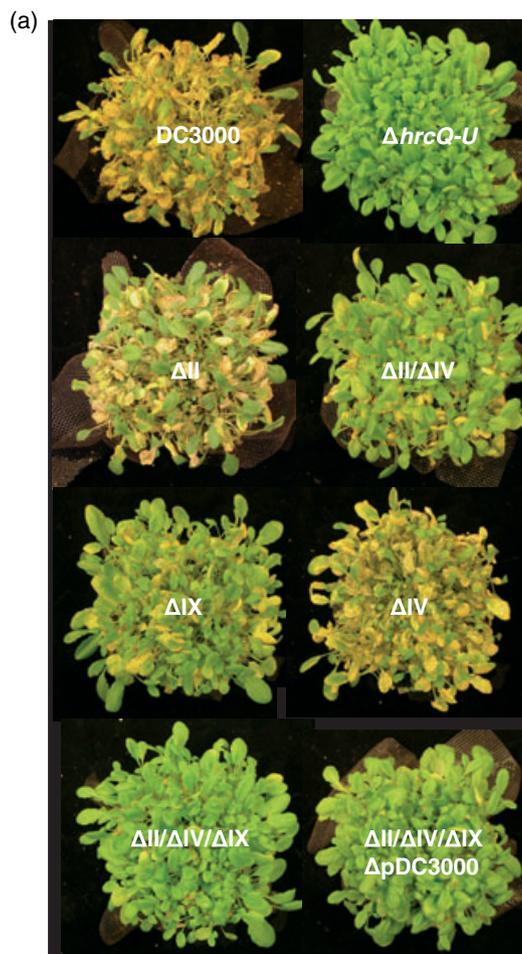


Figure 6. *Pseudomonas syringae* pv. *tomato* DC3000 effector polymutants are reduced in virulence and growth in Arabidopsis Col-0. (a) Arabidopsis Col-0 plants were vacuum infiltrated with the indicated strains at 10^5 CFU ml⁻¹ and photographed 6 days after inoculation. (b) Bacterial growth in Arabidopsis Col-0. Bacteria were vacuum infiltrated at 10^5 CFU ml⁻¹ and populations were measured from nine leaves at 0, 2 and 4 days after inoculation. Error bars indicate the standard deviation of populations measured from three leaves from each of three plants. Means with the same letter were not significantly different at the 5% confidence level based on Duncan's multiple range test. The experiment was repeated three times with similar results.

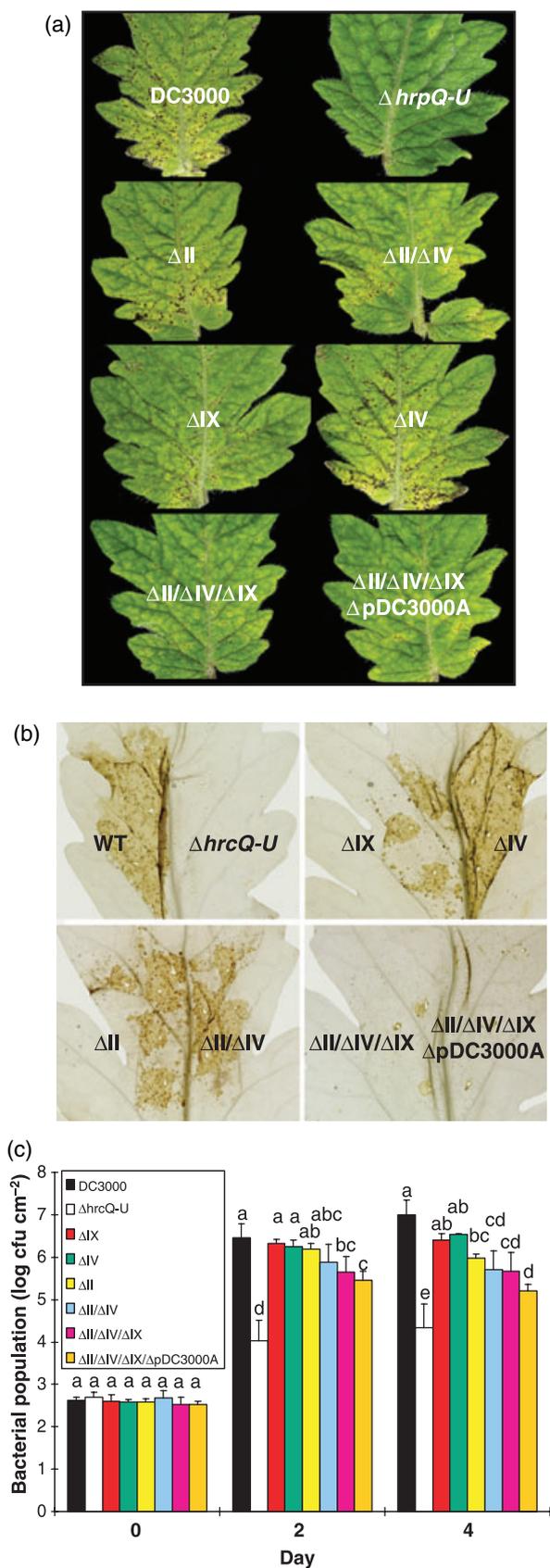


Figure 7. *Pseudomonas syringae* pv. *tomato* DC3000 effector polymutants are reduced in virulence and growth in tomato.

(a) Symptoms in tomato (*S. lycopersicum* cv. Moneymaker) leaves 5 days following inoculation by dipping with bacteria at 10^6 CFU ml⁻¹ carrying the indicated effector gene cluster deletions.

(b) Symptoms in tomato following inoculation with a blunt syringe of bacteria at 10^4 CFU ml⁻¹ carrying the indicated effector gene cluster deletions. Four replicate sets of tomato leaves were inoculated, and 5 days after inoculation the extent of lesion formation in representative leaves was documented by clearing the leaf with Carnoy's solution and then photographing the leaf.

(c) Bacterial growth in tomato. Bacteria were infiltrated at 10^4 CFU ml⁻¹ and populations were measured from three 0.6-cm-diameter leaf discs at 0, 2 and 4 days after inoculation. Error bars indicate the standard deviation of populations measured from three leaf discs from each of three plants. Means with the same letter were not significantly different at the 5% confidence level based on Duncan's multiple range test. The experiment was repeated three times with similar results.

ces visualization of bacterial speck lesions in tomato (Cohn and Martin, 2005). The leaves in Figure 7b, which are representative of several independent experiments, provide further evidence that the mutants fall into three classes regarding virulence in tomato: the ΔIV mutant is not discernibly reduced in virulence; the ΔII and ΔIX mutants show intermediate reduction; and the $\Delta II/\Delta IV/\Delta IX$ and $\Delta II/\Delta IV/\Delta IX/\Delta pDC3000A$ mutants are strongly reduced.

Regarding growth in tomato leaves, all strains containing the ΔII mutation were significantly reduced relative to wild-type DC3000 at 4 days post-inoculation (Figure 7c). The $\Delta II/\Delta IV/\Delta IX/\Delta pDC3000A$ polymutant was significantly reduced relative to the ΔII mutant but still showed significantly more growth than the $\Delta hrcQ-U$ mutant. The residual ability of the polymutant to grow in tomato leaves is consistent with its residual ability to produce scattered speck lesions in dip-inoculated leaves. In summary, in our experiments effector gene cluster IV did not make a significant contribution to the ability of DC3000 to grow or produce lesions in tomato, whereas cluster IX contributed to lesion formation only and cluster II contributed to both growth and lesion formation.

Discussion

We have discovered that *Pto* DC3000 produces multiple effectors that can elicit cell death in *N. benthamiana*, but that deleting just the *hopQ1-1* effector gene enables this model pathogen to extend its host range to an apparent non-host plant species. This observation provides further evidence for the role of effectors in determining host range at the pathovar-species level, and it establishes a useful new disease model for exploring type III effector functions. The latter point was validated by the similar effects of DC3000 effector gene polymutants on disease lesion formation in *N. benthamiana*, *Arabidopsis* and tomato (Table 3). Below we will discuss the nature of non-host resistance, the experimental merits of a DC3000-*N. benthamiana* disease model, factors controlling *P. syringae* host specificity and insights gained from effector polymutants.

Table 3 Summary of phenotypes of DC3000 mutants with effector gene clusters deleted

Mutant (CUCPB)	Cluster deleted	<i>N. benthamiana</i>		Arabidopsis		Tomato	
		Growth	Lesions	Growth	Lesions	Growth	Lesions
5445	II	Transient	None	Reduced	Reduced	Reduced	Reduced
5440	IV	Sustained	Blight/speck	WT	WT	WT	WT
5439	IX	Transient	None	WT	Reduced	WT	Reduced
5448	II/IV	Sustained	Reduced, speck only	Reduced	Few	Reduced	Reduced
5451	II/IV/IX	Sustained	Reduced, speck only	Reduced	Very few	Reduced	Strongly reduced
5452	II/IV/IX DC3000A	Sustained	Strongly reduced speck	Strongly reduced	None	Strongly reduced	Strongly reduced

Type-II non-host resistance

The simplest explanation for type-II non-host resistance against a *P. syringae* pathovar is that it is based on the same *R* gene surveillance of effector repertoires that operates in race-specific host resistance. This hypothesis is grounded on the seminal observation that *Pto* strain PT23 contains multiple effectors that confer avirulence to *P. syringae* pv. *glycinea* in various soybean cultivars, and thus could account for the failure of *Pto* to be a pathogen on soybean (Kobayashi *et al.*, 1989). Although mutagenesis of four of these effectors did not extend the host range of *Pto* to soybean (Lorang *et al.*, 1994), it is possible that additional uncharacterized effectors act as avirulence determinants in *Pto*–soybean interactions. Our results support the concept that type-II non-host resistance and host resistance have the same basis in *R*-gene surveillance of pathogen effector repertoires. Further support for this concept is found in the observation that *Pto* *R*-gene-mediated recognition of AvrPto and AvrPtoB is important in the defense of tomato against several pathovars of *P. syringae* (Lin and Martin, 2007).

The postulated *R* gene that mediates *N. benthamiana* recognition of HopQ1-1 is undefined and therefore its universality in this species is unknown. It is possible that susceptible genotypes exist for *N. benthamiana*. Indeed, the same possibility exists for any plant species showing type-II non-host resistance against a given pathovar of *P. syringae*. It is also possible that strains of *Pto* exist in nature that lack *hopQ1-1* and therefore would represent virulent races on *N. benthamiana*. Because *N. benthamiana* is not a crop plant, nothing is known about its susceptibility in the field to *P. syringae* pathovars or about the genotypic variation in that resistance. However, the lessons learned from a variety of crop plants suggest that species–pathovar interactions are relatively stable. Despite wide planting, a given crop species does not become susceptible to a growing collection of pathovars. For example, *Pta* is the only pathovar causing significant disease (wildfire or angular leaf spot, depending on toxin production) in tobacco (Shew and Lucas, 1991).

There is no reason to expect that the genotypes of *N. benthamiana* used in current research are unusual in their interactions with *Pto*, and our working conclusion is that *N. benthamiana* is normally a non-host for *Pto*.

In this regard, it is important to note that *N. benthamiana* is not susceptible to all *P. syringae* pathovars and showed type-II non-host resistance against *Psy* 61 and *Pph* 1448A, as well as against wild-type *Pto* DC3000. *N. benthamiana* is susceptible to wild-type *Pta* and has recently been reported to be susceptible to *Psy* B728a (Vinatzer *et al.*, 2006). Pathovar *P. syringae* is highly heterogeneous, so the differential virulence of strains B728a and 61 should not be interpreted as indicating race specificity. B728a causes brown spot of bean, and its effector repertoire is about half that of DC3000 (Lindeberg *et al.*, 2006; Vinatzer *et al.*, 2006). Importantly, B728a lacks any member of the HopQ effector family. It is similarly noteworthy that the Δ *hopQ1-1* mutation did not extend the host range of DC3000 to *N. tabacum* (data not shown), which indicates that the mutation does not confer some general virulence benefit to DC3000. Nor does T3SS-delivered HopQ1-1 elicit cell death in tomato (Badel *et al.*, 2006). Indeed, HopQ1-1 shows all of the hallmarks of a typical avirulence protein by conferring an avirulence phenotype to *Pta* and by eliciting an SGT1-dependent HR when delivered by the T3SS into *N. benthamiana*.

N. benthamiana as a useful laboratory host for investigating *Pto* DC3000–plant interactions

N. benthamiana has emerged as an important model in plant biology that has experimental advantages complementary to those of Arabidopsis. *N. benthamiana* is highly amenable to *A. tumefaciens*-mediated transient expression of foreign genes, its large leaves are easily infiltrated with multiple test bacteria, and it is amenable to the powerful technique of VIGS (Baulcombe, 1999; Kamoun *et al.*, 2003). For example, VIGS-based forward genetic screens in *N. benthamiana* have identified plant genes required for R protein perception of *P. syringae* effectors (del Pozo *et al.*, 2004), and *A. tumefaciens*

iens-mediated transient expression has been used to study effectors that suppress such defenses (Abramovitch *et al.*, 2003; Jamir *et al.*, 2004). *Pta* has been used in some of these studies to check the effects of silencing a target gene on a virulent pathogen. However, no strain of *Pta* has been sequenced and relatively little is known about the genetics underlying *Pta* virulence. In contrast, much is known about DC3000, and the development of an *N. benthamiana*-DC3000 disease model will enable the simultaneous use of genetics in both the host and pathogen to peel away layers of interacting factors. The use of VIGS in this report to demonstrate the requirement of SGT1 for HopQ1-1-elicited cell death is one example of the utility of the *N. benthamiana* pathosystem. It is worth highlighting that the *SGT1*-silencing experiment involved delivery of the test effector by a *P. syringae* T3SS expressed in *P. fluorescens*, which permits the effect on the plant to be studied in the absence of other effectors and without potential artifacts associated with *A. tumefaciens*-mediated transient overexpression. Thus, the *N. benthamiana*-DC3000 disease model supports rapid and relatively natural loss-of-function and gain-of-function experiments involving both the host and the pathogen.

Several features of the disease that DC3000 Δ *hopQ1-1* strains cause in *N. benthamiana* are noteworthy. Firstly, the symptoms that develop following dip inoculation at 10^5 CFU ml⁻¹ into *N. benthamiana* are remarkably similar to the bacterial speck symptoms caused by wild-type DC3000 in tomato. Importantly, the ability to quantify speck symptoms is useful in detecting subtle contributions of host and pathogen genes to the disease interaction. Secondly, deletion of effector gene clusters II and IX produced similar reductions in virulence in tomato and Arabidopsis. The similar symptoms and mutant phenotypes suggest that *N. benthamiana* genes found to condition the interaction with DC3000 will be relevant to tomato and Arabidopsis. Thirdly, the defenses of *N. benthamiana* against DC3000 appear to be quantitatively weaker than those of tomato and Arabidopsis. For example, the HR elicited by HopQ1-1 develops relatively slowly and wild-type DC3000 grows significantly during this period. More importantly, the Δ II/ Δ IV/ Δ IX/ Δ pDC3000A mutant is able to grow as well as *Pta* 11528 despite lacking nearly half of its effectors. This contrasts with the strongly reduced growth of this mutant in Arabidopsis and tomato. Thus, DC3000 requires fewer effectors for growth in *N. benthamiana*.

Comparing the diseases and effector repertoires associated with three P. syringae pathovars that are virulent on N. benthamiana

Wild-type *Psy* B728a has recently been shown to be able to cause disease in *N. benthamiana* (Vinatzer *et al.*, 2006). As with DC3000, the ability of B728a to grow and produce symptoms is dependent on the T3SS, but the symptoms are

distinct. B728a causes spreading necrotic lesions that are similar to those caused by *Pta* but differ from the speck symptoms caused by DC3000. Several individual effector genes were mutated in B728a, but none of these mutations reduced virulence in *N. benthamiana*. Comparing the effector repertoires of DC3000 and B728a is a useful first step in understanding the ability of these bacteria to cause disease in *N. benthamiana*. Unfortunately, no sequence data are available for *Pta*. However, a dot-blot analysis suggested that homologs of only three B728a effector genes were present in *Pta*: *hopI1*, *hopAE1* and *hopAG1* (Vinatzer *et al.*, 2006). In addition, this analysis revealed the presence of the harpin-like *hopAH1* and putative translocon component *hrpK1* genes in *Pta*. However, hybridization-based surveys of T3SS effector genes must be interpreted cautiously because they do not differentiate active genes from pseudogenes (which are common in *P. syringae* effector inventories). For example, the *hopAG1* homolog in DC3000 is a pseudogene (Schechter *et al.*, 2006), but we do not know the status of the *Pta* 11528 homolog.

Comparison of the complete effector repertoires of the phylogenetically distinct strains DC3000, B728a and 1448A suggests that all *P. syringae* strains are likely to carry an active member of the *avrE1*, *hopI1*, *hopX1*, *hopAB* and *hopAF* families (Lindeberg *et al.*, 2006). In addition, *hopM1* and *hopAA1-1* are two members of the conserved effector locus that appear universal although they are disrupted in some strains (Lindeberg *et al.*, 2006). *hopH1* is the only variably distributed effector gene that appears active and is shared between B728a and DC3000. Similarly, comparing the effector repertoires of the bean pathogens *Pph* 1448A and *Psy* B728a revealed *hopAE1* as the only effector gene that is not also carried by DC3000 (Lindeberg *et al.*, 2006). A recent analysis of 91 strains from 39 hosts by DNA hybridization, using a DC3000 microarray, also failed to reveal any effector repertoire profiles associated with strains based on their hosts of origin (Sarkar *et al.*, 2006).

Pto DC3000 effector gene polymutant phenotypes, the repertoire of effectors that can elicit cell death and the basis for host specificity

Polymutant phenotypes provide another perspective on the role of effectors in virulence and host specificity, and suggest that the virulence targets of type III effectors may be fundamentally the same in diverse plant species. The universal effectors are unlikely to be specialists for virulence in specific plant hosts, and there is no pattern in the repertoire of variably distributed effectors that correlates with host range. Furthermore, deletions in DC3000 involving two seemingly unrelated gene clusters for variable effectors have generally the same effect on all three host species tested. That is, deletion of cluster II (*hopH1* and *hopC1*) from DC3000 reduced virulence in *N. benthamiana* and reduced

both lesion formation and growth in Arabidopsis and tomato. Thus, the contribution of these two genes to virulence is clearly not plant specific. Similarly, deleting effector gene cluster IX (*hopAA1-2*, *hopV1*, *hopAO1* and *hopG1*) strongly reduced virulence but not growth in all three test plants. Furthermore, although these four genes contribute demonstrably to the virulence of DC3000 in *N. benthamiana*, they are lacking from B728a (although *hopAA1-2* is a paralog of a universal effector). The simplest explanation for these observations is that *P. syringae* pathovars acquire highly variable and redundant effector repertoires that have an innate potential to promote disease in a wide range of plant species, but promiscuity is thwarted by R-protein-mediated surveillance. The observation that effectors such as HopA1 can elicit genotype-specific resistance without an HR raises the possibility that even type-I non-host resistance against *P. syringae* may have the same basis in anti-effector surveillance (Gassmann, 2005).

Although several DC3000 effectors showed a potential to elicit cell death in *N. benthamiana* in tests involving T3SS-proficient *P. fluorescens*, only HopQ1-1 was found to act as an avirulence determinant. It is possible that some of these effectors would not elicit cell death in natural infections involving DC3000, where they would be expressed from native promoters and translocated in competition with other effectors. It is also possible that the cell killing observed with the T3SS-proficient *P. fluorescens* indicates avirulence activity that is normally masked by other effectors when the entire repertoire is delivered (Jackson *et al.*, 1999). Several effectors with the ability to suppress defense-associated programmed cell death have been reported in DC3000 (Abramovitch *et al.*, 2003; Jamir *et al.*, 2004). Our discovery here of multiple potential avirulence determinants highlights the potential importance of such suppressors in the effector repertoire. In this regard, it is interesting that all three of the effectors in the conserved effector locus (AvrE1, HopM1 and HopAA1-1) acted as potential avirulence determinants when tested in T3SS-proficient *P. fluorescens*, but are clearly not functioning as avirulence determinants in *hopQ1-1*-deficient DC3000 in *N. benthamiana*. This observation is consistent with a model in which core effectors involved in the interdiction of basal defense pathways are protected from R-protein surveillance by suppressor effectors (which may be dispensable and exchangeable in the face of surveillance).

Effector gene repertoires in *P. syringae* are now thought to be highly dynamic components of the genome. Analysis of sequenced genomes indicates that effector genes are horizontally acquired and also commonly disrupted by frameshifts or insertions of mobile genetic elements (Greenberg and Vinatzer, 2003; Lindeberg *et al.*, 2006). Furthermore, exposure to host defenses associated with R-gene-mediated race-specific resistance has recently

been shown to select for loss of an effector gene with avirulence activity (Pitman *et al.*, 2005). Given these observations and our finding that the loss of a single effector gene can extend the host range of *Pto* DC3000 to a new host species, it is puzzling that host specificity at the species-pathovar level appears relatively stable in the field. One explanation is that although a DC3000 *hopQ1-1* mutant may cause disease in *N. benthamiana* plants in the laboratory, the mutant lacks multiple adaptations for virulence on *N. benthamiana* in the field. According to this model, a spontaneous mutation of *hopQ1-1* in a field strain of *Pto* would not have a persistent benefit, even if *N. benthamiana* were widely planted. Another possibility is that effector repertoires have evolved to comprise interdependent components. Rapid advances in our ability to sequence and characterize effector repertoires in *P. syringae* strains that are tested on multiple plant species and studied in crop fields should help us understand whether incompatible effector repertoires are the primary factor limiting host range, or are more of an indicator of an underlying lack of fitness on non-host species. Ultimately a better understanding of the interactions of *P. syringae* and plants in agricultural and natural ecosystems will be needed.

Experimental procedures

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table S1, except that the pML123 derivatives used to express effector genes in *P. fluorescens* are given in Table 1 along with their source. Additional effector genes that were cloned into pML123 for this study were constructed as previously described (Jamir *et al.*, 2004), and primer sequences are available upon request. *E. coli* Top10 and DH5 α were used for general cloning and Gateway[®] manipulations. *E. coli* was grown in Luria-Bertani (LB) broth (Hanahan, 1985) at 37°C. *P. syringae* and *P. fluorescens* strains were grown on King's B (KB) medium at 30°C (King *et al.*, 1954). Antibiotics were used at the following final concentrations in $\mu\text{g ml}^{-1}$: ampicillin (Ap), 100; kanamycin (Km), 50; gentamicin (Gm), 10; rifampicin (Rif), 50; spectinomycin (Sp), 50; tetracycline (Tc), 10; and cycloheximide (Cx), 2. For marker exchange, Km, Gm and Sp were used at half concentration.

Recombinant DNA techniques

DNA manipulations and PCR were performed according to standard procedures (Innis *et al.*, 1990; Sambrook *et al.*, 1989). Oligonucleotide primers for sequencing or PCR were purchased from Integrated DNA Technology (<http://www.idtdna.com>). PCR was performed with either ExTaq (Takara Bio Inc., <http://www.takara-bio.com>) or Deep Vent polymerase (New England Biolabs, <http://www.neb.com>). All DNA sequencing was carried out at the Cornell Bioresource Center with an ABI 3700 automated DNA sequencer (Applied Biosystems, <http://www.appliedbiosystems.com>). DNA sequences were analyzed with the VECTOR NTI software package (Infomax; Invitrogen, <http://www.invitrogen.com>).

Pto DC3000 effector gene cluster deletions

Unmarked deletions were constructed in each of the three chromosomal effector gene clusters using PCR-amplified flanking sequences (see Table S2 for primers). Primers P1963/P1946 and P1947/P1964 were used to PCR amplify 1.0-kb regions flanking cluster IX. The flanks were joined by splicing by overlap extension (SOEing) PCR and TOPO[®] cloned into pENTR/D-TOPO (Horton *et al.*, 1989). The FRT Sp/Sm^R cassette was amplified from pCPP5242 with primers P1696/P1697 and cloned between the flanks at a primer-introduced *Bam*HI site. The entry vector was then LR recombined (using Gateway LR Clonase) with pCPP5301 to create pCPP5397. Similarly, the 1.7- and 1.5-kb regions flanking cluster IV were amplified with primers P2285/P2286 and P2287/P2288, respectively. The flanks were joined by SOEing PCR and TOPO[®] cloned into pENTR/SD/D-TOPO. The FRT Gm^R cassette was amplified from pCPP5209 with primers P2293/P2294 and cloned between the flanks at a primer-introduced *Hind*III site. The entry vector was then LR recombined with pCPP5301 to create pCPP5398. Also, similarly, the 1.5- and 1.3-kb regions flanking cluster II were amplified with primers P2289/P2290 and P2291/P2292, respectively. The flanks were joined by SOEing PCR and TOPO[®] cloned into pENTR/SD/D-TOPO. An FRT Gm^R cassette was amplified from pCPP5209 with primers P1483/P1484 and cloned between the flanks at a primer-introduced *Xho*I site. The entry vector was then LR recombined with pCPP5301 to create pCPP5399.

Plasmids carrying the *hop* cluster deletions were transferred into DC3000 and derivative strains by conjugation using an *E. coli* 517-1 donor (Siman *et al.*, 1983) and then marker-exchanged into the chromosome as previously described (Alfano *et al.*, 1996). Plasmid pCPP5264 (*Flp*⁺) was then introduced into each mutant by conjugation to delete the FRT cassettes, leaving an 84-bp FRT scar. To construct polymutants, the same process was repeated for each deletion. Independently constructed deletions produced the same phenotype *in planta*. Deletion of clusters IX, IV and II was confirmed by PCR primers P1967/P1968, P1970/1971 and P1973/1974, respectively. All of the deletions in polymutants were confirmed by simultaneous use of all of the relevant primers. To complement the Δ II mutation, PCR primers P1975 and P1736 were used to clone the *hopC1-hopH1* gene cluster from DC3000 into pENTRSD/D to produce pCPP5656. The *hopC1-hopH1* gene cluster was subsequently LR recombined into broad-host-range vector pBS46, a Gateway-ready vector adapted from pBBR1MC55 (Kovach *et al.*, 1995), to produce pCPP5657.

Deletion of *Pto* DC3000 *hopQ1-1*

To make CUCPB5460, the *hopQ1-1* mutant of DC3000, 1.5- and 1.0-kb regions flanking *hopQ1-1* were amplified by PCR with the primers P2295/P2296 and P2297/P2298, respectively. These two fragments were ligated via primer-introduced *Pst*I sites and cloned into the mobile suicide vector pK18*mobsacB* (Schafer *et al.*, 1994). The resulting vector, pCPP5608, was electrotransformed into *E. coli* S17-1. The plasmid was transferred from *E. coli* S17-1 into DC3000 by conjugation. Integrants were selected with Km and then plated onto KB plates containing 10% sucrose for 2 days at 25°C to counter-select the integration. Km-sensitive colonies were screened by PCR using the primers P2299 and P2300. To complement the Δ *hopQ1-1* mutation, the *hopQ1-1* gene carried in pCPP3373 (Schechter *et al.*, 2004) was LR recombined into broad-host-range vector pBS46 to produce pCPP5655, which expresses *hopQ1-1* from a vector *Npt* promoter and generates a C-terminal fusion of the protein product with an HA tag.

Plant growth and virulence assays

Fully expanded and healthy leaves of 8-week post-germination tobacco (*N. tabacum* cv. Xanthi), 6-week post-germination *N. benthamiana*, 4-week post-germination tomato (*S. lycopersicum* cv. Moneymaker) plants and 4-week post-germination *Arabidopsis thaliana* Col-0 were used for virulence assays. *N. benthamiana*, tomato and tobacco plants were grown under greenhouse conditions and transferred to the laboratory 1 day prior to inoculation with a blunt syringe, or were transferred to a growth chamber with 95% humidity at 25°C with 12-h illumination 1 day prior to inoculation by dipping. *Arabidopsis* was grown and incubated in a growth chamber at 22°C with 12-h illumination. Plants were inoculated by blunt syringe as previously described (Alfano *et al.*, 1996). Strains were inoculated at 10⁸ CFU ml⁻¹ for HR assays and at 10⁴ CFU ml⁻¹ for virulence assays. For *Arabidopsis* vacuum infiltration, bacteria were diluted to 10⁵ CFU ml⁻¹ in water containing 0.01% Silwet L-77. Plants were dipped upside down in 200 ml of bacterial suspension and a vacuum was applied to 58 kPa followed by a slow release to infiltrate the leaves uniformly. For *N. benthamiana* and tomato dip-inoculation, bacteria were diluted to 10⁶ or 10⁵ CFU ml⁻¹ in water containing 0.02% Silwet L-77. Plants were submerged upside down in 1 L of bacterial suspension and swirled for 30 s. *N. benthamiana* plants were then incubated in a growth chamber with 12-h illumination and 95% humidity at 25°C. Tomato plants were incubated in a growth chamber with 12-h illumination and 50% humidity at 25°C. To aid visualization of lesions in tomato leaves in some experiments, leaves were destained using Carnoy's fluid (10% acetic acid, 30% chloroform and 60% ethanol) prior to photography. To measure bacterial growth, three leaves from *Arabidopsis* plants or three leaf discs from *N. benthamiana* and tomato leaves were ground in 300 ml 10 mM MgCl₂ and 100 mM sucrose, and serial dilutions were spotted onto KB medium with Rif and Cx. CFU were counted 2 days after incubation at 28°C.

Virus-induced gene silencing

The TRV vector and pTRV2::*SGT1* were described previously (Liu *et al.*, 2002; Peart *et al.*, 2002). Cultures of *A. tumefaciens* GV2260 (4 ml) containing pTRV1, pTRV2 or pTRV2::*SGT1* were grown for 16–18 h in LB broth supplemented with 100 µg ml⁻¹ Rif and 30 µg ml⁻¹ Km. Cells were pelleted, washed and resuspended in infiltration buffer [10 mM MgCl₂, 10 mM 2-(*N*-morpholine)-ethanesulphonic acid (MES), pH 5.5, 150 mM acetosyringone]. Three hours after induction at room temperature, *A. tumefaciens* containing pTRV1 and pTRV2 (\pm *SGT1* insert) were mixed in a 1:1 ratio to a final OD₆₀₀ of 0.3. Leaves and cotyledons of 2-week-old *N. benthamiana* seedlings were infiltrated using a blunt syringe. Plants were then grown for 4 weeks to allow silencing to occur.

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Expression of *hopQ1-1* in *trans* restores avirulence in *Nicotiana benthamiana* to *Pseudomonas syringae* pv. *tomato* Δ *hopQ1-1* mutant CUCPB5460.

Figure S2. Silencing *SGT1* in *Nicotiana benthamiana* blocks elicitation of cell death by HopQ1-1.

Figure S3. *Nicotiana benthamiana* is susceptible to *Pseudomonas syringae* pv. *tabaci* (Pta) 11528 and *P. syringae* pv. *syringae* (Psy) B728a, but displays type-II non-host resistance against *P. syringae* pv. *phaseolicola* (Pph) 1448A and *P. syringae* pv. *syringae* (Psy) 61.

Figure S4. Wild-type growth in Arabidopsis leaves is restored to *Pseudomonas syringae* pv. *tomato* Δ II mutant CUCPB5445 by *hopH1* and *hopC1* carried on plasmid pCPP5657.

Table S1. Strains and plasmids used in this study.

Table S2. Primers used to construct and analyze mutations.

This material is available as part of the online article from <http://www.blackwell-synergy.com>.

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