USING NATURAL VARIATION TO STUDY THE EVOLUTION OF THE TOMATO RESISTANCE GENE PTO AND THE PSEUDOMONAS SYRINGAE POPULATION PRESENT IN NEW YORK

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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January 2017

USING NATURAL VARIATION TO STUDY THE EVOLUTION OF THE TOMATO RESISTANCE GENE PTO AND THE PSEUDOMONAS SYRINGAE POPULATION PRESENT IN NEW YORK

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Cornell University 2017

The use of natural variation is a powerful tool to study the evolution of plant resistance genes and pathogen effectors. In tomato, the Pto protein kinase interacts directly with two *Pseudomonas syringae* pv. *tomato* (*Pst*) effectors, AvrPto and AvrPtoB. This direct interaction is mediated by the binding of each effector through both a shared and a unique interface with Pto. The presence of two unique interfaces suggested that the recognition of these two effectors by Pto might have evolved independently. We conducted a screen of wild tomato accessions for their ability to mount effector-triggered immunity upon recognition of AvrPto or AvrPtoB to seek evidence of natural variation that would shed further light on how Pto-like kinases recognize and respond to two structurally different effectors. Our screen of wild relatives of tomatoes uncovered 22 accessions of *Solanum chmielewskii* (*Schm*) that recognize only AvrPtoB. Through further molecular characterization we found that a single histidine-to-aspartate substitution at position 193 in the activation domain of *Schm* Pto-2677 was sufficient to confer recognition of AvrPto in plant cells. The reciprocal substitution of aspartate-to-histidine-193 in Pto abolished AvrPto recognition, confirming the importance of this residue for signaling in response to AvrPto. Our results reveal that there are not only distinct binding interfaces involved in the Pto response to these effectors, but that there is also a difference in downstream signaling.

Based on recent worldwide collections of *Pst* isolates it is known that race 1 strains have

displaced race 0 strains and are now the most common strains found in the field. Race 0 strains express AvrPto or AvrPtoB and elicit resistance in *Pto*-expressing tomato lines, whereas race 1 strains lack these effectors and do not elicit resistance. We screened *Pst* isolates from infected field tomato plants across New York (NY) in 2015 and characterized them for their virulence and for the presence of specific effectors. We found that all isolates encode a functional AvrPto, which can be recognized by Pto. However, this recognition is 'masked' during later stages of infection, allowing development of mild bacterial speck symptoms in *Pto*-expressing tomatoes when vacuum infiltrated with high bacterial populations and under laboratory conditions. Our study demonstrates that introgression of *Pto* is still a viable strategy to manage bacterial speck of tomatoes in NY.

BIOGRAPHICAL SKETCH

Christine Maria Kraus was born and raised in Lima, Peru. After completing the German high school in 2003, she moved to Vienna, Austria to continue her education. Christine attended the University of Vienna were she received her Bachelor and Master of Science in Molecular Biology in 2011. For her Master thesis, Christine joined the laboratory of Prof. Gregory Martin as a visiting scientist for one year where she worked on the characterization of the tomato protein Bti9 as a potential microbe-associated molecular pattern receptor of tomato. It was during this year that she decided to continue studying the molecular basis of plant-microbe interactions. Christine joined the Department of Plant Pathology and Plant-Microbe Biology at Cornell University in 2011 to continue her research under the supervision of Dr. Martin. Her worked focused on the use of natural variation present in wild tomato accessions as well as *Pseudomonas syringae* field isolates to identify and characterize key proteins involved in this plant-microbe interaction.

ACKNOWLEDGMENTS

I would like to thank my advisor Dr. Gregory Martin for giving me the opportunity to work in his lab as well as for all the guidance that shaped me to be a better scientist. His trust gave me the courage to test new hypotheses, conduct original experiments and to present my data to the public. I am grateful to Dr. Alan Collmer, Dr. Susan McCouch and Dr. Larry Smart for being more than just part of my special committee but also for being great mentors.

Many scientists at BTI and Cornell for their scientific help and friendship. I was very lucky to be part of the BTI Postgraduate Society (PGS) and the Plant Pathology Graduate Association (PPGSA); I enjoyed every event we organized and I am very thankful for all the great people I met there.

I am very appreciative for all my lab colleagues, for their great support, and for sharing their knowledge with me. My special thanks goes to Dr. Johannes Mathieu, Dr. Patrick Boyle and Dr. Patricia Manosalva for truly showing me that science is about hard work and curiosity even if that means staying in the lab until late hours in the night. You all made this experience special and thank you for still remaining genuine friends.

Foremost, I would like to thank my family for always being there for me and for all your unconditional support throughout my life.

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LIST OF ABBREVIATIONS

CBEL	cellulose binding elicitor lectin
CERK1	Chitin elicitor receptor kinase 1
EF-Tu	Elongation factor Tu
EFR	EF-Tu receptor
EIX	Ethylene-inducing xylanase
ETI	Effector-triggered immunity
FLS2	Flagellum sensing 2
HR	Hypersensitive response
IP	Invasion pattern
IPR	Invasion pattern receptor
LRR-RLK	Leucine rich repeat receptor like kinases
MAMPS	Microbe-associated molecular patterns
MPK4	Mitogen-activated protein kinase 4
NB-LRR	Nucleotide binding site-leucine rich repeat
Nep1	necrosis- and ethylene-inducing-like proteins 1
PCD	Programmed cell death
Prf	*Pseudomonas* resistance and fenthion sensitivity

PRR	Pattern recognition receptor
Pst	*Pseudomonas syringae* pv. *tomato*
PTI	Pattern-triggered immunity
Pto	Resistance against *P. syringae* pv. *tomato*
QRL	Quantitative Resistance Loci
R	Resistance
RIN4	Resistance to *Pseudomonas maculicula* protein 1 interacting protein 4
ROS	Reactive oxygen species
Rpg1b	Resistance to *Pseudomonas glycinea* 1b
Rpg1r	Resistance to *Pseudomonas glycinea* 1r
RPS2	Resistance to *Pseudomonas syringae* protein 2
RPS4	Resistance to *Pseudomonas syringae* 4
RPT2	Resistance to *Pseudomonas syringae* pv. *tomato*
RRS1	Resistant to *Ralstonia solanacearum* 1
Rsb	Resistance suppressed by AvrPtoB C terminus
Schm	*Solanum chmielewskii*
SUMM2	Suppressor of mkk1 mkk2
T3SS	Type III secretion system
TGase	Calcium-dependent cell transglutaminase

CHAPTER 1

INVESTIGATING PLANT IMMUNITY AND BACTERIAL PATHOGENICITY USING THE TOMATO-*PSEUDOMONAS SYRINGAE* PATHOSYSTEM

1.1 INTRODUCTION

Pathogens are defined as microorganisms that can be detrimental to the fitness of their host causing serious epidemics in humans, animal or plant populations. The human immune response to pathogens is relatively well characterized and understood. While our knowledge of the interplay between the plant immune system and its perception of pathogens still falls short of that of its human counterpart, major advances in our understanding of the perception of plant pathogens and the following immune response in plants have been made during the past decade. One of the biggest differences between human and plant immune systems is that plants do not have an adaptive immune system based on specialized, motile cells. Instead, plants rely on pre-existing and inducible defense mechanisms, forming an elaborate multilayer surveillance system that is present in each single cell (Maekawa et al., 2011).

The constitutive or "integral" defenses that constitute the first level of protection are physical barriers such as a thick cell wall, waxy epidermal cuticles and bark which makes it difficult for microbes to access the intracellular compartments where nutrients are stored (Agrios, 1997). Additionally, plants also produce a large and diverse number of antifungal or antimicrobial compounds. These form chemical barriers that can be deadly to microbes when they come in contact with these compounds (Agrios, 1997). In contrast to the integral defense, which is to a certain extent always present as part of the normal plant body plan, inducible defenses require a stimulus to be activated and are divided into

two layers of immune response referred as to as pattern-triggered immunity (PTI) or effector-triggered immunity (ETI) (Chisholm et al., 2006; Dangl and Jones, 2001).

The immune system of plants can be divided into two layers of defense responses

PTI relies on recognition of conserved molecules of essential microbial structures known as microbe-associated molecular patterns (MAMPS) by cell membrane localized immune receptors (Altenbach and Robatzek, 2007). These pattern recognition receptors (PRRs) can specifically recognize one of these molecules and trigger a PTI response, either autonomously or with the help of an accessory cytoplasmic regulatory protein (Nurnberger and Kemmerling, 2009). The best-studied MAMPs are bacterial flagellin (Felix et al., 1999), elongation factor Tu (EF-Tu) (Kunze et al., 2004), and the fungal cell wall molecule chitin (Baureithel et al., 1994; Ito et al., 1997). All these molecules are parts of structures essential for the survival of members of their specific microbial kingdoms, leading to their conservation among members of this clade. This conservation makes them excellent microbial recognition targets for the plant using PRRs such as FLS2, EFR and CERK1 respectively (Altenbach and Robatzek, 2007; Boller and Felix, 2009; Miya et al., 2007; Zipfel et al., 2006; Zipfel et al., 2004). MAMPS can also be present in non-pathogenic microbes that do not pose a threat to the plant host. Consequently, the response is relatively mild and consists mostly in cell wall fortification at the site of MAMP detection and release of reactive oxygen species (ROS) to the outside of the cell. Nonetheless, these measures are sufficient to inhibit infections by potentially virulent but non-adapted pathogens.

PTI is viewed as a general host resistance response recognizing widely conserved MAMPS; however, several studies have shown that some recognized MAMPs can be present only in a somewhat narrow group of pathogens (Bittel and Robatzek, 2007). The rice Xa21 PRR confers immunity against *Xanthomonas oryzae* pv. *oryzae* (*Xoo)* strains by recognizing a 17 base pair sulfonated epitope of the *Xoo* protein Ax21. Although

Ax21 is present in other *Xanthomonas* species, it is not sulfonated and thus not recognized by Xa21 (Lee et al., 2006; Pruitt et al., 2015). Pep-13 is a MAMP derived from a calcium-dependent cell transglutaminase (TGase) protein that is conserved in *Phytophthora* species, but not in other oomycetes (Brunner et al., 2002). There are also plant PRRs that are restricted to a very narrow group of hosts. For example the PRR involved in detecting cold shock protein in *Solanum,* and FLS3, the receptor responsible for detecting flg28 epitope from flagellin, is only present even in the smaller subgroup *Solanoideae* (Hind et al., 2016; Wang et al., 2016)

A conceptual hallmark of MAMPs is strong structural conservation necessitated by the fitness penalty that alteration would incur. Nevertheless, it has been observed that the recognition of a MAMP by a PRR exerts selective pressure to alter the recognized epitopes. The bacterial flagellum epitope flg22 can be recognized by FLS2 (Gomez-Gomez and Boller, 2000; Zipfel et al., 2004). This system is well studied because FLS2 is widely conserved across both monocotyledonous and dicotyledonous plant species, including the model systems tomato and *Arabidopsis*. It has been shown that significant variation exists between wild tomato accessions and heirloom tomatoes in their ability to recognize flg22 from *Pseudomonas syringae* pv. *tomato* (*Pst*) (Veluchamy et al., 2014). Likewise, when flg22 epitopes of diverse bacteria were compared, they elicited different degrees of ROS production in tomatoes. A closer look at the epitope peptide sequences revealed amino acid variability (Felix et al., 1999; Pfund et al., 2004). These examples point to a flexible immune response by PTI, molded by continuous host-pathogen co-evolution.

The last line of defense is known as effector-triggered immunity or ETI. Successful pathogens express virulence proteins known as effectors that are translocated into the plant cell to suppress PTI and to manipulate host cellular pathways to their advantage. Resistance (R) proteins monitor the cell for direct or indirect evidence of an invasive

pathogen by detecting effectors either directly or by the biochemical activities that underlie their function inside the host cells. Recognition of effectors by R proteins triggers a strong response, often involving programmed cell death (PCD) as part of a process called hypersensitive response (HR) to limit pathogen spread (Dodds and Rathjen, 2010; Greenberg and Yao, 2004; Lam et al., 2001). In essence plant cells detecting the presence of effectors commit suicide to inhibit or halt microbial proliferation.

ETI is thought to be a pathosystem-specific immune response eliciting strong selective pressure on both pathogens and hosts to co-evolve. However, there is evidence of *R* genes that can recognize effectors from diverse microbes, even across kingdoms. For example, maize Rxo1 triggers an HR response after recognition of the bacterial *X. oryzae* pv. *oryzicola* AvrRxo1 effectors, as well as an unrelated and unknown *Burkholderia andropogonis* effector (Zhao et al., 2005; Zhao et al., 2004); the *Arabidopsis* R protein duo RRS1 and RPS4 confers resistance not only against the fungal pathogen *Colletotrichum higgindisnum,* but also against *Ralstonia solanacearum*, a bacterial wilt pathogen (Narusaka et al., 2009).

Host-pathogen coevolution

Plants are constantly exposed to many pathogens including viruses, bacteria, fungi and nematodes. While they developed defense mechanisms to keep infectious agents at bay, the pathogens are also under a selective pressure to overcome these defenses and re-gain the ability to infect the host to get access to nutrients. This constant “back and forth” co-evolution has been represented nicely by the zigzag model proposed by Jones and Dangl in 2006 (Jones and Dangl, 2006).

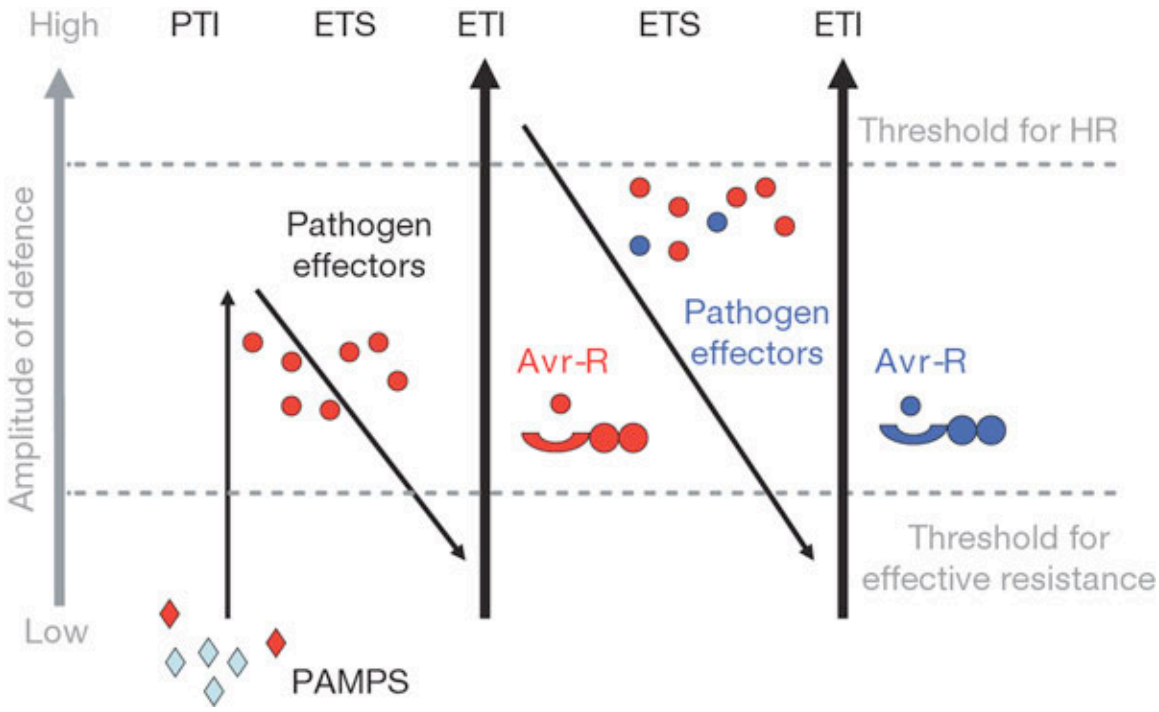

Figure 1.1. Zig-Zag model proposed by Dangl and Jones for ETI and PTI (Jones and Dangl, 2006).

This model formalized the delivery of virulence effectors into the host cell as a measure to suppress the activation of PTI after its activation in response to the detection of pathogen MAMPS by plant cells PRRs (Guo et al., 2009). Plants were then proposed to have evolved dedicated detector R proteins in response, to perceive secreted effectors and thus trigger a strong, secondary ETI response. Some pathogens have evolved a novel set of effectors that can interfere with and prevent ETI signaling, reestablishing the chances for infection (Abramovitch et al., 2003; Abramovitch and Martin, 2004; Jamir et al., 2004; Rosebrock et al., 2007). This new set of effectors could again be recognized by a new set of R proteins and so forth. This constant battle puts evolutionary pressure on both the host and the pathogen to counter each other. The formalization of these evolutionary processes in the zigzag model has helped the plant pathology community to represent these dynamics in an accessible visual form. However, the hierarchical representation of the different levels of the plant immune system can lead to the misconception that PTI

and ETI are separate events both mechanistically and in their evolutionary emergence, with PTI representing the more 'basal' and evolutionary 'older' immune system layer, and ETI coming into play later and having evolved more recently.

In reality, the plant immune system can be viewed as a continuum. Nep1-like proteins in bacteria, oomycetes and fungi, and cerato-platanin protein from *Botrytis cinerea* are great examples of secreted proteins contributing to the virulence of the pathogen and therefore satisfy the criteria to be classified as effectors, but that additionally contain epitopes recognized by plant PRRs (Böhm et al., 2014; Oome et al., 2014). Other examples are the MAMP CBEL, a cell wall glycoprotein present in *Phytophthora parasitica* var. *nicotianae* (Khatib et al., 2004), ethylene-inducing xylanase (EIX) from *Trichoderma viride* (Brunner et al., 2002; Nurnberger et al., 1994) and the harpin protein HopZ from *Pst* (Engelhardt et al., 2008; Kvitko et al., 2009; Kvitko et al., 2007; Lee et al., 2001), all of which cause a strong HR-like response in tobacco and *Arabidopsis*, a response usually associated with PCD after effector recognition. All these examples highlight pathogen molecules with dual function in PTI and ETI, blurring the separation between these two immunity levels in their conservation as well as the strength of the response (Thomma et al., 2011).

Another problem with the simplicity of the zigzag model is that it does not apply to host interactions with non-biotrophic pathogens. To address these shortcomings, Cook and colleagues recently proposed a more relaxed model called the invasion model. Host extracellular or intracellular receptors termed invasion pattern receptors (IPR) detect either host- or microbial derived ligands that indicate a potential infection, called invasion pattern (IP). This model represents the plant immune system as a continuum, as opposed to the two separate immune responses of the zigzag model. In the invasion model, any molecule could serve as an IP that can be recognized by an IPR. The chances of a ligand becoming an IP increase with the importance of that molecule for the

pathogen (physiology, virulence, etc.). The model states that all IPRs can be subcategorized depending on their immune response (weak to strong) and conservation (specific to common), but that ultimately all receptors are there to detect the presence of pathogens and stop invasion (Cook et al., 2015). The zigzag model is a great model to test single interactions, but for further development of the area of plant-microbe interactions, new models that take into consideration quantitative and qualitative data are needed (Cook et al., 2015; Pritchard and Birch, 2014).

The advent of novel experimental approaches on whole genome, transcriptome and proteome level, combined with refined computational techniques to efficiently manage and analyze the resultant amounts of data, has the potential to allow us to compare plant immune reactions to different pathogens or even to multiple pathogens at the same time, a situation closer to what is happening in the field under normal conditions. With the decreasing cost of high-throughput "omics" technologies, scientists are now able to ask these big questions. A good example is the attempt to compare ETI to PTI responses after *Pst* infection in tomato. The data showed that there is indeed overlap between the ETI- and PTI transcriptomes, but that there are also unique gene expression changes associated with each of these immune responses (Pombo et al., 2014; Rosli et al., 2013). In conclusion, there is sufficient evidence demonstrating that ETI and PTI responses cannot always be distinguished. Both responses can be weak or strong, depending on the molecule recognized, the receptor and possibly also environmental conditions.

Two modes of plant recognition

Many R proteins conferring resistance to viral, bacterial and fungal pathogens in addition to nematodes and insect pests have been identified in plants. *R* genes mostly encode intracellular proteins belonging to the nucleotide binding site-leucine rich repeat (NB-LRR) group or less commonly, the leucine rich repeat receptor like kinases (LRR-RLKs). Both groups share the leucine rich repeat domain, which is frequently involved in the

formation of protein–protein interactions. However, *R* genes that do not encode an LRR domain have also been described (Caplan et al., 2008; Eitas and Dangl, 2010). For example, the tomato *R* genes *Pto* and *Fen* encode kinases that can confer resistance to certain *Pseudomonas syringae* strains (Martin et al., 1993; Rosebrock et al., 2007). Both Pto and Fen interact with the NB-LRR protein Prf to trigger an ETI response (Gutierrez et al., 2010; Salmeron et al., 1996). Recognition of effectors by R proteins can occur either through direct physical interaction or indirectly by the detection of the target (plant protein) modification induced by the effector (Jones and Dangl, 2006). In each case, perception of pathogen attack is followed by an immune response to combat disease.

Some examples of resistance proteins that directly interact with their cognate effector are L and M in flax and N in tobacco (Catanzariti et al., 2010; Dodds et al., 2006; Krasileva et al., 2010; Ueda et al., 2006). Another group of proteins that directly interacts with effectors act as decoys to detect pathogen invasion; these are proteins that mimic the effector target to "lure" the effector into interacting with them. A very well studied example supporting the decoy model is the interaction between Pto and Fen and their corresponding effector(s) AvrPto and AvrPtoB in tomato (Kim et al., 2002). Pto and Fen appear to mimic the kinase domain of PRRs, the real target of these *Pst* effectors (He et al., 2006; Martin, 2012; Shan et al., 2008; Xiang et al., 2008). The *Arabidopsis*-interacting NB-LRR pair, RRS1-R/ RPS4, confers resistance to both effectors AvrRPS4 from *Pst* and PopP2 from *Ralstonia solanacearum*. In this case, RRS1-R directly interacts with the effectors through a C-terminal WRKY domain (Le Roux et al., 2015).

In many cases R proteins do not interact directly with the effector(s), but instead detect the presence of the virulence proteins by monitoring modifications of the actual plant target(s). This has been formalized as of the "guard hypothesis" (Chisholm et al., 2006; Jones and Dangl, 2006). One example for indirect recognition of effectors is the detection of RIN4 modification by the *Pseudomonas* effectors AvrRpt2, AvrB and AvrRpm1 by

two different R proteins, RPT2 and RPS2, in *Arabidopsis* (Axtell and Staskawicz, 2003; Kim et al., 2005; Mackey et al., 2002). These two effectors and the modification of RIN4 are also monitored in soybean by the R proteins Rpg1b and Rpg1r, suggesting guarding of RIN4 as a common theme for the detection of these effectors (Ashfield et al., 1995; Ashfield et al., 2014). A second example is the R-protein SUMM2 that guards the modification of MPK4 (Zhang et al., 2012). The advantage of this indirect mechanism is that plants had to evolve a smaller set of *R* genes to only monitor key host targets that many diverse effectors act upon as opposed to a distinct *R* gene for each effector.

***Pseudomonas syringae* – tomato pathosystem as model system to study ETI**

The interaction between *Pseudomonas syringae* pv. *tomato* and tomato plants has long been an active area of research. The presence of virulent and avirulent bacterial strains on one side and resistant and susceptible tomato species on the other side made it a perfect model for the investigation of plant pathogen interactions. Tomato has been coevolving with the pathogen for a long time, producing resistant tomato species that allow the investigation of PTI and ETI and provide insights into the evolutionary arms' race that unfolded in between the pathogen and its host.

Over the past three decades, the resistance gene *Pto* has been widely used to control *Pst* infections, especially in processing tomatoes (Pedley and Martin, 2003). *Pto* encodes a cytoplasmic serine/threonine kinase that confers resistance against strains of *Pseudomonas* that express the effector genes AvrPto and / or AvrPtoB. It was originally discovered in *Solanum pimpinellifolium*, one of 12 wild related species of tomato, and was isolated by map-based cloning (Martin et al., 1993). It is a member of small gene family containing up to 6 genes that likely evolved through sequential duplication, followed by sequence divergence in this region (Martin et al., 1993). Prf, a NB-LRR, is embedded in the *Pto* region and is essential for Pto-triggered immunity after detection of AvrPto or AvrPtoB (Salmeron et al., 1996). Pto interaction with these two effectors has

been extensively studied. Pto and Prf are suggested to be in a stable complex, with conformational changes of Pto induced by AvrPto or AvrPtoB acting as a signal for Prf to activate the hypersensitive response (Dong et al., 2009; Mucyn et al., 2006; Xing et al., 2007).

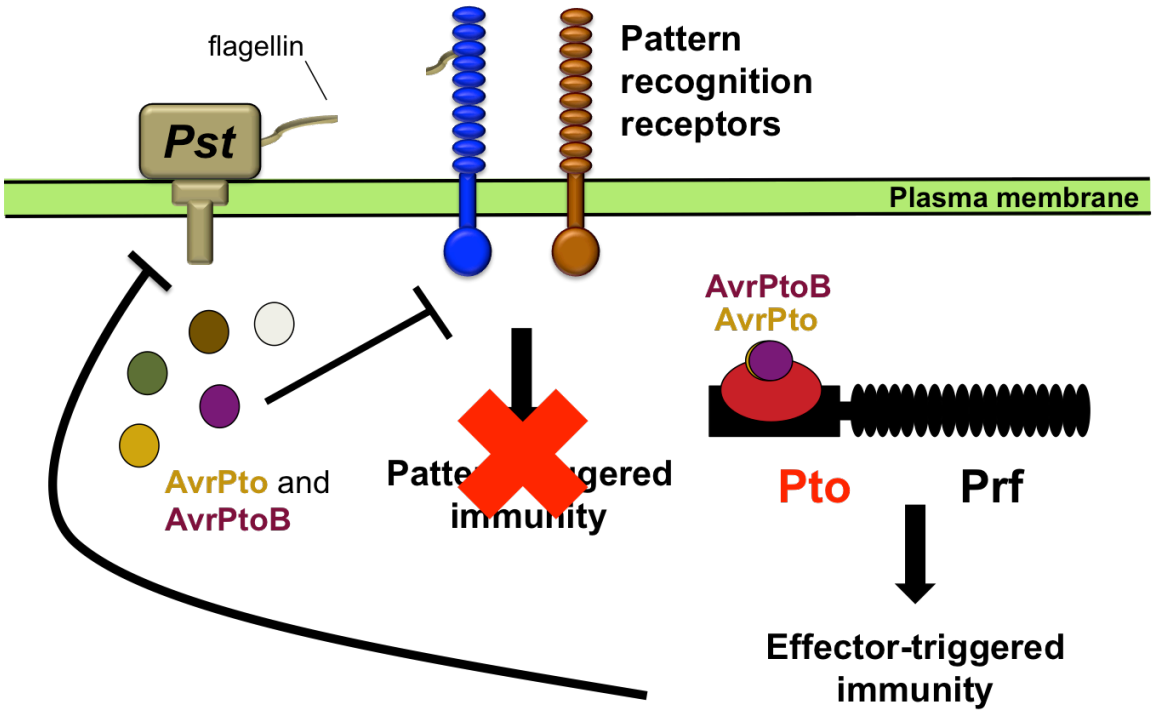

Figure 1.2. Cartoon representing the stages of *Pst* infection of plant leaves expressing the *Pto* resistance gene.

Fen, also called PtoB, is another family member of the *Pto* locus. Fen shares 80% amino acid identity with Pto, but cannot recognize AvrPto or full-length AvrPtoB (Kim et al., 2002). Further analysis demonstrated that Fen could trigger an HR response to AvrPtoB lacking its C-terminal E3-ligase domain (Rosebrock et al., 2007). This Pto-independent detection is referred to as Rsb (Resistance suppressed by AvrPtoB C terminus) and is highly conserved in wild tomato accessions and tobacco (Abramovitch et al., 2003; Kraus et al., 2016; Rosebrock et al., 2007). The wide occurrence of Rsb might indicate that the *Fen* gene arose before the *Pto* gene in the *Solanum* species (Riely and Martin, 2001).

AvrPtoB can target Fen and Pto for proteasomal degradation by ubiquitination. The Fen-interacting domain of AvrPtoB, a domain that can also be recognized by Pto, is proximal to the C-terminal E3 ubiquitin ligase domain. However, Pto can also detect AvrPtoB through a specific Pto-interacting domain, more distal to the C-terminus, and thus evade degradation (Mathieu et al., 2014). No role in host immunity or any other host process has been ascribed to any other member of the *Pto* gene family to date.

The co-crystal structure of Pto interacting with AvrPto and AvrPtoB revealed that the interaction between Pto and the two effectors is mediated by two interfaces (Dong et al., 2009). The P+1 loop acts as a shared binding site and mutations in this domain of Pto suggest that it is important for the negative regulation of immune signaling; many mutations in this region cause a gain of function and induce a constitutive HR (Bernal et al., 2005; Dong et al., 2009; Rathjen et al., 1999; Wu et al., 2004; Xing et al., 2007). The second interface is specific for each recognized effector. Particular mutations in the specific interfaces only abolish the interaction of the specific effector, whereas substitutions on the shared interface abolish recognition of both effectors in a yeast two-hybrid system (Dong et al., 2009). However, the mutants could never be tested in plants, because these substitutions cause Pto auto-activation as demonstrated in *N. benthamiana*. While differences in the interaction of Pto with the two effectors are well established, so far no difference in downstream signaling following detection has been found. As of now, the consensus is that Pto has a negative effect on Prf signaling. Upon interaction with the effectors, Prf "senses" the disturbance of Pto, which activates a downstream signaling.

Solanum lycopersicum **(tomato)**

Tomato (*Solanum lycopersicum)* is a versatile model crop to study plant immune responses; it has a relatively small diploid genome (Arumuganathan et al., 1991) and is easy to grow in greenhouses without the need for too much space. Tomato plants are self-

pollinating, but it is still possible to produce crosses between wild and cultivated species. They are easily propagated by seed or clonally and have a relatively short generation time compared to many other crop plants (Pedley and Martin, 2003). In recent years several tomato genome sequences have become publicly available, facilitating genetic and molecular analyses (http://www.tomatogenome.net).

Tomato plants are amenable to molecular work, as they are easily transformable, and many of the immunity related assays established for *Arabidopsis* also work in tomatoes. In contrast to *Arabidopsis*, tomato is highly susceptible to a wide variety of pathogens. Major diseases of tomatoes can be caused by at least 24 fungi, 7 bacteria, 10 viruses, 3 viroids, and multiple nematodes, making it possible to study all aspects of plant immune responses and plant-microbe interactions (Arie et al., 2007). In fact, many of the major breakthrough identifications of proteins involved in plant immunity have been described first in tomato (Jones et al., 1994; Martin et al., 1993; Salmeron et al., 1996).

The germplasm of cultivated tomato varieties shows relatively little genetic variation - the result of its inbreeding mating system and severe genetic bottlenecks that are hypothesized to have occurred before, during and after tomato domestication (Bai and Lindhout, 2007; Grandillo et al., 2011). This is an important impediment for tomato improvement, since breeding for enhanced traits relies on sufficient genetic variation to find new genes. In contrast to their domesticated counterparts, wild tomatoes are a rich source of genetic variation. There are 12 described wild tomato species. These species, native to South America, are adapted to a wide variety of environmental regions such temperate dessert, Andean highlands, tropical forest and arid rocky outcrops of lava close to the ocean shoreline. Each species has a distinct geographic distribution, reflecting a wide adaptation to diverse habitats differing in temperature range, altitude, annual precipitation and soil composition. They also contain important genes involved in fruit size and yield and resistance against biotic and abiotic stresses. Wild tomato accessions

have been used in tomato breeding programs since the 1940s and, except for a few cases, all *R* genes were derived from this wild tomato ancestry (Bai and Lindhout, 2007; Grandillo et al., 2011; Peralta et al., 2008). Furthermore, tomato belongs to the *Solanaceae* family, a large and diverse family including other economically important crops like potato, pepper and eggplant. Tomato researchers have access to a large collection of cultivated and wild tomatoes that can be ordered at the C. M. Rick Tomato Genetics Resource Center (TGRC), UC Davis (http://tgrc.ucdavis.edu).

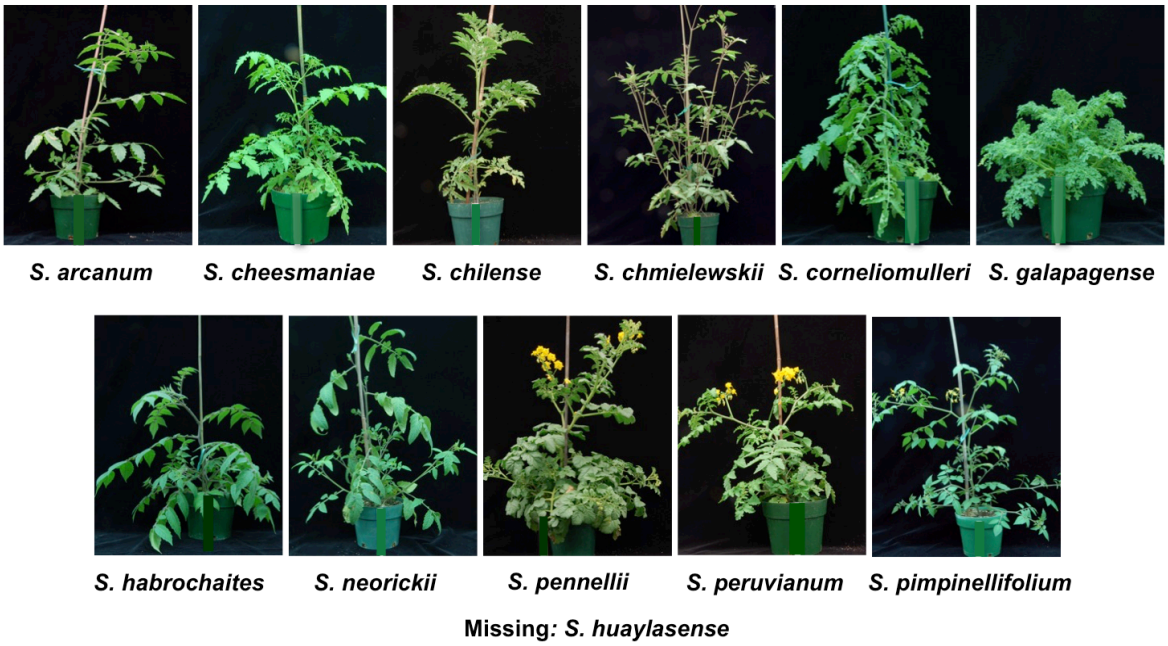

Figure 1.3. Wild relatives of tomato.

The cultivated tomato is the second most important vegetable crop worldwide after potatoes, with a total world production of more than 160 million tons (FAOSTAT 2012; http://faostat.fao.org). The USA is the second biggest tomato producer after China, accounting for 14.1 million tons with a value of $10.86 billion dollars (USDA; https://www.ers.usda.gov/topics/crops/vegetables-pulses/tomatoes.aspx). This popular fruit is rich in vitamins and antioxidants and an important source of β-carotene (provitamin A) and vitamin C. Tomato production is divided into two main markets,

processing and fresh market. Fresh market tomato varieties are grown in most US states for the local markets, with California and Florida being the biggest producers of fresh market tomatoes at commercial scale, followed by Ohio, Virginia, Georgia, and Tennessee. Processing tomatoes, on the other hand, are mostly grown on commercial acreage in California, which accounts for more than 90% of harvested processing tomatoes (USDA; https://www.ers.usda.gov/topics/crops/vegetables-pulses/tomatoes.aspx).

***Pseudomonas syringae* pv. *tomato* the casual agent of bacterial speck in tomato**

Pst is the causal agent of bacterial speck in tomato. Symptoms appear as small necrotic lesions on leaves, stem, and green tomato fruits and can have a great impact on crop marketability and yield (Jones, 1991; Pedley and Martin, 2003). The ability of *Pst* and other bacterial pathogens to infect and multiply in plant leaves depends on the secretion of virulence proteins through a type III secretion system (T3SS) directly into the plant cells to suppress host cellular pathways (Alfano and Collmer, 2004; Chang et al., 2005; Dean, 2011). To date, 94 different *Pseudomonas* virulence effectors have been described, with each bacterial strain harboring a subset of about 30 of them (Baltrus et al., 2011; Buell et al., 2003; Lindeberg et al., 2012).

Figure 1.4. Bacterial speck on tomato caused by *Pseudomonas syringae* pv. *tomato*.

Pst with a mutated T3SS grows poorly in plants and cannot cause disease symptoms, indicating the importance of the effector repertoire for virulence (Roine et al., 1997). While we do not yet understand the function of all effectors, several are delivered into the cell to suppress plant defense responses activated during PTI (Chisholm et al., 2006; Cunnac et al., 2009; Guo et al., 2009). Although deletion of most individual *Pst* effectors typically does not have an effect on bacterial virulence and the ability to grow and produce symptoms, combined Δ*avrPto*Δ*avrPtoB* mutations significantly reduce the ability of *Pst* to grow in tomato (Badel et al., 2006; Chang et al., 2005; Kvitko et al., 2009; Lin and Martin, 2005; Mudgett, 2005; Petnicki-Ocwieja et al., 2002; Schechter et al., 2004).

AvrPto is a small (18 kDa) hydrophilic protein first identified in 1992 based on its avirulence function in tomato plants expressing Pto and Prf (Ronald et al., 1992). Once delivered into the plant cell cytoplasm, it appears to be targeted to the membrane (Boyle et al., 2016; Shan et al., 2000), where it binds to the kinase domains of PRRs and inhibits their kinase activity (Xiang et al., 2008). The AvrPto knockout strain remained avirulent

in *Pto* carrying tomato lines, suggesting the presence of a second Pto-specific avirulence gene. It was not until 2002 that a second *Pst* effector, now referred to as AvrPtoB, was identified (Kim et al., 2002).

AvrPtoB is much larger (59 kDa) and interacts with PRRs through two structurally distinct virulence domains, a N-terminal region that inhibits kinase activity of PRRs much like AvrPto (Cheng et al., 2011; Shan et al., 2008) and a C-terminal E3 ubiquitin ligase domain (Abramovitch et al., 2006; Janjusevic et al., 2006) that has been reported to mark FLS2, CERK1 and probably other PRRs for degradation by the proteasome; however this has been only demonstrated with overexpression experiments in *N. benthamiana* (Gimenez-Ibanez et al., 2009; Gohre et al., 2008). In contrast, the E3 ligase function has been shown to be dispensable for the interaction with and the inhibition of PRRs and other co-receptors under natural expression levels, questioning whether ubiquitination and degradation are indeed necessary for the inhibition of the recognition complex by AvrPtoB (Cheng et al., 2011; Shan et al., 2008).

Pto was initially reported in the late 1970s and was quickly introgressed into several tomato cultivars (Pedley and Martin, 2003). While Pto-resistance is effective against race 0 *Pst* strains such as DC3000 and JL1065, it is ineffective in triggering an immune response against the more virulent race 1 strains (Lin et al., 2006). Races in *Pst* are defined by the ability of Pto to mount an effective immune response (Arredondo and Davis, 2000). Race 1 strains evade this recognition by suppressing AvrPto and AvrPtoB recognition (Kunkeaw et al., 2010; Lin et al., 2006). In the case of T1, the best studied *Pst* race 1 strain, *avrPto* is absent, and AvrPtoB protein expression is post-transcriptionally suppressed by an unknown mechanism (Almeida et al., 2009; Lin et al., 2006). A study comparing *Pst* isolates worldwide demonstrated that the population has shifted towards the more virulent race 1 strains, a situation that is affecting tomato growers (Cai et al., 2011). Since Pto is not a viable solution to stop infection with race 1

strains, researchers are employing wild tomato accessions in an attempt to discover new resistance loci against race 1 strains. *Solanum habrochaites* accessions have promising Quantitative Resistance Loci (QRL) that confer partial resistance to race 0 strains (Bao et al., 2015; Thapa et al., 2015). Even though each of these QRL by itself might not provide the same level of resistance as the single dominant gene does for race 0 strains, their combined effect plays a quantitative role in the ability of tomatoes to withstand infection by race 1 strains, and in combination with the use of proper plant management practices can be useful against the new hyper-virulent *Pst* strains.

Aims of this thesis

Tomato-*Pst* is one of the best-studied pathosystem for several reasons: The genomes of several representatives of both organisms have been sequenced, and a large collection of tomato cultivars and wild species, as well as a massive collection of *Pst* isolates, are available to researchers. Due to these advantages as well as others mentioned earlier, the *Pst* – tomato model is a great system to study basic plant immunity and bacterial pathogenicity. At the same time, bacterial speck of tomato constitutes an economically important disease that is important to study and monitor.

Bacterial speck is a common disease of tomato in the northern states of the USA and in Canada, most likely due to the often mild and rainy summers generating suitable conditions for its multiplication and spread. Although almost no commercial processing tomatoes are grown in New York (NY), significant amounts of fresh market tomatoes are grown for local markets. Most of these cultivars do not carry *Pto* and in recent years tomato growers experienced substantial losses due to bacterial speck. The year 2015 had a very cool and rainy summer which presented the perfect environmental conditions for a bacterial speck outbreak in NY in several tomato fields, demonstrating that if the environmental conditions are conducive to disease *Pst* causes severe economic damage. The lack of *Pto* resistance, the continuing presence of bacterial speck in NY tomato field

and the knowledge of the emergence of highly virulent race 1 strains worldwide presented a strong case for the proper study of the *Pst* population present in this area.

The work presented in chapter 2 of this dissertation provides evidence that natural variation can be used to detect and study co-evolution between a host *R* gene and its pathogen effectors. Specifically, a screen of wild tomato accessions for their ability to recognize *Pst* delivering AvrPto or AvrPtoB discovered variation between wild tomato species in their capability to detect these effectors. Further molecular characterization demonstrated that a region of Pto distinct from the effector interfaces, referred to as the activation loop, plays an important role in the response to AvrPto, but not to AvrPtoB. This led to the discovery that a single amino acid in the activation loop region, D193, is essential for downstream signaling in response to AvrPto recognition.

In chapter 3, I describe an evaluation of several *Pst* isolates present in NY in 2015 for their molecular virulence. Using diagnostic oligonucleotides and PCR, I demonstrate that these isolates are more closely related to race 1 strains, but that their virulence is attenuated because of a functional AvrPto protein recognized by Pto, as is typical in race 0 strains. Importantly, delayed speck symptoms do appear in vacuum infiltrated plants, indicating a possible new effector that is able to suppress downstream Pto signaling after AvrPto recognition. The data indicate that, at least in NY, the introgression of *Pto* into fresh market tomato cultivars might provide a viable strategy for resistance against the current outbreaks of bacterial speck.

1.2 References

Abramovitch, R.B., Janjusevic, R., Stebbins, C.E., and Martin, G.B. (2006). Type III effector AvrPtoB requires intrinsic E3 ubiquitin ligase activity to suppress plant cell death and immunity. Proc Natl Acad Sci USA 103:2851-2856.

Abramovitch, R.B., Kim, Y.-J., Chen, S., Dickman, M.B., and Martin, G.B. (2003). *Pseudomonas* type III effector AvrPtoB induces plant disease susceptibility by inhibition of host programmed cell death. EMBO J 22:60-69.

Abramovitch, R.B., and Martin, G.B. (2004). Strategies used by bacterial pathogens to suppress plant defenses. Curr Opin Plant Biol 7:356-364.

Agrios, G.N. (1997). Plant Pathology. San Diego: Academic Press.

Alfano, J.R., and Collmer, A. (2004). Type III secretion system effector proteins: double agents in bacterial disease and plant defense. Annu Rev Phytopathol 42:385-414.

Almeida, N.F., Yan, S., Lindeberg, M., Studholme, D.J., Schneider, D.J., Condon, B., Liu, H., Viana, C.J., Warren, A., Evans, C., et al. (2009). A draft genome sequence of *Pseudomonas syringae* pv. *tomato* T1 reveals a type III effector repertoire significantly divergent from that of *Pseudomonas syringae* pv. *tomato* DC3000. Mol Plant-Microbe Interact 22:52-62.

Altenbach, D., and Robatzek, S. (2007). Pattern recognition receptors: from the cell surface to intracellular dynamics. Mol Plant-Microbe Interact 20:1031-1039.

Arie, T., Takahashi, H., Kodama, M., and Teraoka, T. (2007). Tomato as model plant for plant-pathogen interactions. Plant Biotechnol J 24:135-147.

Arredondo, C.R., and Davis, R.M. (2000). First report of *Pseudomonas syringae* pv. *tomato* race 1 on tomato in California. Plant Disease 84:371.

Arumuganathan, K., Slattery, J.P., Tanksley, S.D., and Earle, E.D. (1991). Preparation and flow cytometric analysis of metaphase chromosomes of tomato. Theor Appl Genet 82:101-111.

Ashfield, T., Keen, N., Buzzell, R., and Innes, R. (1995). Soybean resistance genes specific for different *Pseudomonas syringae* avirulence gene are allelic, or closely linked, at the RPG1 locus. Genetics 141:1597-1604.

Ashfield, T., Redditt, T., Russell, A., Kessens, R., Rodibaugh, N., Galloway, L., Kang, Q., Podicheti, R., and Innes, R.W. (2014). Evolutionary relationship of disease

resistance genes in soybean and *Arabidopsis* specific for the *Pseudomonas syringae* effectors AvrB and AvrRpm1. Plant Physiol 166:235-251.

Axtell, M.J., and Staskawicz, B.J. (2003). Initiation of RPS2-specified disease resistance in *Arabidopsis* is coupled to the AvrRpt2-directed elimination of RIN4. Cell 112:369-377.

Badel, J.L., Shimizu, R., Oh, H.S., and Collmer, A. (2006). A *Pseudomonas syringae* pv. *tomato avrE1/hopM1* mutant is severely reduced in growth and lesion formation in tomato. Mol Plant-Microbe Interact 19:99-111.

Bai, Y., and Lindhout, P. (2007). Domestication and breeding of tomatoes: what have we gained and what can we gain in the future? Ann Bot (Lond) 100:1085-1094.

Baltrus, D.A., Nishimura, M.T., Romanchuk, A., Chang, J.H., Mukhtar, M.S., Cherkis, K., Roach, J., Grant, S.R., Jones, C.D., and Dangl, J.L. (2011). Dynamic evolution of pathogenicity revealed by sequencing and comparative genomics of 19 *Pseudomonas syringae* isolates. PLoS Pathog 7:e1002132.

Bao, Z., Meng, F., Strickler, S.R., Dunham, D.M., Munkvold, K.R., and Martin, G.B. (2015). Identification of a candidate gene in *Solanum habrochaites* for resistance to a race 1 strain of *Pseudomonas syringae* pv. *tomato*. Plant Genome 8.

Baureithel, K., Felix, G., and Boller, T. (1994). Specific, high affinity binding of chitin fragments to tomato cells and membranes: Competitive inhibition of binding by derivatives of chitooligosaccharides and a Nod factor of *Rhizobium*. J Biol Chem 269:17931–17938.

Bernal, A.J., Pan, Q., Pollack, J., Rose, L., Kozik, A., Willits, N., Luo, Y., Guittet, M., Kochetkova, E., and Michelmore, R.W. (2005). Functional analysis of the plant disease resistance gene *Pto* using DNA shuffling. J Biol Chem 280:23073-23083.

Bittel, P., and Robatzek, S. (2007). Microbe-associated molecular patterns (MAMPs) probe plant immunity. Curr Opin Plant Biol 10:335–341.

Böhm, H., Albert, I., Oome, S., Raaymakers, T.M., Van den Ackerveken, G., and Nürnberger, T. (2014). A conserved peptide pattern from a widespread microbial virulence factor triggers pattern-induced immunity in *Arabidopsis*. PLoS Pathog 10.

Boller, T., and Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annu Rev Plant Biol 60:379-406.

Boyle, P.C., Schwizer, S., Hind, S.R., Kraus, C.M., De la Torre Diaz, S., He, B., and Martin, G.B. (2016). Detecting N-myristoylation and S-acylation of host and pathogen proteins in plants using click chemistry. Plant methods 12.

Brunner, F., Rosahl, S., Lee, J., Rudd, J.J., Geiler, C., Kauppinen, S., Rasmussen, G., Scheel, D., and Nurnberger, T. (2002). Pep-13, a plant defense-inducing pathogen-associated pattern from *Phytophthora transglutaminases*. EMBO J 21:6681–6668.

Buell, C.R., Joardar, V., Lindeberg, M., Selengut, J., Paulsen, I.T., Gwinn, M.L., Dodson, R.J., Deboy, R.T., Durkin, A.S., Kolonay, J.F., et al. (2003). The complete genome sequence of the *Arabidopsis* and tomato pathogen *Pseudomonas syringae* pv. *tomato* DC3000. Proc Natl Acad Sci USA 100:10181-10186.

Cai, R.M., Lewis, J., Yan, S.C., Liu, H.J., Clarke, C.R., Campanile, F., Almeida, N.F., Studholme, D.J., Lindeberg, M., Schneider, D., et al. (2011). The plant pathogen *Pseudomonas syringae* pv. *tomato* is genetically monomorphic and under strong selection to evade tomato immunity. PLoS Pathog 7:e1002130.

Caplan, J., Padmanabhan, M., and Dinesh-Kumar, S.P. (2008). Plant NB-LRR immune receptors: from recognition to transcriptional reprogramming. Cell Host Microbe 3:126-135.

Catanzariti, A.M., Dodds, P.N., Ve, T., Kobe, B., Ellis, J.G., and Staskawicz, B.J. (2010). The AvrM effector from flax rust has a structured C-terminal domain and interacts directly with the M resistance protein. Mol Plant-Microbe Interact 23:49-57.

Chang, J.H., Urbach, J.M., Law, T.F., Arnold, L.W., Hu, A., Gombar, S., Grant, S.R., Ausubel, F.M., and Dangl, J.L. (2005). A high-throughput, near-saturating screen for type III effector genes from *Pseudomonas syringae*. Proc Natl Acad Sci USA 102:2549-2554.

Cheng, W., Munkvold, K.R., Gao, H., Mathieu, J., Schwizer, S., Wang, S., Yan, Y.B., Wang, J., Martin, G.B., and Chai, J. (2011). Structural analysis of *Pseudomonas*

syringae AvrPtoB bound to host BAK1 reveals two similar kinase-interacting domains in a type III effector. Cell Host Microbe 10:616-626.

Chisholm, S.T., Coaker, G., Day, B., and Staskawicz, B.J. (2006). Host-microbe interactions: shaping the evolution of the plant immune response. Cell 124:803-814.

Cook, D.E., Mesarich, C.H., and Thomma, B.P.H.J. (2015). Understanding plant immunity as a surveillance system to detect invasion. Annu Rev Phytopathol 53:541-563.

Cunnac, S., Lindeberg, M., and Collmer, A. (2009). *Pseudomonas syringae* type III secretion system effectors: repertoires in search of functions. Curr Opin Microbiol 12:53-60.

Dangl, J.L., and Jones, J.D.G. (2001). Plant pathogens and integrated defence responses to infection. Nature 411:826-833.

Dean, P. (2011). Functional domains and motifs of bacterial type III effector proteins and their roles in infection. FEMS Microbiol Rev 35:1100-1125.

Dodds, P.N., Lawrence, G.J., Catanzariti, A.M., Teh, T., Wang, C.I., Ayliffe, M.A., Kobe, B., and Ellis, J.G. (2006). Direct protein interaction underlies gene-for-gene specificity and coevolution of the flax resistance genes and flax rust avirulence genes. Proc Natl Acad Sci USA 103:8888-8893.

Dodds, P.N., and Rathjen, J.P. (2010). Plant immunity: towards an integrated view of plant-pathogen interactions. Nat Rev Genet 11:539-548.

Dong, J., Xiao, F., Fan, F., Gu, L., Cang, H., Martin, G.B., and Chai, J. (2009). Crystal structure of the complex between *Pseudomonas* effector AvrPtoB and the tomato Pto kinase reveals both a shared and a unique interface compared with AvrPto-Pto. Plant Cell 21:1846-1859.

Eitas, T.K., and Dangl, J.L. (2010). NB-LRR proteins: pairs, pieces, perception, partners, and pathways. Curr Opin Plant Biol 13:472-477.

Engelhardt, S., Lee, J., Gäbler, Y., Kemmerling, B., Haapalainen, M., Li, C., Wei, Z., Keller, H., Joosten, M., Taira, S., et al. (2008). Separable roles of the *Pseudomonas syringae* pv. *phaseolicola* accessory protein HrpZ1 in ion-conducting pore formation and activation of plant immunity. Plant J 57:706–717.

Felix, G., Duran, J.D., Volko, S., and Boller, T. (1999). Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. Plant J 18:265-276.

Gimenez-Ibanez, S., Ntoukakis, V., and Rathjen, J.P. (2009). The LysM receptor kinase CERK1 mediates bacterial perception in *Arabidopsis*. Plant Signal Behav 4:539-541.

Gohre, V., Spallek, T., Haweker, H., Mersmann, S., Mentzel, T., Boller, T., de Torres, M., Mansfield, J.W., and Robatzek, S. (2008). Plant pattern-recognition receptor FLS2 is directed for degradation by the bacterial ubiquitin ligase AvrPtoB. Curr Biol 18:1824-1832.

Gomez-Gomez, L., and Boller, T. (2000). FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. Molecular cell 5:1003-1011.

Grandillo, S., Chetelat, R.T., Knapp, S., Spooner, D.M., Peralta, I.E., Cammareri, M., Perez, P., Termolino, P., Chiusano, M.L., Ercolano, M.R., et al. (2011). From *Solanum sect. Lycopersicon*. In: Wild crop relatives: genomic and breeding resources. Vegetables (1st edition)--Kole, C., ed. Berlin, Heidelberg, New York: Springer. 129-215.

Greenberg, J.T., and Yao, N. (2004). The role and regulation of programmed cell death in plant-pathogen interactions. Cell Microbiol 6:201-211.

Guo, M., Tian, F., Wamboldt, Y., and Alfano, J.R. (2009). The majority of the type III effector inventory of *Pseudomonas syringae* pv. *tomato* DC3000 can suppress plant immunity. Mol Plant-Microbe Interact 22:1069-1080.

Gutierrez, J.R., Balmuth, A.L., Ntoukakis, V., Mucyn, T.S., Gimenez-Ibanez, S., Jones, A.M., and Rathjen, J.P. (2010). Prf immune complexes of tomato are oligomeric and contain multiple Pto-like kinases that diversify effector recognition. Plant J 61:507-518.

He, P., Shan, L., Lin, N.C., Martin, G.B., Kemmerling, B., Nurnberger, T., and Sheen, J. (2006). Specific bacterial suppressors of MAMP signaling upstream of MAPKKK in *Arabidopsis* innate immunity. Cell 125:563-575.

Hind, S.R., Strickler, S.R., Boyle, P.C., Dunham, D.M., Bao, Z., O'Doherty, I.M., Baccile, J.A., Hoki, J.S., Viox, E.G., Clarke, C.R., et al. (2016). Tomato receptor

FLAGELLIN-SENSING 3 binds flgII-28 and activates the plant immune system. Nat Plants 2.

Ito, Y., Kaku, H., and Shibuya, N. (1997). Identification of a high-affinity binding protein for N-acetylchitoologosaccharide elicitor in the plasma membrane of suspension-cultured rice cells by affinity labeling. Plant J 12:347–356.

Jamir, Y., Guo, M., Oh, H.S., Petnicki-Ocwieja, T., Chen, S., Tang, X., Dickman, M.B., Collmer, A., and Alfano, J.R. (2004). Identification of *Pseudomonas syringae* type III effectors that can suppress programmed cell death in plants and yeast. Plant J 37:554-565.

Janjusevic, R., Abramovitch, R.B., Martin, G.B., and Stebbins, C.E. (2006). A bacterial inhibitor of host programmed cell death defenses is an E3 ubiquitin ligase. Science 311:222-226.

Jones, D.A., Thomas, C.M., Hammond-Kosack, K.E., Balint-Kurti, P.J., and Jones, J.D.G. (1994). Isolation of the tomato *Cf-9* gene for resistance to *Cladosporium fulvum* by transposon tagging. Science 266:789-793.

Jones, J.B. (1991). Bacterial speck. In: Compendium of tomato diseases--Jones, J.B., Jones, J.P., Stall, R.E., and Zitter, T.A., eds. St. Paul, MN: APS Press. 26-27.

Jones, J.D., and Dangl, J.L. (2006). The plant immune system. Nature 444:323-329.

Khatib, M., Lafitte, C., Esquerré-Tugayé, M., Bottin, A., and Rickauer, M. (2004). The CBEL elicitor of *Phytophthora parasitica* var. *nicotianae* activates defence in *Arabidopsis thaliana* via three different signalling pathways. New Phytol 162:501–510.

Kim, M.G., da Cunha, L., McFall, A.J., Belkhadir, Y., DebRoy, S., Dangl, J.L., and Mackey, D. (2005). Two *Pseudomonas syringae* type III effectors inhibit RIN4-regulated basal defense in Arabidopsis. Cell 121:749-759.

Kim, Y.J., Lin, N.-C., and Martin, G.B. (2002). Two distinct *Pseudomonas* effector proteins interact with the Pto kinase and activate plant immunity. Cell 109:589-598.

Krasileva, K.V., Dahlbeck, D., and Staskawicz, B.J. (2010). Activation of an *Arabidopsis* resistance protein is specified by the in planta association of its leucine-rich repeat domain with the cognate oomycete effector. Plant Cell 22:2444-2458.

Kraus, C.M., Munkvold, K.R., and Martin, G.B. (2016). Natural variation in tomato reveals differences in the recognition of AvrPto and AvrPtoB effectors from *Pseudomonas syringae*. Mol Plant 9:639–649.

Kunkeaw, S., Tan, S., and Coaker, G. (2010). Molecular and evolutionary analyses of *Pseudomonas syringae* pv. *tomato* race 1. Mol Plant-Microbe Interact 23:415-424.

Kunze, G., Zipfel, C., Robatzek, S., Niehaus, K., Boller, T., and Felix, G. (2004). The N terminus of bacterial elongation factor Tu elicits innate immunity in *Arabidopsis* plants. Plant Cell 16:3496-3507.

Kvitko, B.H., Park, D.H., Velasquez, A.C., Wei, C.F., Russell, A.B., Martin, G.B., Schneider, D.J., and Collmer, A. (2009). Deletions in the repertoire of *Pseudomonas syringae* pv. *tomato* DC3000 type III secretion effector genes reveal functional overlap among effectors. PLoS Pathog 5:e1000388.

Kvitko, B.H., Ramos, A.R., Morello, J.E., Oh, H.S., and Collmer, A. (2007). Identification of harpins in *Pseudomonas syringae* pv. *tomato* DC3000, which are functionally similar to HrpK1 in promoting translocation of type III secretion system effectors. J Bacteriol 189:8059–8072.

Lam, E., Kato, N., and Lawton, M. (2001). Programmed cell death, mitochondria and the plant hypersensitive response. Nature 411:848-853.

Le Roux, C., Huet, G., Jauneau, A., Camborde, L., Tremousaygue, D., Kraut, A., Zhou, B., Levaillant, M., Adachi, H., Yoshioka, H., et al. (2015). A recepetor pair with an integrated decoy converts pathogen disabling of transcription factors to immunity. Cell 161:1074-1088.

Lee, J., Klüsener, B., Tsiamis, G., Stevens, C., Neyt, C., Tampakaki, A.P., Panopoulos, N.J., Joachim Nöller, J., Weiler, E.W., Cornelis, G.R., et al. (2001). HrpZPsph from the plant pathogen *Pseudomonas syringae* pv. *phaseolicola* binds to lipid bilayers and forms an ion-conducting pore in vitro. Proc Natl Acad Sci USA 98:289–294.

Lee, S.W., Han, S.W., Bartley, L.E., and Ronald, P.C. (2006). Unique characteristics of Xanthomonas oryzae pv. oryzae AvrXa21 and implications for plant innate

immunity. Proceedings of the National Academy of Sciences of the United States of America 103:18395-18400.

Lin, N.C., Abramovitch, R.B., Kim, Y.J., and Martin, G.B. (2006). Diverse AvrPtoB homologs from several *Pseudomonas syringae* pathovars elicit Pto-dependent resistance and have similar virulence activities. Appl Environ Microbiol 72:702-712.

Lin, N.C., and Martin, G.B. (2005). An *avrPto/avrPtoB* mutant of *Pseudomonas syringae* pv. *tomato* DC3000 does not elicit Pto-mediated resistance and is less virulent on tomato. Mol Plant-Microbe Interact 18:43-51.

Lindeberg, M., Cunnac, S., and Collmer, A. (2012). *Pseudomonas syringae* type III effector repertoires: last words in endless arguments. Trends Microbiol 20:199-208.

Mackey, D., Holt, B.F., Wiig, A., and Dangl, J.L. (2002). RIN4 interacts with *Pseudomonas syringae* type III effector molecules and is required for RPM1-mediated resistance in *Arabidopsis*. Cell 108:743-754.

Maekawa, T., Kufer, T.A., and Schulze-Lefert, P. (2011). NLR functions in plant and animal immune systems: so far and yet so close. Nat Immunol 12:817-826.

Martin, G.B. (2012). Suppression and activation of the plant immune system by *Pseudomonas syringae* effectors AvrPto and AvrPtoB. In: Effectors in Plant-Microbe Interactions--Martin, F., and Kamoun, S., eds. Oxford, UK.: Wiley-Blackwell. 123-154.

Martin, G.B., Brommonschenkel, S.H., Chunwongse, J., Frary, A., Ganal, M.W., Spivey, R., Wu, T., Earle, E.D., and Tanksley, S.D. (1993). Map-based cloning of a protein kinase gene conferring disease resistance in tomato. Science 262:1432-1436.

Mathieu, J., Schwizer, S., and Martin, G.B. (2014). Pto kinase binds two domains of AvrPtoB and its proximity to the effector E3 ligase determines if it evades degradation and activates plant immunity. PLoS Pathog 10:e1004227.

Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., Narusaka, Y., Kawakami, N., Kaku, H., and Shibuya, N. (2007). CERK1, a LysM receptor

kinase, is essential for chitin elicitor signaling in *Arabidopsis*. Proc Natl Acad Sci USA 104:19613-19618.

Mucyn, T.S., Clemente, A., Andriotis, V.M., Balmuth, A.L., Oldroyd, G.E., Staskawicz, B.J., and Rathjen, J.P. (2006). The tomato NBARC-LRR protein Prf interacts with Pto kinase in vivo to regulate specific plant immunity. Plant Cell 18:2792-2806.

Mudgett, M.B. (2005). New insights to the function of phytopathogenic bacterial type III effectors in plants. Annu Rev Plant Biol 56:509-531.

Narusaka, M., Shirasu, K., Noutoshi, Y., Kubo, Y., Shiraishi, T., Iwabuchi, M., and Narusaka, Y. (2009). RRS1 and RPS4 provide a dual Resistance-gene system against fungal and bacterial pathogens. Plant J 60:218-226.

Nurnberger, T., and Kemmerling, B. (2009). PAMP-triggered basal immunity in plants. Adv Bot Res 51:1377?

Nurnberger, T., Nennstiel, D., Jabs, T., Sacks, W., Hahlbrock, K., and Scheel, D. (1994). High affinity binding of a fungal oligopeptide elicitor to parsley plasma membranes triggers multiple defense responses. Cell 78:449-460.

Oome, S., Raaymakers, T.M., Cabral, A., Samwel, S., Böhm, H., Albert, I., Nürnberger, T., and Van den Ackerveken, G. (2014). Nep1-like proteins from three kingdoms of life act as a microbe-associated molecular pattern in Arabidopsis. Proc Natl Acad Sci USA 111:16955–16960.

Pedley, K.F., and Martin, G.B. (2003). Molecular basis of Pto-mediated resistance to bacterial speck disease in tomato. Annu Rev Phytopathol 41:215-243.

Peralta, I.E., Spooner, D.M., and Knapp, S. (2008). Systematic botany monographs *Solanum* (*Solanaceae*). United States of America: Amer Society of Plant Taxonomists.

Petnicki-Ocwieja, T., Schneider, D.J., Tam, V.C., Chancey, S.T., Shan, L., Jamir, Y., Schechter, L.M., Janes, M.D., Buell, C.R., Tang, X., et al. (2002). Genomewide identification of proteins secreted by the Hrp type III protein secretion system of *Pseudomonas syringae* pv. *tomato* DC3000. Proc Natl Acad Sci USA 99:7652-7657.

Pfund, C., Tans-Kersten, J., Dunning, F.M., Alonso, J.M., Ecker, J.R., Allen, C., and Bent, A.F. (2004). Flagellin is not a major defense elicitor in *Ralstonia*

*solanacearum c*ells or extracts applied to *Arabidopsis thaliana*. Mol Plant-Microbe Interact 17:696-706.

Pombo, M.A., Zheng, Y., Fernandez-Pozo, N., Dunham, D.M., Fei, Z., and Martin, G.B. (2014). Transcriptomic analysis reveals tomato genes whose expression is induced specifically during effector-triggered immunity and identifies the Epk1 protein kinase which is required for the host response to two bacterial effector proteins. Genome biology.

Pritchard, L., and Birch, P.R. (2014). The zigzag model of plant–microbe interactions: is it time tomove on? Mol Plant Pathol 15:865-870.

Pruitt, R.N., Schwessinger, B., Joe, A., Thomas, N., Liu, F., Albert, M., Robinson, M.R., Chan, L.G., Luu, D., Chen, H., et al. (2015). The rice immune receptor XA21 recognizes a tyrosine-sulfated protein from a Gram-negative bacterium. Science Advances.

Rathjen, J.P., Chang, J.H., Staskawicz, B.J., and Michelmore, R.W. (1999). Constitutively active Pto induces a Prf-dependent hypersensitive response in the absence of AvrPto. EMBO J 18:3232-3240.

Riely, B.K., and Martin, G.B. (2001). Ancient origin of pathogen recognition specificity conferred by the tomato disease resistance gene Pto. Proc Natl Acad Sci USA 98:2059-2064.

Roine, E., Wei, W., Yuan, J., Nurmiaho-Lassila, E.L., Kalkkinen, N., Romantschuk, M., and He, S.Y. (1997). Hrp pilus: An *hrp*-dependent bacterial surface appendage produced by *Pseudomonas syringae* pv. *tomato* DC3000. Proc Natl Acad Sci USA 94:3459-3464.

Ronald, P.C., Salmerson, J.M., Carland, F., M., and Staskawicz, B.J. (1992). The cloned avirulence gene *avrPto* induces disease resistance in tomato cultivars containing the *Pto* resistance gene. J Bacteriol 174:1604-1611.

Rosebrock, T.R., Zeng, L., Brady, J.J., Abramovitch, R.B., Xiao, F., and Martin, G.B. (2007). A bacterial E3 ubiquitin ligase targets a host protein kinase to disrupt plant immunity. Nature 448:370-374.

Rosli, H.G., Zheng, Y., Pombo, M.A., Zhong, S., Bombarely, A., Fei, Z., Collmer, A., and Martin, G.B. (2013). Transcriptomics-based screen for genes induced by

flagellin and repressed by pathogen effectors identifies a cell wall-associated kinase involved in plant immunity. Genome biology 14:R139.

Salmeron, J.M., Oldroyd, G.E., Rommens, C.M., Scofield, S.R., Kim, H.S., Lavelle, D.T., Dahlbeck, D., and Staskawicz, B.J. (1996). Tomato *Prf is* a member of the leucine-rich repeat class of plant disease resistance genes and lies embedded within the *Pto* kinase gene cluster. Cell 86:123-133.

Schechter, L.M., Roberts, K.A., Jamir, Y., Alfano, J.R., and Collmer, A. (2004). *Pseudomonas syringae* type III secretion system targeting signals and novel effectors studied with a Cya translocation reporter. J Bacteriol 186:543-555.

Shan, L., He, P., Li, J., Heese, A., Peck, S.C., Nurnberger, T., Martin, G.B., and Sheen, J. (2008). Bacterial effectors target the common signaling partner BAK1 to disrupt multiple MAMP receptor-signaling complexes and impede plant immunity. Cell Host Microbe 4:17-27.

Shan, L., He, P., Zhou, J.-M., and Tang, X. (2000). A cluster of mutations disrupt the avirulence but not the virulence function of AvrPto. Mol Plant-Microbe Interact 13:592-598.

Thapa, S.P., Miyao, E.M., Davis, R.M., and Coaker, G. (2015). Identifcation of QTLs controlling resistance to *Pseudomonas syringae* pv. *tomato* race 1 strains from the wild tomato, *Solanum habrochaites* LA1777. Theor Appl Genet 128:681–692.

Thomma, B.P., Nurnberger, T., and Joosten, M.H. (2011). Of PAMPs and effectors: the blurred PTI-ETI dichotomy. Plant Cell 23:4-15.

Ueda, H., Yamaguchi, Y., and Sano, H. (2006). Direct interaction between the tobacco mosaic virus helicase domain and the ATP-bound resistance protein, N factor during the hypersensitive response in tobacco plants. Plant Mol Biol 61:31–45.

Veluchamy, S., Hind, S.R., Dunham, D.M., Martin, G.B., and Panthee, D.R. (2014). Natural variation for responsiveness to flg22, flgII-28, and csp22 and *Pseudomonas syringae* pv. *tomato* in heirloom tomatoes. PLoS One 2.

Wang, L., Albert, M., Einig, E., Fürst, U., Krust, D., and Felix, G. (2016). The pattern-recognition receptor CORE of *Solanaceae* detects bacterial cold-shock protein. Nat Plants.

Wu, A.J., Andriotis, V.M., Durrant, M.C., and Rathjen, J.P. (2004). A patch of surface-exposed residues mediates negative regulation of immune signaling by tomato Pto kinase. Plant Cell 16:2809-2821.

Xiang, T., Zong, N., Zou, Y., Wu, Y., Zhang, J., Xing, W., Li, Y., Tang, X., Zhu, L., Chai, J., et al. (2008). *Pseudomonas syringae* effector AvrPto blocks innate immunity by targeting receptor kinases. Curr Biol 18:74-80.

Xing, W., Zou, Y., Liu, Q., Liu, J., Luo, X., Huang, Q., Chen, S., Zhu, L., Bi, R., Hao, Q., et al. (2007). The structural basis for activation of plant immunity by bacterial effector protein AvrPto. Nature 449:243-247.

Zhang, Z., Wu, Y., Gao, M., Zhang, J., Kong, Q., Liu, Y., Ba, H., Zhou, J., and Zhang, Y. (2012). Disruption of PAMP-induced MAP kinase cascade by a *Pseudomonas syringae* effector activates plant immunity mediated by the NB-LRR protein SUMM2. Cell Host Microbe 11:253-263.

Zhao, B., Lin, X., Poland, J., Trick, H., Leach, J., and Hulbert, S. (2005). A maize resistance gene functions against bacterial streak disease in rice. Proc Natl Acad Sci USA 102:15383-15388.

Zhao, B.Y., Ardales, E., Brasset, E., Claflin, L.E., Leach, J.E., and Hulbert, S.H. (2004). The Rxo1/Rba1 locus of maize controls resistance reactions to pathogenic and non-host bacteria. Theor Appl Genet 109:71-79.

Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J.D., Boller, T., and Felix, G. (2006). Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. Cell 125:749-760.

Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E.J., Jones, J.D., Felix, G., and Boller, T. (2004). Bacterial disease resistance in *Arabidopsis t*hrough flagellin perception. Nature 428:764-767.

CHAPTER 2

NATURAL VARIATION IN TOMATO REVEALS DIFFERENCES IN THE RECOGNITION OF AVRPTO AND AVRPTOB EFFECTORS FROM *PSEUDOMONAS SYRINGAE*[1]

2.1 Abstract

The Pto protein kinase from *Solanum pimpinellifolium* interacts with *Pseudomonas syringae* effectors AvrPto or AvrPtoB to activate effector-triggered immunity. The previously solved crystal structures of the AvrPto-Pto and AvrPtoB-Pto complexes revealed that Pto binds each effector through both a shared and a unique interface. Here we use natural variation in wild species of tomato to further investigate Pto recognition of these two effectors. One species, *Solanum chmielewskii*, was found to have many accessions that recognize only AvrPtoB. The Pto ortholog from one of these accessions was responsible for recognition of AvrPtoB and it differed from *Solanum pimpinellifolium* Pto by just 14 amino acids, including two in the AvrPto-specific interface, glutamate-49/glycine-51. Converting these two residues to those in Pto (histidine-49/valine-51) did not restore recognition of AvrPto. Subsequent experiments revealed that a single substitution of a histidine-to-aspartate at position 193 in Pto, which is not near the AvrPto-specific interface, was sufficient for conferring recognition of AvrPto in plant cells. The reciprocal substitution of aspartate-to-histidine-193 in Pto abolished AvrPto recognition, confirming the importance of this residue. Our results reveal new aspects about effector recognition by Pto and demonstrate the value of using natural variation to understand the interaction between resistance proteins and pathogen effectors.

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[1] This chapter was published in modified form in Molecular Plant (2016, 9:5) and was written by Christine M. Kraus, Kathy R. Munkvold and Gregory B. Martin.

2.2 Introduction

The interaction of tomato with *Pseudomonas syringae* pv. *tomato* (*Pst*), which can result in bacterial speck disease, is an established model system for investigating the molecular basis of bacterial pathogenesis and the plant immune system. In this interaction, microbe-associated molecular patterns from *Pst* are bound by host pattern recognition receptors (PRRs) triggering a signaling pathway leading to pattern-triggered immunity (PTI), which effectively suppresses growth of the pathogen (Chinchilla et al., 2007; Gomez-Gomez and Boller, 2000; Mueller et al., 2012; Robatzek et al., 2007; Shan et al., 2008; Zipfel et al., 2004). Two sequence-divergent effector proteins, AvrPto and AvrPtoB, are translocated by *Pst* into the host cell where they interfere with the kinase domains of PRR complexes, resulting in enhanced growth of the pathogen (Cheng et al., 2011; He et al., 2006; Shan et al., 2008; Xiang et al., 2008). Both of these effectors are modular, with each having two discrete domains with distinct virulence activities; AvrPtoB also has a C-terminal E3 ligase domain that suppresses effector-triggered immunity (ETI) (Chang et al., 2001; Cheng et al., 2011; Mathieu et al., 2014; Shan et al., 2000a; Shan et al., 2000b; Wulf et al., 2004).

Tomato has evolved mechanisms to recognize one virulence domain of AvrPto and both virulence domains of AvrPtoB, leading in each case to activation of ETI (Abramovitch et al., 2003; Mathieu et al., 2014; Rosebrock et al., 2007; Shan et al., 2000b). These recognition events involve an NB-LRR protein Prf and members of the clustered *Pto* gene family that encodes host cytoplasmic protein kinases, some of which appear to have evolved to mimic (i.e., act as a decoy of) the kinase domains of PRRs and BAK1 (Cheng et al., 2011; Lin and Martin, 2007; Martin, 2012; Salmeron et al., 1996). One member of this family, Fen, binds variants of AvrPtoB that lack the E3 ligase domain that normally targets this kinase for degradation and acts with Prf to induce ETI. This recognition event is referred to as Rsb (Recognition suppressed by AvrPtoB C-terminus (Abramovitch et

al., 2003; Rosebrock et al., 2007). Another member of the kinase family, Pto, binds either the 'CD loop' in the core domain of AvrPto or the Fen- or Pto-interaction domain (PID and FID) of AvrPtoB, each of which target PRR kinase domains (Cheng et al., 2011; Gimenez-Ibanez et al., 2009; Gohre et al., 2008; Mathieu et al., 2014; Shan et al., 2008; Xiang et al., 2008; Xiao et al., 2007; Zeng et al., 2012). The Pto family has three other members (PtoA, PtoC, PtoD), but a role for these in immunity or any other host process is unknown (Chang et al., 2002; Pedley and Martin, 2003; Riely and Martin, 2001).

Pto was originally discovered in a wild relative of tomato, *Solanum pimpinellifolium*, one of 12 species of wild relatives of tomato native to western South America (Martin et al., 1993; Peralta et al., 2008). Accessions belonging to each of these species have the *Pto* gene family and *Pto* family orthologs between species are more similar in sequence than are paralogs within the species (Chang et al., 2002; Riely and Martin, 2001; Rose et al., 2005). Clustered gene families with members highly similar to *Pto* are present in other Solanaceous species including *Nicotiana benthamiana*, tobacco, pepper and potato suggesting that the *Pto* family arose prior to *Solanum* speciation (Bombarely et al., 2012; Grube et al., 2000; Vleeshouwers et al., 2001).

Solution of the crystal structures of the AvrPto-Pto and AvrPtoB-Pto complexes revealed that each pathogen-host protein interaction involves both a common and an effector-specific interface (Dong et al., 2009; Xing et al., 2007). Substitutions in amino acids of Pto at the shared interface abolish the interaction with both effectors in a yeast two-hybrid system, whereas substitutions in each unique interface abolish interaction only with the cognate individual effector (Dong et al., 2009). The expectation that such substitutions would abolish effector recognition in plant cells could not be tested because the substitutions caused Pto auto-activation (constitutive cell death) in *N. benthamiana* (Dong et al., 2009; Xing et al., 2007). The discovery of effector-specific interfaces suggested that natural variation might exist in the Pto family in wild relatives of tomato

that would shed further light on how Pto-like kinases recognize and respond to AvrPto and AvrPtoB. We therefore screened wild relatives of tomato for their response specifically to these effectors and found *Solanum chmielewskii* (*Schm)* accessions that are susceptible to *Pst* expressing AvrPto but resistant to those expressing AvrPtoB. Molecular characterization of this phenotype revealed that a region of Pto distinct from the effector-interfaces plays an important role in the response to AvrPto.

2.3 Results

Solanum chmielewskii accessions are resistant to *Pst* delivering AvrPtoB, but susceptible to *Pst* delivering AvrPto

To investigate ETI-mediated natural variation for *Pst* resistance in tomato, we screened accessions of wild tomato species by syringe infiltrating different leaflets in the same leaf with the following *Pst* strains: 1) DC3000 wild type (having both *avrPto* and *avrPtoB*); 2) DC3000Δ*avrPto*; 3) DC3000Δ*avrPtoB*; or 4) DC3000Δ*avrPto*Δ*avrPtoB*. Plants were scored for symptoms of speck disease 3-4 days later. All six of the *Schm* accessions tested initially were resistant to DC3000 wild type and to the *Pst* strain with AvrPtoB only (strain DC3000Δ*avrPto*), but were susceptible to the strain with AvrPto only (DC3000Δ*avrPtoB*) and to the *Pst* strain DC3000Δ*avrPto*Δ*avrPtoB* lacking both effectors. A subsequent targeted screen of other *Schm* accessions identified an additional 16 that recognized only AvrPtoB (3 *Schm* accessions recognized neither effector). *Schm* only occurs in Andean Valleys in the Apurimac, Ayacucho and Cuzco regions within a geographically restricted area in southern Peru, and the accessions we tested derived from all three of these regions (Peralta et al., 2008) (**Figure 2.1A**).

We focused on one *Schm* accession, LA2677, as representative of the AvrPtoB-specific phenotype, to investigate the molecular basis of AvrPtoB-specific recognition. Whole plants of LA2677 were vacuum-infiltrated with *Pst* strains DC3000Δ*avrPto* or

DC3000Δ*avrPtoB* and scored for disease 3-4 days later. Bacterial speck disease developed only in LA2677 plants that were infiltrated with DC3000 lacking *avrPtoB* (**Figure 2.1B**). As expected, the tomato variety Rio Grande-PtoR (RG-PtoR), which expresses *Pto* from *Solanum pimpinellifolium*, showed no disease symptoms upon infiltration with either of the DC3000 strains and RG-prf3 (RG-PtoR with a deletion in the *Prf* gene) developed disease upon infiltration with both DC3000 strains. To examine whether these results reflected *Pst* growth in leaves, tissue samples were taken immediately (day 0) and at day 3 after infiltration to measure bacterial populations (**Figure 2.1C**). As expected, both bacterial strains grew poorly in RG-PtoR, but multiplied to high levels in RG-prf3. In the case of LA2677, DC3000Δ*avrPtoB* reached a population size comparable to that in RG-prf3. DC3000Δ*avrPto* reached a population size four orders of magnitude lower and below even that observed in RG-PtoR (**Figure 2.1C**). These experiments therefore confirmed that LA2677 is able to recognize AvrPtoB, but not AvrPto, to effectively activate ETI and suppress bacterial speck disease.

Bacterial speck resistance elicited by AvrPtoB in LA2677 requires Prf

To examine whether AvrPtoB-specific recognition in LA2677 involves the Pto/Prf pathway, as expected, we used virus-induced gene silencing (VIGS). Due to their sequence similarity it was not possible to silence individual *Pto* family members, so instead a Tobacco Rattle Virus (TRV) vector containing a fragment of *Prf* that was developed and verified previously was used to infect LA2677 seedlings; a TRV construct carrying a fragment of *E. coli* (*EC1*) served as a control (Ekengren et al., 2003; Rosli et al., 2013).

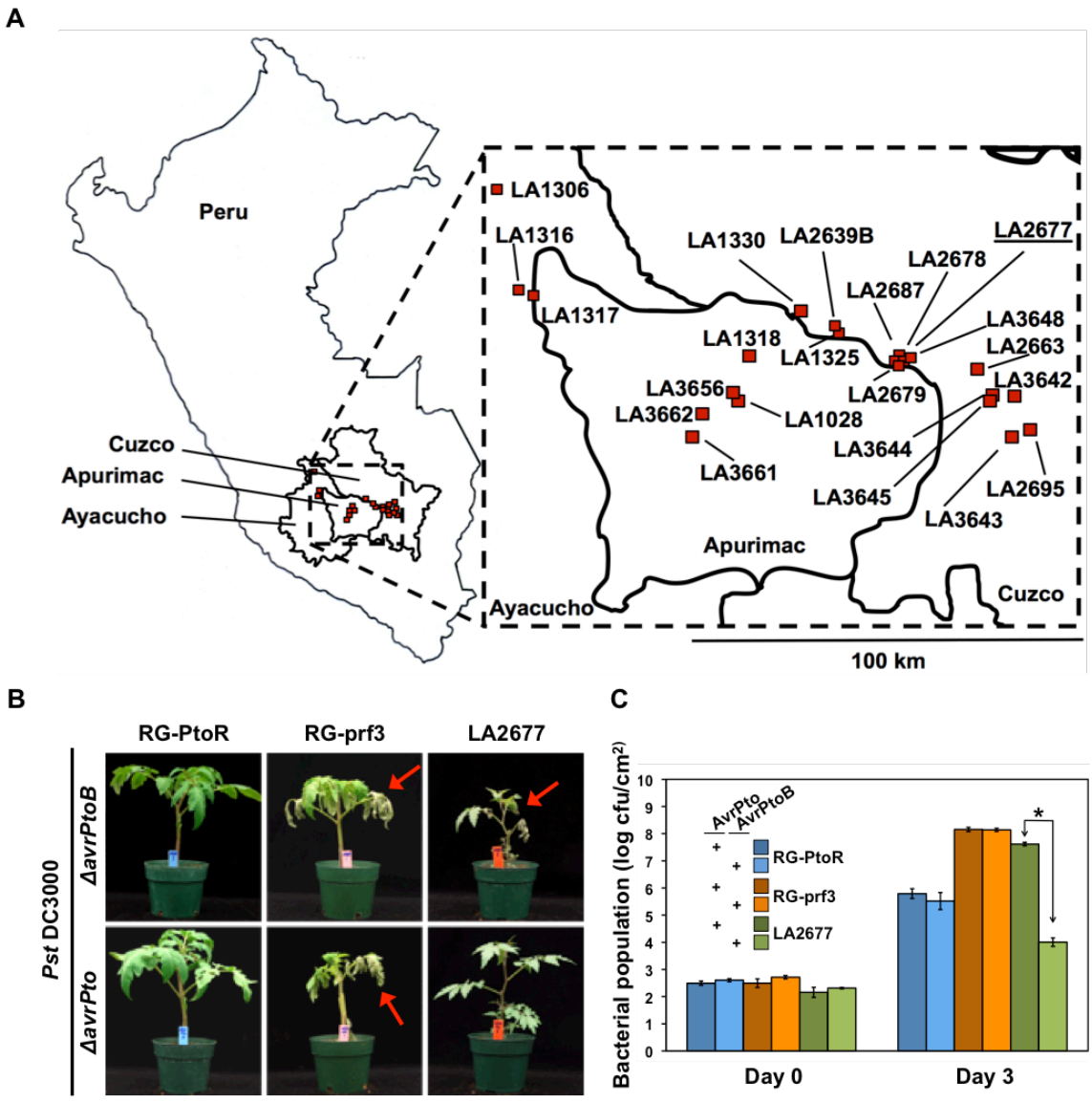


Figure 2.1. *S. chmielewskii* accessions are resistant to *P. s. pv. syringae (Pst*) strains that translocate AvrPtoB but not AvrPto into the plant cell. **(A)** Map showing the geographical locations in three regions of southern Peru where the *S. chmielewskii* accessions (red squares) were originally collected that recognize only AvrPtoB (information from TGRC: tgrc.ucdavis.edu). **(B)** Plants were vacuum infiltrated with 5 x 10^4 CFU/mL *Pst* DC3000Δ*avrPto* (expresses AvrPtoB) or DC3000Δ*avrPtoB* (expresses AvrPto) strains and photographed four days after inoculation. Red arrows point to disease symptoms. **(C)** Bacterial populations were measured in plants inoculated as in part B three hours (day 0) and three days after inoculation. The experiments were repeated 4 times and panels B and C depict results from a representative experiment using three biological replicates per strain. Data are presented as mean ± SD. The asterisk denotes a statistically significant difference (p value < 0.05).

Four weeks after TRV infection the plants were vacuum-infiltrated with DC3000Δ*avrPto* and four days later were scored for symptoms of speck disease. LA2677 plants silenced for *Prf* developed typical symptoms of speck disease, whereas the *EC1* control plants showed no disease (**Figure 2.2A**). These results indicate that the recognition of full-length AvrPtoB in LA2677 requires *Prf* and therefore likely involves a member of the *Pto* gene family.

Three *Pto* gene family members are expressed in LA2677 leaves

Using two sets of primers designed to amplify all five of the *Pto* family members, we succeeded in cloning and sequencing genes of four different *Pto* family members from LA2677 genomic DNA. A maximum likelihood tree placed each LA2677 *Pto* gene with its ortholog in the tomato variety Rio Grande-PtoR (RG-PtoR; these orthologs are from the *S. pimpinellifolium* chromosomal segment present in this variety and are referred to hereafter as *Pto*, *Fen* and *PtoC* and *PtoD*) (Chang et al., 2002; Riely and Martin, 2001). The *Schm* genes are referred to as *Pto*-2677, *Fen*-2677, *PtoC*-2677, and *PtoD*-2677 (**Figure 2.2B**). To determine whether these genes are expressed, primers specific for each LA2677 gene were designed and tested first to verify that they amplified the expected product from genomic DNA, which they did (**Figure 2.2C**). RNA was then extracted from LA2677 leaves, cDNA was synthesized and used in an RT-PCR reaction with the gene-specific primers. Transcripts of only *Pto-2677*, *Fen-2677*, and *PtoD-2677* were detected indicating that these genes, but not *PtoC*-2677, are expressed in leaves of LA2677 (**Figure 2.2C**).

Pto-2677 physically interacts with AvrPtoB in a yeast two-hybrid system

To gain further insight into which of the three *Pto* family members expressed in LA2677 might be involved in the AvrPtoB-specific resistance, we performed a pairwise yeast two-hybrid analysis. The *Pto-2677*, *Fen-2677*, and *PtoD-2677* genes, as well as their orthologs from the tomato variety RG-PtoR (*Pto, Fen, PtoD*), were cloned into a bait

vector and tested against full-length and truncated versions of AvrPtoB (**Figure 2.3A**). As previously reported, Fen interacts with the Fen-interaction domain (FID) of AvrPtoB only when the E3 ubiquitin ligase has either been mutated, as in the case of an E3 ligase loss-of-function (E3-LOF) protein, or deleted, as in the case of the E3 ubiquitin ligase truncated variant, $AvrPtoB_{1-387}$ (Abramovitch et al., 2003; Mathieu et al., 2014). Pto interacts with two domains of AvrPtoB, the FID as well as a unique domain for Pto, within amino acids 1-307, referred to as the Pto-interaction domain (PID) (Abramovitch et al., 2003). PtoD does not interact with any of the AvrPtoB variants (Rosebrock et al., 2007). We observed that the LA2677 Pto family members interacted with AvrPtoB in an identical manner as their orthologs in RG-PtoR (**Figure 2.3A**). Although Fen-2677 had some auto-activation background, the darker blue patches indicated this protein interacted with E3-LOF and $AvrPtoB_{1-387}$, but not with full-length AvrPtoB that has an active E3 ubiquitin ligase, nor with $AvrPtoB_{1-307}$, which lacks the FID (**Figure 2.3A**). Pto-2677 interacted with all of the AvrPtoB variants and, notably, was the only one of the three LA2677 Pto proteins that interacted with full-length AvrPtoB. PtoD-2677 did not interact with any of the variants (**Figure 2.3A**). Proteins of all the Pto family members were expressed well in yeast (**Figure 2.4A**).

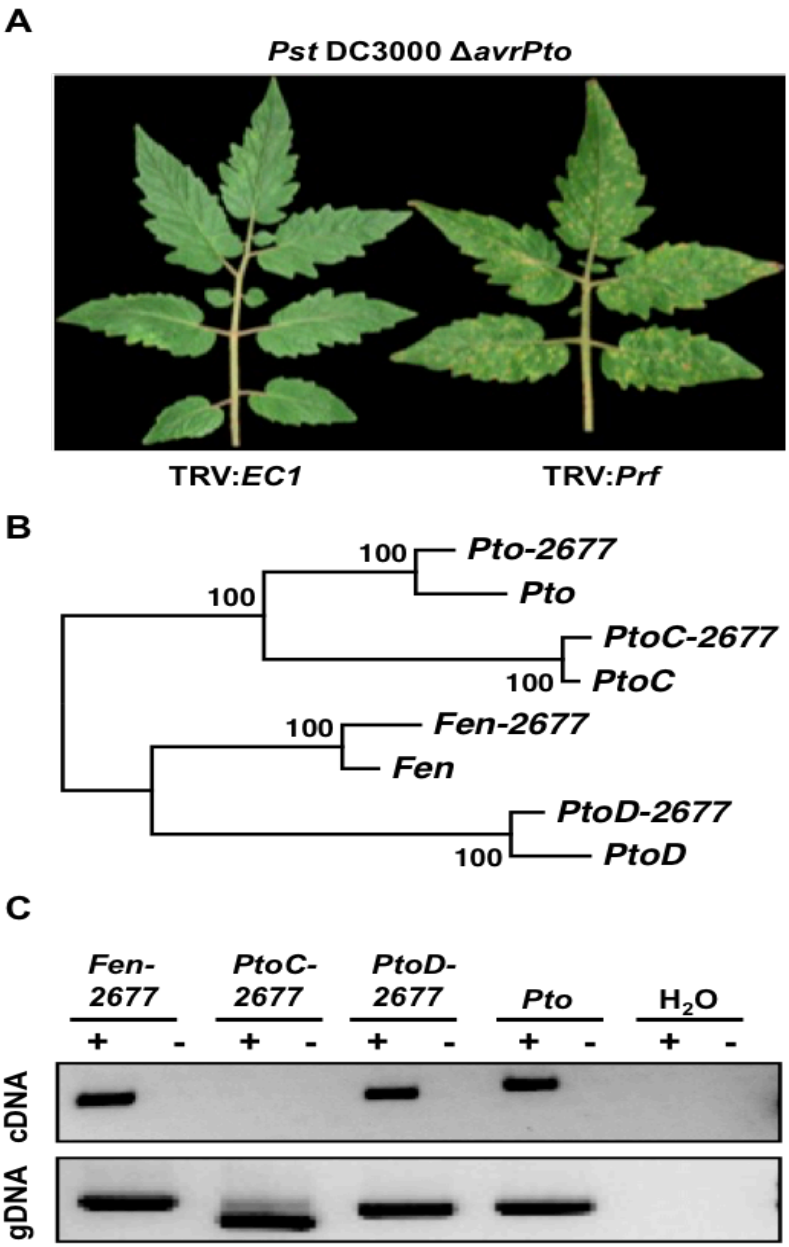


Figure 2.2. LA2677 recognition of AvrPtoB is dependent on Prf. **(A)** Leaves silenced for *Prf* or with the control *EC1* were vacuum infiltrated with 10^5 CFU/mL *Pst* DC3000Δ*avrPto* (expresses AvrPtoB) and symptoms of bacterial speck disease (appearing only on the TRV:Prf plants) were photographed **4** days later. **(B)** A maximum likelihood tree comparing the aligned nucleotide sequences of the *Pto* family members present in LA2677 and their homologs in RG-PtoR. The tree is unrooted and the number of supporting bootstrap values for 100 replications is shown. **(C)** Reverse transcriptase-PCR using primers specific for each *Pto* member. +, with reverse transcriptase (RT); -, no RT control. cDNA, complementary DNA; gDNA, genomic DNA.

Pto-2677 triggers AvrPtoB-specific cell death in *Nicotiana benthamiana* leaves

We next examined whether any of the LA2677 Pto proteins would cause AvrPtoB-specific immunity-associated cell death in leaves of *Nicotiana benthamiana*, a model species for plant-pathogen studies (Bombarely et al., 2012). For these experiments, we utilized *Agrobacterium*-mediated transient expression ('agroinfiltration'), which is a robust method to study Pto-mediated recognition of both *Pst* effectors as long as the tomato Prf protein is also co-expressed in the system (Mucyn et al., 2006). Expression of the Fen protein alone in this system can trigger cell death so each LA2677 Pto family member was first tested for this possibility. Although each of the LA2677 Pto proteins was expressed well in *N. benthamiana* leaves, none of the proteins triggered cell death on their own, even seven days after agroinfiltration (**Figure 2.4B,C**). Consistent with the results from the yeast two-hybrid analysis, expression of either Pto or Pto-2677, together with AvrPtoB and Prf, caused cell death in *N. benthamiana* leaves (**Figure 2.3B**).

AvrPto interacts in yeast with Pto-2677 but the expression of the effector in LA2677 leaves does not trigger cell death

Our results at this stage indicated that Pto-2677 is responsible for the AvrPtoB-mediated resistance observed in LA2677, but they left unanswered the question of why this protein is unable to activate ETI in response to AvrPto. A comparison of the amino acid sequence of Pto with Pto-2677 revealed that they differ by just 14 residues (4% of the 320 residues) (**Figure 2.5A**). All amino acids known to be important for the physical interaction with AvrPtoB are conserved (Dong et al., 2009). However, two amino acids known to be involved in the Pto interaction with AvrPto, histidine (H)-49 and valine (V)-51, have substitutions in Pto-2677 (glutamic acid (E)-49 and glycine (G)-51) (**Figure 2.5A**) (Dong et al., 2009; Xing et al., 2007). These same substitutions occur in Fen, which is also unable to interact with AvrPto. Nevertheless, we found that Pto-2677 was able to interact with AvrPto in the yeast-two hybrid system. The interaction was abolished by the I96A

substitution in AvrPto, an amino acid that is essential for interaction with Pto (**Figure 2.5B**) (Devarenne et al., 2006).

The interaction of AvrPto with Pto-2677 was unexpected based on the inability of LA2677 to recognize AvrPto when it is translocated from *Pst*. To test whether Pto-2677 might respond to AvrPto when the effector is overexpressed, we syringe-infiltrated leaves of LA2677 or RG-PtoR with *Agrobacterium* carrying constructs for the overexpression of AvrPto, AvrPtoB or yellow fluorescent protein (YFP) and monitored for immunity-associated cell death. Consistent with the phenotypes we observed in the natural *Pst* – LA2677 interaction (**Figure 2.1B**), AvrPto did not elicit immunity-associated cell death, whereas, as expected, AvrPtoB did (**Figure 2.5C**). Also as expected, both effectors elicited cell death in leaves of RG-PtoR. These results therefore indicate that although Pto-2677 might interact with AvrPto in plant cells, the interaction does not activate an effective immune response in LA2677.

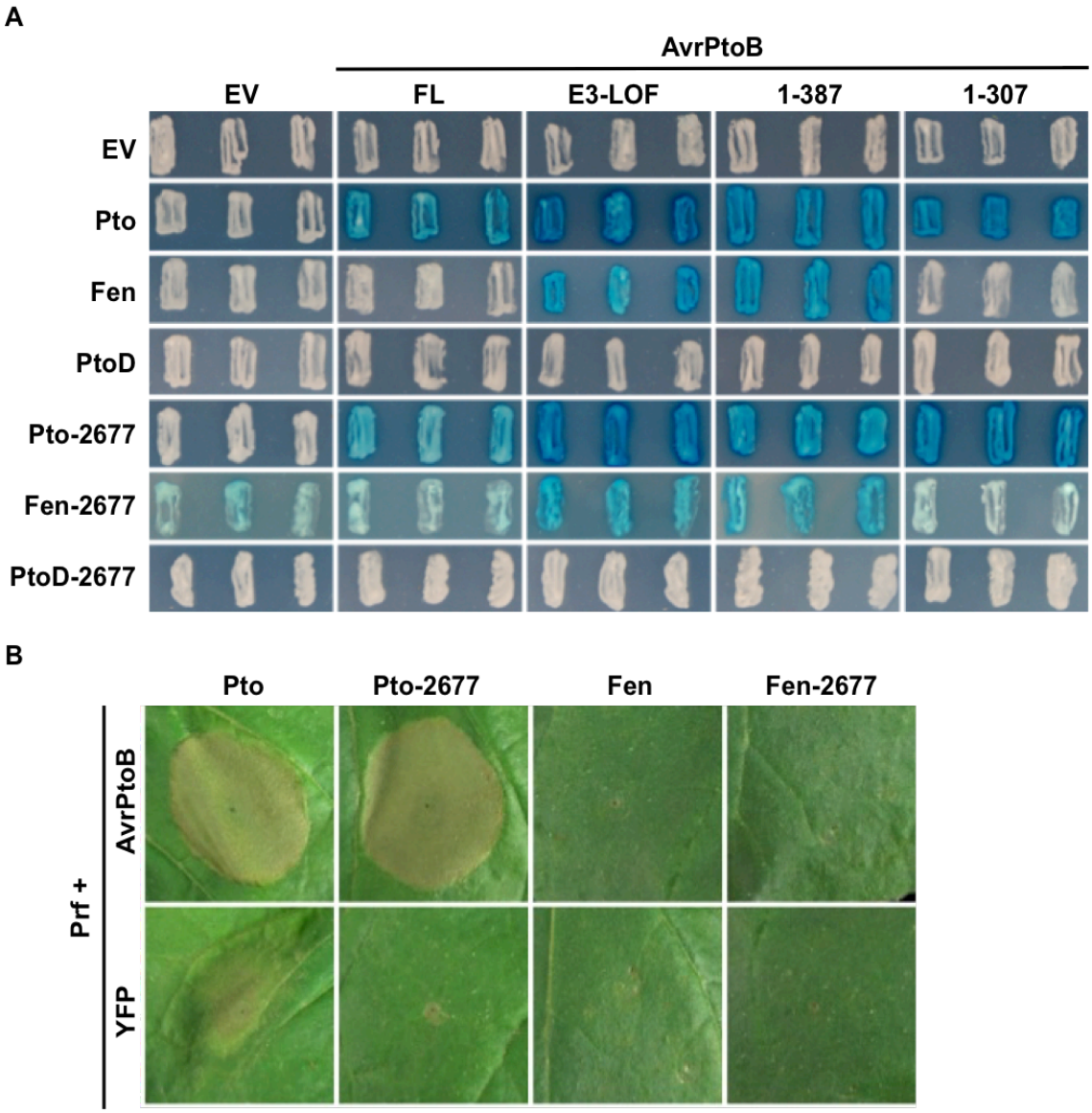
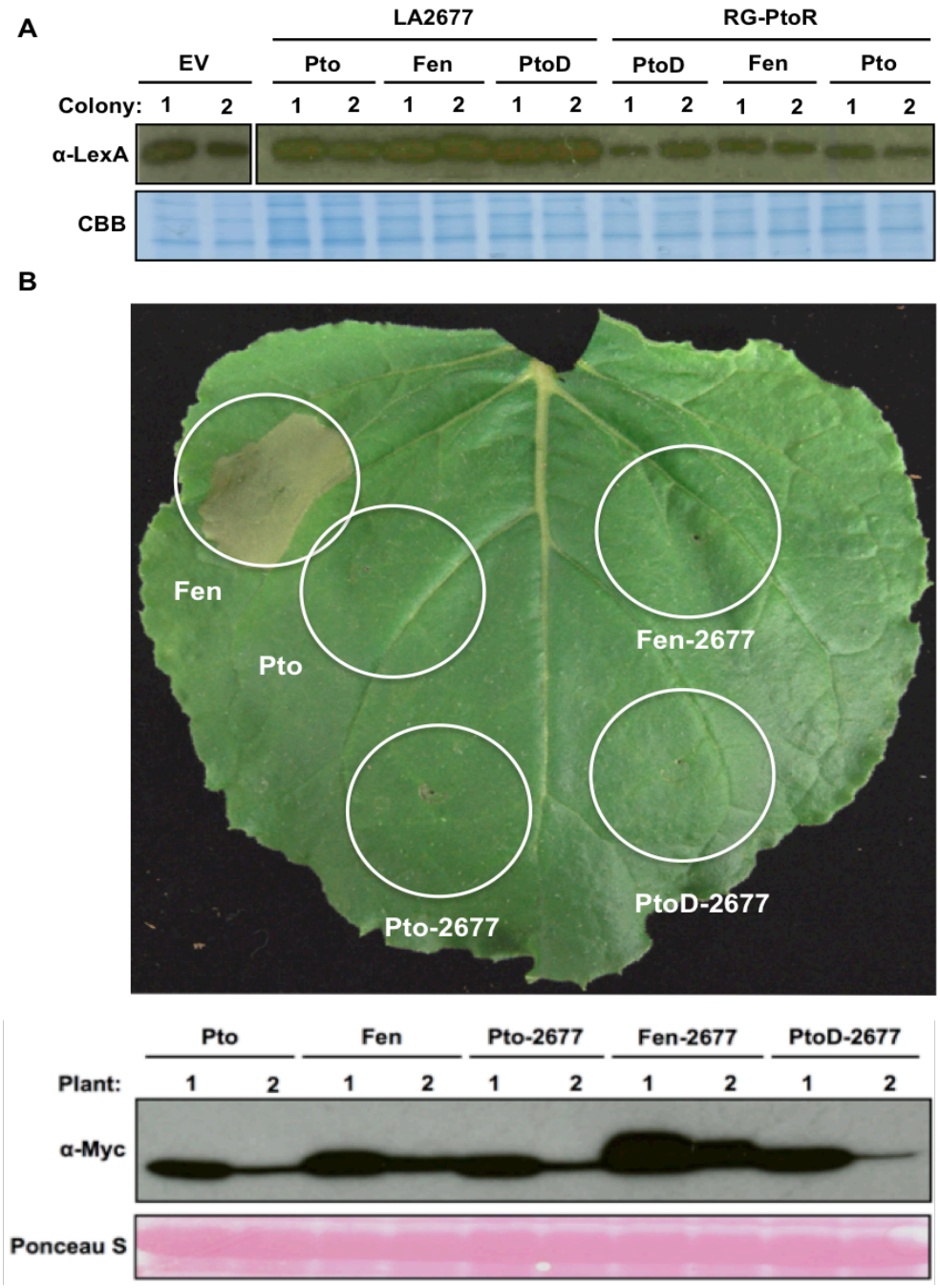


Figure 2.3. Pto-2677 interacts with AvrPtoB in yeast and activates AvrPtoB-specific cell death in leaves. **(A)** Pairwise yeast two-hybrid analyses testing interaction of Pto family members (bait vector) with full-length AvrPtoB and truncated forms of AvrPtoB (prey vector). Blue patches indicate a positive interaction. Photographs were taken 26 hours after patching, except for Fen-2677, which was photographed after 16 hours due to strong auto-activation. EV, empty vector; FL, full-length; E3-LOF, E3 ubiquitin ligase loss-of-function; 1-387 and 1-307, $AvrPtoB_{1-387}$ and $AvrPtoB_{1-307}$ truncations, respectively (Table S1). **(B)** Pto and Fen from LA2677 or RG-PtoR were co-expressed with Prf and either AvrPtoB or yellow fluorescent protein (YFP) in *N. benthamiana* leaves using Agrobacterium-mediated transient transformation. Photographs were taken 5 days later.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α-Myc
Ponceau S

Figure 2.4 (*previous page*). Expression of LA2677 Pto family members in yeast and *N. benthamiana*. **(A)** Expression of Pto proteins fused to LexA in yeast (related to Figure 3A). The mass of the empty vector (EV) construct (i.e., LexA) is 26 kDa and for the different Pto:LexA proteins is 59 kDa. 1.5 mL yeast at an $OD_{600} = 0.6$ was boiled in Laemmli sample buffer and proteins were detected using an anti-LexA antibody. Western blot gels show samples taken from two biological replicates for each protein expressed. Staining with Coomassie brilliant blue (CBB) is shown as a loading control. **(B)** The three Pto proteins that are expressed in LA2677 (see Figure 2B), as well as Pto and Fen from RG-PtoR were expressed in *N. benthamiana* leaves using *Agrobacterium*-mediated transient transformation. Photographs were taken 5 days after infiltration. Leaf samples were taken 48 hours after infiltration and proteins were isolated for Western blot analysis. All proteins are fused to a Myc epitope and proteins were detected using an anti-c-Myc-HRP rabbit polyclonal antibody. Predicted size for all proteins is approximately 45 kDa. Western blot shows samples taken from two biological replicates. Ponceau staining was performed to demonstrate equal loading.

Substitutions in residue 49 and 51 in Pto-2677 are not sufficient to activate an AvrPto-mediated immune response

We next tested whether simply changing the E49 and G51 residues in Pto-2677 to the H49 and V51 of Pto was sufficient to confer recognition of AvrPto in *N. benthamiana* **(Figure 2.6A)**. These substitutions in Pto-2677, however, were not sufficient to allow for recognition of AvrPto as no cell death was observed (**Figure 2.6B)**. We proceeded to use this Pto-2677(E49H/G51V) construct to make additional substitutions in some of the other 12 residues that differ between Pto and Pto-2677. These new variants of Pto-2677 were then tested for their ability to cause immunity-associated cell death after co-expression with Prf and either AvrPtoB or AvrPto. We used the co-crystal structure of the Pto-AvrPto complex (PDB# 2QKW) as well as a previous paper on the DNA shuffling of Pto family members to aid in determining which Pto-2677 amino acids to alter (Bernal et al., 2005; Xing et al., 2007) **(Figure 2.6A)**. Of six constructs tested, only one, in which the Pto-2677 histidine residue (H) at position 193 was changed to aspartic acid (D) (Pto-2677[E49H/G51V/H193D]), caused cell death in *N. benthamiana* leaves upon co-expression with AvrPto and Prf (**Figure 2.6B, Figure 2.7A**). All of the Pto-2677 variant proteins were expressed as shown by Western blot and were functional based on their

ability to trigger cell death in leaves when co-expressed with AvrPtoB and Prf (**Figure 2.6B, Figure 2.7B**).

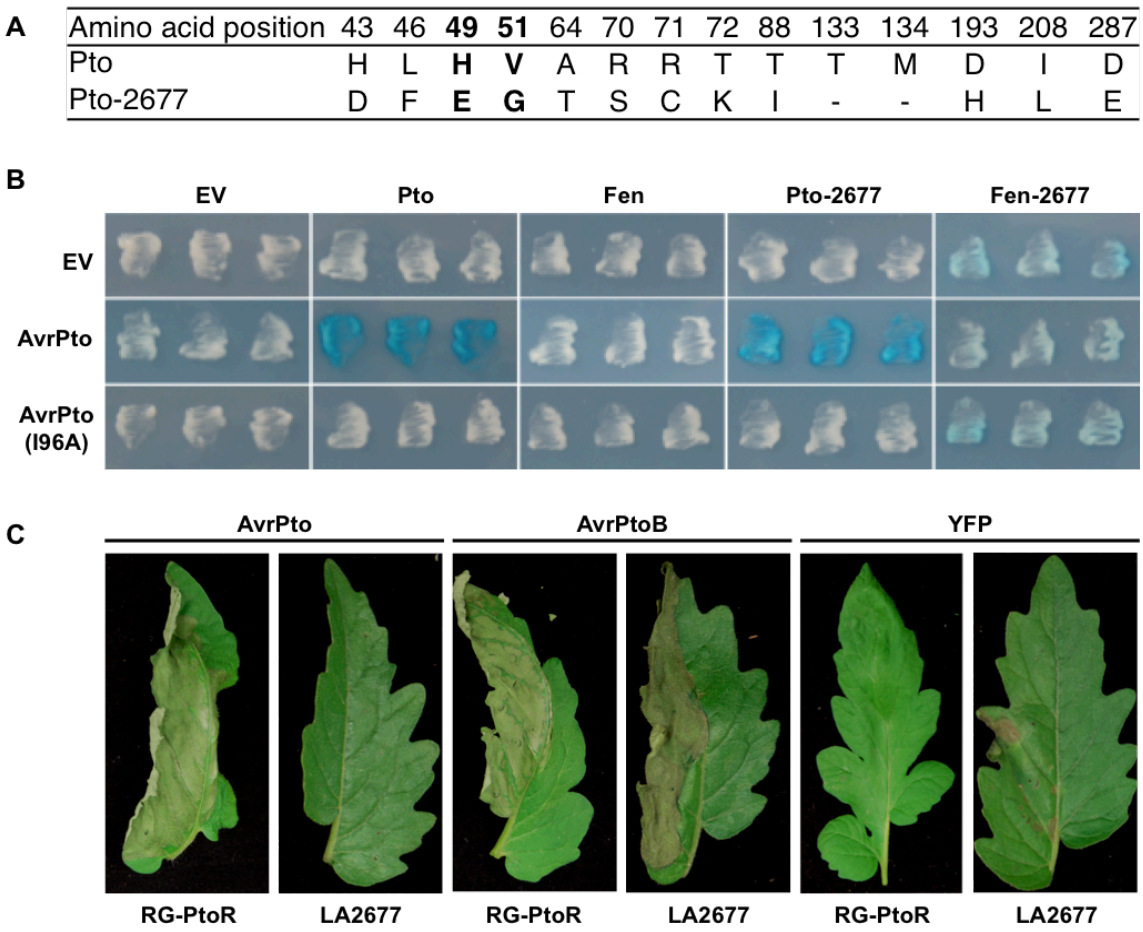


Figure 2.5. Pto proteins from LA2677 and RG-PtoR differ in amino acid sequence and in their ability to recognize AvrPto in leaves. **(A)** Representation of the 14 amino acid differences between Pto-2677 and Pto. Marked in bold are the two residues, H49 and V51, known to be important for AvrPto recognition and signaling. **(B)** Pairwise yeast-two hybrid analyses testing the interaction of Pto and Fen from LA2677 and RG-PtoR (bait vector) with AvrPto and a known AvrPto mutant (AvrPto (I96A)) that does not interact with Pto in yeast as a control. Blue patches indicate a positive interaction. Photographs were taken 18 hours after patching, except for Fen-2677, which was photographed 16 hours after patching because of auto-activation at later time points. EV, empty vector. **(C)** AvrPto, AvrPtoB or YFP were expressed in leaves of RG-PtoR or LA2677 using Agrobacterium-mediated transient expression and photographs were taken 3 days later. Cell death indicates effective recognition of the effector and subsequent activation of the immune signaling pathway.

Aspartic acid-193 plays an essential role in recognition of AvrPto

To gain further insight into the importance of the aspartic acid-193 residue in Pto for AvrPto recognition, we generated a Pto-2677(H193D) construct and tested it transiently in *N. benthamiana* for its ability to cause immunity-associated cell death. In *N. benthamiana* leaf areas co-expressing Pto-2677(H193D), Prf and AvrPto, we observed cell death in ~60% of our experiments (11/18 infiltrations from 6 independent experiments; **Figure 2.6B**), and its appearance was often delayed by two days as compared to cell death triggered by AvrPto-Pto-2677(E49H/G51V/H193D) or AvrPto-Pto, in which we observed cell death in 100% of the infiltrated areas. These observations suggest that H193 plays an important role in AvrPto recognition and, consistent with previous reports, H49/V51 are also needed for the robust response to AvrPto (Dong et al., 2009; Xing et al., 2007).

We next tested whether D193 in Pto also plays a role in responding to AvrPto in *N. benthamiana* leaves. We observed that while co-expression of Pto(D193H) with AvrPtoB and Prf triggered cell death as expected, the D193H substitution completely abolished its ability to cause AvrPto-specific cell death (**Figure 2.6C**). D193 residue is therefore required for Pto to signal an immune response upon recognition of AvrPto. Importantly, AvrPtoB is still recognized by all of the Pto-2677 and Pto mutants (**Figure 2.6B, Figure 2.6C**), demonstrating that Pto either recognizes AvrPto and AvrPtoB differently, or that there are differences in the downstream signaling pathway subsequent to effector recognition.

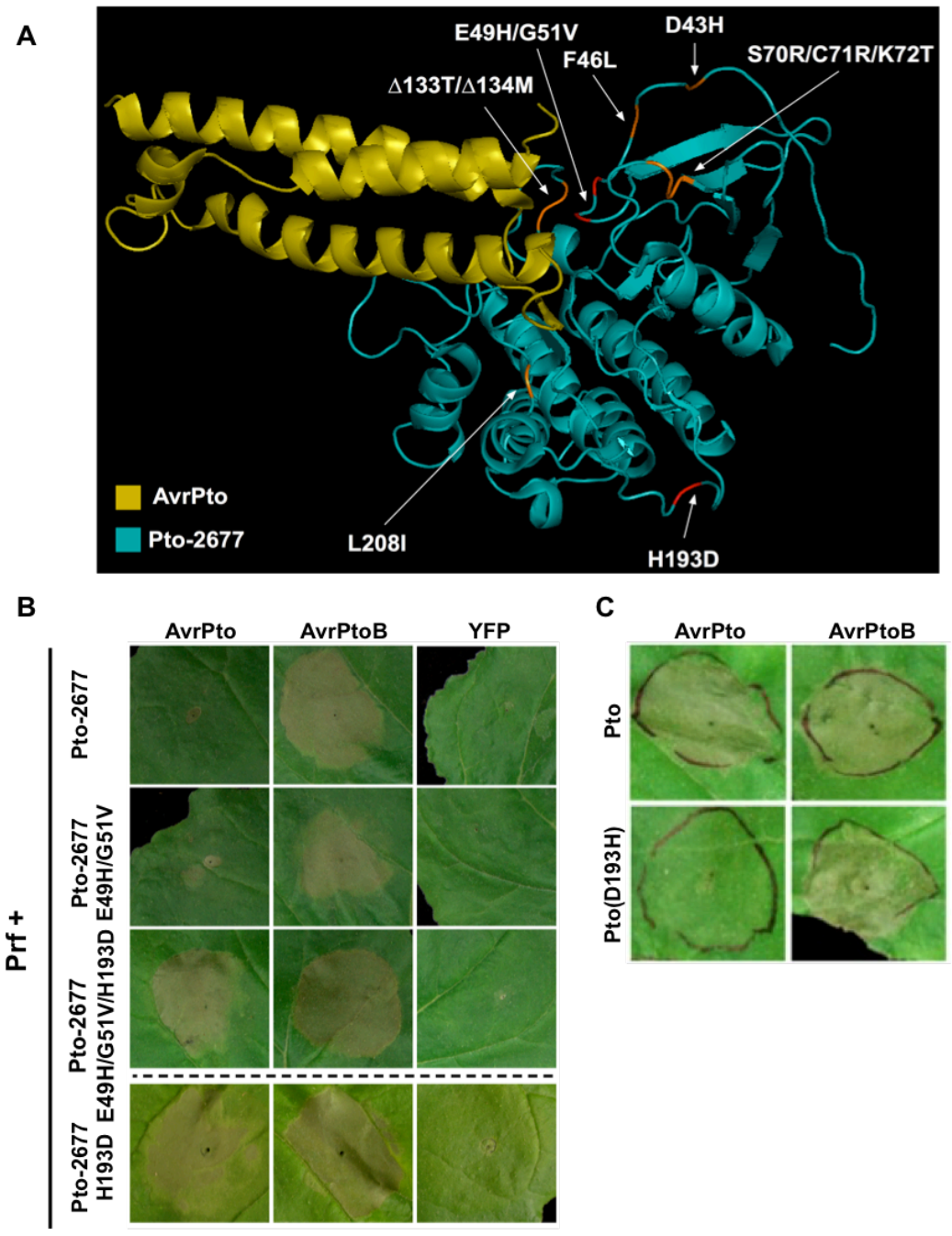

A
E49H/G51V
D43H
F46L
S70R/C71R/K72T
Δ133T/Δ134M
AvrPto
Pto-2677
L208I
H193D
B
AvrPto
AvrPtoB
YFP
Prf +
Pto-2677
Pto-2677 E49H/G51V
Pto-2677 E49H/G51V/H193D
Pto-2677 H193D
C
AvrPto
AvrPtoB
Pto
Pto(D193H)

Figure 2.6 (*Previous page*). Aspartic acid-193 in Pto is required for AvrPto-specific cell death in *N. benthamiana.* (A) Predicted crystal structure of Pto-2677 (cyan) interacting with AvrPto (gold) based on the co-crystal structure of the Pto-AvrPto complex (PDB# 2QKW). Highlighted in orange are the residues that were changed in Pto-2677 and which were not involved in AvrPto-specific responses. Highlighted in red are the two residues at the AvrPto-specific interface (E49/G51 as well as the third amino acid (H193), a substitution at which was required to allow Pto-2677 recognition of and signaling in response to AvrPto. All residues were changed in Pto-2677 to the amino acid present in Pto at the same position (see Figure 4A). The open-source molecular visualization system PyMOL was used to generate the image based on coordinates in PDB # 2QKW. (B) Pto-2677 variants were co-expressed with one of the effectors or YFP and tomato Prf in *N. benthamiana* leaves using *A. tumefaciens*-mediated transient expression. Photographs were taken 5 days after infiltration. This experiment was repeated several times with identical results and representative results are shown. The Pto-2677(H193D) experiment was performed separately multiple times, as indicated by the dashed line; cell death was visible in 60% of our infiltrations; photographs in these cases were taken 7 days after infiltration. (C) Pto and Pto(D193H) and tomato Prf were co-expressed with AvrPto or AvrPtoB in *N. benthamiana* leaves by *Agrobacterium*-mediated transient transformation. Pto(D193H) was unable to trigger immunity-associated cell death in areas co-infiltrated with AvrPto.

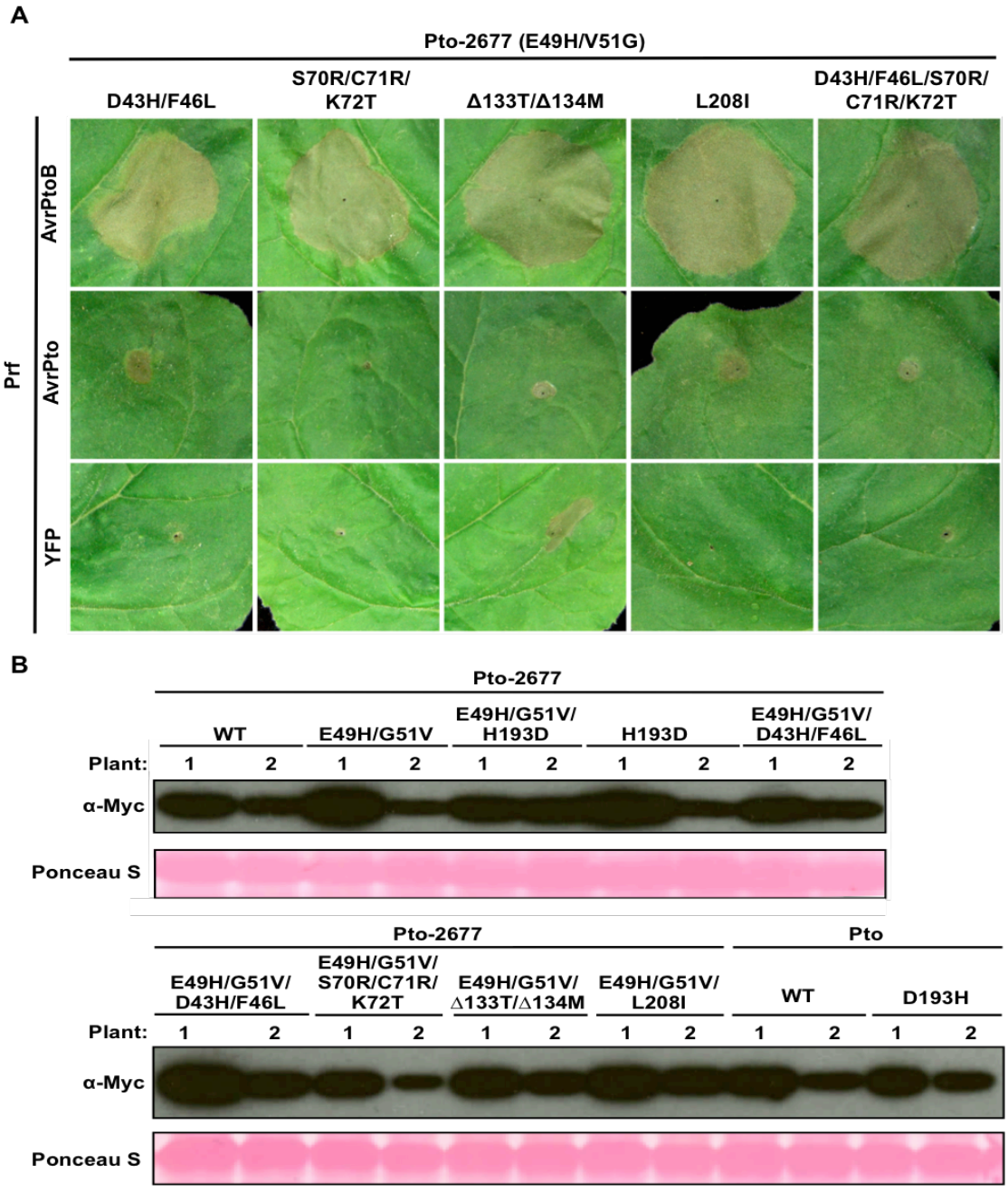


Figure 2.7. Substitutions of eight amino acid residues that differ between Pto-2677 and Pto did not confer the ability to recognize AvrPto. **(A)** Pto-2677 variants (already carrying the E49H/G51V substitutions) were co-expressed with Prf and AvrPto, AvrPtoB or YFP using *Agrobacterium*-mediated transient expression in *N. benthamiana* leaves. Photographs were taken 5 days after infiltration. This experiment was repeated several times with identical results. **(B)** Protein expression and accumulation for all the mutants used in Figure 5AB and S2A were confirmed by Western blot. Samples were taken 48 hours after infiltration and proteins were detected as in Figure S1B.

2.4 Discussion

When the crystal structures of the AvrPto-Pto and AvrPtoB complexes were solved they revealed both a shared and a unique interface for each effector interaction (Dong et al., 2009; Xing et al., 2007). This raised the possibility that natural variants of Pto might exist that interact with one effector and not the other. In fact, our screen of wild relatives of tomatoes uncovered examples of this in 22 accessions of *Schm* which recognize only AvrPtoB. Our screen also identified three accessions that were susceptible to wild type DC3000, revealing there is some natural variation for *Pst* recognition in this species. *Schm* occurs in a geographically restricted area in southern Peru and its ability to recognize only AvrPtoB might suggest that *Pst* strains it has been exposed to over its evolutionary history have this effector and not AvrPto. Unfortunately, nothing has been reported of the effector repertoires of *Pst* strains that are endemic to Peru and a fuller understanding of the *Pst* selection pressures that might have been or are still exerted on the wild relatives of tomato will require collection and characterization of *Pst* strains from this region.

By a series of experiments, we determined that the ability of LA2677 to recognize AvrPtoB is due to its Pto ortholog acting with Prf. The Pto-2677 protein has all of the residues known from studies with Pto to be required for interaction with AvrPtoB. Interestingly, a *Pst* strain expressing AvrPtoB reached lower population levels in LA2677 that it does in RG-PtoR which expressed Pto/Prf from *Solanum pimpinellifolium*. The reason for this is unknown and could be due to several, not mutually exclusive, possibilities including the presence of non-Pto-related resistance factors in LA2677, higher expression of Pto-2677, stronger binding of AvrPtoB by Pto-2677, or a more robust downstream response perhaps due to an enhanced interaction of Pto-2677 with Prf from LA2677.

Our experiments with the various LA2677 *Pto* gene family members also revealed that Fen-2677, in contrast to Fen, does not cause cell death by itself when expressed in leaves of *N. benthamiana*. It has been proposed that this Fen constitutive signaling in *N. benthamiana* requires an active kinase activity, and that Fen auto-activation might be inhibited by physical interaction with the tomato Prf protein (Mucyn et al., 2009). As is the case with Fen, Fen-2677 interacted only with AvrPtoB variants that have the FID but which lack E3 ligase activity, suggesting Fen-2677 is also degraded by AvrPtoB-mediated ubiquitination (Rosebrock et al., 2007). Fen-2677 differs from Fen by just 8 amino acids and in the future it will be interesting to determine if one or more of these differences renders the Fen-2677 kinase inactive or if they allow *N. benthamiana* Prf or another repressor protein in this species to bind more strongly to Fen-2677 and hence control Fen activity.

We found that LA2677 is not resistant to *Pst* expressing AvrPto and that *Agrobacterium*-mediated overexpression of AvrPto in leaves of LA2677 did not cause immunity-associated cell death. It was therefore unexpected that Pto-2677 interacted with AvrPto in our yeast-two hybrid experiments. It is possible this is an artifact of the yeast two-hybrid system which involves overexpression of proteins and localization to the yeast nucleus. However, the fact that AvrPto(I96A) does not interact with Pto-2677 indicates the interaction relies on the well-characterized CD loop of the effector (Martin, 2012). If AvrPto and Pto-2677 also physically interact in plant cells then it raises the intriguing question as to why the interaction does not trigger an effective immune response. One possibility is that there are not only distinct binding interfaces involved in the Pto response to these effectors (Dong et al., 2009), but that Pto also undergoes different structural changes depending on the recognized effector that affect its subsequent signaling properties. There is currently no evidence that AvrPto and AvrPtoB affect Pto signaling abilities differently, nor is it known which residues of Pto are involved in the

interaction with Prf. X-ray crystallography of the complex between Pto and Prf together with each effector will ultimately be needed to understand the structural aspects of this multiple-protein interaction.

It has been reported previously that the *Pto* gene from *Schm* accessions LA1306, LA2695, and LA3653 was able to cause immunity-associated cell death when agroinfiltrated into transgenic *N. benthamiana* highly expressing AvrPto (Rose et al., 2005). The same report also presented evidence that *Schm* accessions LA1306 and LA2695 are resistant to *Pst* strain T1 delivering AvrPto. However, we found that LA1306, LA2695, and LA3653 were susceptible to DC3000Δ*avrPtoB*, which delivers AvrPto. Further, in our hands, LA2695 had moderate resistance to T1, but this resistance was independent of the presence of AvrPto. It is possible that LA1306 is also resistant to T1 independently of AvrPto, but this remains to be tested. The use of different vectors, which express the proteins at different levels in *N. benthamiana,* as well as differences in *Pst* strains and possibly disease assay conditions in tomato are the most likely explanation for these discrepancies.

The fact that Pto-2677 differs by just 14 amino acids from Pto made it potentially straightforward to investigate why Pto-2677 is unable to confer resistance to *Pst* expressing AvrPto, although our experiments led to a surprising result. We initially focused on the two residues H49/V51, which in Pto are known to be required for the interaction with AvrPto, and which are not conserved in Pto-2677. Previous work demonstrated that the variant Pto(H49E/V51D) does not interact with AvrPto *in vitro* and in yeast, however, transient expression of either Pto(H49E/V51D) or Pto(H49E/V51G) without a corresponding effector protein caused cell death in leaves of *N. benthamiana* and prevented characterization of these variants in plant cells (Dong et al., 2009; Xing et al., 2007). In contrast, Pto-2677, which has E49/G51, did not cause cell death by itself and it should be possible in the future to determine which of the remaining 12 non-

conserved residues in Pto-2677 might inhibit the constitutive cell death that is observed with Pto(H49E/V51D) and Pto(H49E/V51G). Such information could shed light on how Pto activity is negatively regulated. Conversion of E49/G51 in Pto-2677 to those residues present in Pto (i.e., Pto-2677[E49H/G51V]) was insufficient to elicit immunity-associated cell death in leaves co-expressing AvrPto. This observation, along with the interaction of Pto-2677 with AvrPto in yeast, potentially suggested that H49/V51 are not as important as thought for interaction with Pto. The findings also indicated that one or more other residues in Pto, which differ in Pto-2677, are needed for AvrPto triggered immunity.

Subsequent experiments involving individual replacement of 9 additional residues in Pto-2677 with the cognate residue in Pto identified aspartic acid-(D)193 as being essential to allowing Pto-2677 to respond to AvrPto. The Pto-2677(H193D) substitution alone conferred the ability to respond to AvrPto with immunity-associated cell death in *N. benthamiana* although this response was less robust and delayed as compared with the AvrPto-Pto-2677(E49H/G51V/H193D)-mediated cell death which interestingly restored robust AvrPto-mediated cell death. This observation indicates that the H49/V51 residues do, in fact, contribute to the AvrPto-specific response. Supporting the importance of D193, a substitution changing only this residue in Pto (D193H) was sufficient to severely compromise its ability to recognize AvrPto. Importantly, Pto(D193H) and Pto-2677(H193D) were still able to interact with AvrPtoB and cause AvrPtoB-specific immunity-associated cell death, thus demonstrating that histidine-193 residue does not interfere with Pto function in general, but instead has a specific effect on its activity in response to AvrPto.

The discovery that D193 plays such an important role in the response of Pto to AvrPto was surprising because, based on the crystal structure of the AvrPto-Pto complex, this residue is not located within or near the AvrPto-specific interface or the shared interface involving the P+1 loop, the two domains of Pto that have been shown to be involved in

interaction with AvrPto and regulation of the immune response that follows (Dong et al., 2009; Wu et al., 2004; Xing et al., 2007). However, it is noteworthy that D193 lies inside the activation loop of Pto, a domain known to be important for Pto downstream signaling. According to one model, homodimerization of Prf brings at least two Pto molecules in close proximity, but keeps them in an inactive conformation complex (Ntoukakis et al., 2013). AvrPto or AvrPtoB, upon binding to a monophosphorylated Pto molecule (sensor), triggers a de-repression of the P+1 loop. This change activates through Prf the second Pto molecule (helper) in the complex, which then transphosphorylates the activation loop of the first Pto at a second position, thereby activating the Pto/Prf complex for downstream immune signaling (Ntoukakis et al., 2013). Aspartic acid is not phosphorylated, but under neutral pH contributes a negative charge to the domain. It is conceivable that it is not the individual phosphorylation events at specific residues, but rather the sum of negative charges in this domain (which can be increased by phosphorylation) that is the determining factor in the activation of Pto by AvrPto, a hypothesis that needs to be tested.

An earlier analysis of 16 Pto orthologs from accessions belonging to five *Solanum* species found that all have an aspartic acid (D) at position 193 (Rose et al., 2005) and therefore the non-conservative change from the charged aspartic acid to the polar histidine residue observed in Pto-2677 does not appear to be a common occurrence. In the future, as more *Pto* sequences become available from genome sequencing projects, it will be interesting to determine the most plausible ancestral sequence for the *Pto* gene. Much additional work is needed to understand the structural and functional contribution of D193 to the AvrPto-specific response, but we hypothesize that this residue might play a role in the protein-protein interaction interface in Pto that is required for downstream signaling via Prf in response to AvrPto, but not AvrPtoB.

There is a previous report that the recognition of an effector protein can be altered by specific substitutions in a host protein on a site distant from the effector target interaction surface (Qi et al., 2014). The *Pseudomonas syringae* pv. *phaseolicola* effector AvrPphB targets and cleaves host receptor-like cytoplasmic kinases including AVRPPHB Suceptible1 (PBS1). Upon cleavage of PBS1 at the apex of its activation loop, another protein RESISTANCE TO PSEUDOMONAS SYRINGAES 5 (RPS5) is activated initiating a strong ETI response. Amino acid substitutions in a specific loop of PBS1 located on the opposite side of the cleavage site abolished PBS1-mediated activation of by RPS5. Further, analogous changes in the most similar PBS1-like protein PBL27, which normally cannot trigger an ETI response by RPS5 after cleavage by AvrPphB, to the amino acid sequence present in the PBS1 loop was sufficient to confer RPS5 recognition (Qi et al., 2014).

In summary, we relied on natural variation in wild relatives of tomato to identify and characterize an ortholog of Pto that recognizes AvrPtoB but not AvrPto. This led to the discovery that D193 in Pto plays a previously unknown but critical role in the recognition of AvrPto but not AvrPtoB. Our results demonstrate the utility of using natural variation in tomato wild relatives to study pathogen recognition mechanisms and they lay the foundation for investigating the molecular basis for how D193 plays an effector-specific role in ETI.

2.5 Materials and Methods

Plant material

Solanum and *Nicotiana benthamiana* plants were grown in a greenhouse with 16 h light / 8 h dark at a temperature of 24°C during daylight / 22°C at night and 65% humidity. Seeds of cultivated tomatoes and *N. benthamiana* were germinated in trays and seedlings transplanted two to three weeks later, respectively, into larger pots containing Cornell

Plus Mix soil (0.16 m^3 peat moss, 0.34m^3 vermiculite, 2.27 kg lime, 2.27 kg Osmocote Plus15-9-12 and 0.54 kg Uni-Mix 11-5-11; Everris, Israeli Chemicals Ltd). Wild *Solanum* species were treated with half-strength bleach and germinated on 3M Whatman filter paper. Soon after germination, plants were transferred to small trays and three weeks later into larger pots with Sunshine MVP soil mix (Sun Gro Horticulture). Seeds of all wild tomato accessions were obtained from the Tomato Genetic Resource Center at the University of California, Davis (http://tgrc.ucdavis.edu/). Tomato variety Rio Grande-PtoR (RG-PtoR; *Pto/Pto*, *Prf/Prf*), which has the *Pto* haplotype from *S. pimpinellifolium* was used as a resistant control and Rio Grande-prf3 (RG-prf3; *Pto/Pto, prf3/prf3*), a mutant of RG-PtoR with a non-functional *Prf* gene due to an 1.1 kb deletion (Salmeron et al., 1994; Salmeron et al., 1996), was used as a susceptible line for all tomato experiments.

***Pst* DC3000 strains and the wild tomato species screen**

Accessions of wild tomato were screened for their ability to recognize the *Pst* effectors AvrPto and AvrPtoB using the following *Pst* DC3000 strains: 1) wild type DC3000 which has AvrPto and AvrPtoB; 2) DC3000Δ*avrPto* (lacking AvrPto, but having AvrPtoB) (Ronald et al., 1992); 3) DC3000Δ*avrPtoB* (lacking AvrPtoB, but having AvrPto) (Lin and Martin, 2005); and 4) DC3000Δ*avrPto*Δ*avrPtoB* (lacking both effectors) (Lin and Martin, 2005). Strains were grown on King's B agar plates containing the appropriate antibiotics for two days at 30°C. The bacterial lawn was scraped from the plates and suspended in 10 mM $MgCl_2$ buffer. Tubes were centrifuged for 10 min and pellets were resuspended in fresh buffer. The OD_{600} was initially adjusted to 0.5 (~ 5 x 10^8 colony-forming units (CFU) per mL). Subsequent bacterial suspensions were prepared at a final concentration of 5 x 10^4 CFU/mL and the different bacterial suspensions were syringe-infiltrated into separate leaflets of the same leaf for each plant.

The development of disease symptoms was monitored for up to one week after infiltrations.

Virulence assay in tomato

Pst DC3000*ΔavrPto* and DC3000*ΔavrPtoB* mutants were prepared as described above for screening wild tomato species. Whole plants were vacuum-infiltrated as described previously (Mathieu et al., 2014). Plants used in the VIGS experiments were vacuum-infiltrated using a bacterial suspension of 5 x 10^5 CFU/mL. Bacterial population assays were carried out as described previously (Cheng et al., 2011). Infiltrated plants were kept in a climate controlled growth chamber (24°C day and 20°C night temperature) and scored for disease symptoms 3-4 days later.

Virus-induced gene silencing (VIGS) of *Prf*

The *Prf* gene in tomato was silenced using a Tobacco Rattle Virus (TRV)-based VIGS system (Burch-Smith et al., 2004; Nguyen et al., 2010). *PDS* (*phytoene desaturase*) was silenced as a positive control for the efficiency of the VIGS and *EC1,* which carries a fragment of *E. coli* DNA*,* was used as a negative control for the experiments (Rosli et al., 2013). The construction of the plasmids was carried out as described previously (Ekengren et al., 2003). *Agrobacterium tumefaciens* cultures containing the TRV constructs were prepared as described earlier (Velasquez et al., 2009). Cotyledons of 9-day-old Rio Grande-PtoR and 14-day-old LA2677 plants were syringe infiltrated with a 1:1 mixture of *Agrobacterium* strains carrying pTRV1 or pTRV2 containing the gene fragment of interest. Plants were kept in a growth chamber with 20°C day and 18°C night temperatures at 50% relative humidity with a 16-h light / 8-h dark photoperiod until the *PDS* control plant leaves showed signs of photobleaching. Plants were transferred to 24°C day and 20°C night temperatures until they reached the right size, around 30 cm, to be vacuum-infiltrated.

Preparation of genomic DNA and sequencing of *Pto* genes

DNA was isolated from two of the smallest leaves using the CTAB method (Doyle and Doyle, 1987). The DNA pellets were air dried and resuspended in 25-100 µl TE. Samples were left at 4°C overnight to allow the pellet to dissolve completely. 1 µl of 10 mg/mL RNaseA was added to the samples, followed by a 37°C incubation period for 1 hr. Samples were stored at -20°C until further use. Two sets of primers were used to clone the different *Pto* genes from LA2677: Primers F: 5'-ATGGGAAGCAAGTATTCTAA-3' and R: 5'-AAATAACAGACTCTTGGAGA-3' match the 3' and 5' end of the *Pto* gene in RG-PtoR and also *PtoC* and *PtoF* due to their similarity at the beginning and end of their sequence. For amplification of *Fen* and *PtoD*, also very similar in their 3' and 5' prime ends, primers F: 5'-ATGGGAAGCAAGTATTCCAA-3' and R: 5'-ATTCAGGATCATCTTGAAT-3' were used. The PCR protocol used was as described previously (Rose et al., 2005). Products were resolved on agarose gels and purified using Wizard® SV Gel and PCR Clean-Up System and cloned into the pGEM®-T easy vector system following the manufacturer's instructions (Promega). The LA2677 Pto sequences have been deposited in Genbank (Pto-2677: accession no. KT225557; Fen-2677: KT225558; PtoC-2677: KT225559; PtoD-2677: KT225560).

cDNA synthesis and RT-PCR

RNA was extracted using the Plant RNA Purification Reagent (Invitrogen) according to the manufacturer's instructions. Isolated RNA was treated with RQ1 DNase (Promega) for 1 h at 37°C. To ensure high quality of the RNA, samples were additionally purified using an RNeasy ® Mini column (Qiagen) following the manufacturer's protocol. cDNA was synthesized using the SuperScript® III First-Strand Synthesis System (Invitrogen) according to manufacturer's instructions. Transcripts of *Pto* family members were RT-PCR amplified using Phusion HF (Fisher). Primers used for each gene analyzed were: Fen2677: 5'-GAAACAGAAATTGAGATTCTCTCATTTTGC-3' and

3’-CATAGCCTGCGTCTCATCATC-5’,

PtoC: 5’-GAAAAGGCATAATTGTGACTCCCA-3’ and

3’-TTC CTTTCACGTCTGTGCTTAC-5’,

PtoD: 5’-CGAACAGAAATTGAGATACTCTCACAC-3’ and

3’-CAGCTAAACTGATCATATGTGAACGAC-5’,

Pto: 5’-CATAAATGATGCTTTAAGCTCGAGTTATCT-3’ and

3’-TTGATGGAGCTCAGTCCCTT-5’. The PCR protocol used was as described previously (Rose et al., 2005).

Phylogenetic analysis

A phylogenetic analysis of the *Pto* genes from tomato RG-PtoR and *S. chm* LA2677 was performed by generating an unrooted maximum likelihood tree with Mega v. 6.06-mac using a Tamura 3-parameter substitution model and gamma-rate distribution (Hall, 2013; Tamura, 1992; Tamura et al., 2013). Nearest-Neighbor-Interchange was used as the heuristic tree search method with 100 bootstrap samples.

Yeast-two hybrid assay

The LexA yeast-two hybrid (Y2H) system was employed to study the interactions of the different LA2677 Pto proteins with AvrPto and AvrPtoB. pEG202 was used as the bait vector and pEG4-5 as the prey vector (Golemis et al., 2008). Y2H was performed using the protocol described previously (Mathieu et al., 2014). Development of blue patches was monitored for 12 to 42 h.

***Agrobacterium*-mediated transient protein expression**

For infiltrations of *N. benthamiana* leaves, *Agrobacterium tumefaciens* strain GV3101 with helper plasmid pMP90 was used. For tomato leaf infiltrations, *Agrobacterium* strain 1D1249 was used, because GV3101 causes necrosis when infiltrated into tomato leaves (Wroblewski et al., 2005). *Agrobacterium* competent cells were transformed by

electroporation. Single colonies were tested for the presence of the construct by colony PCR. For the assay, *Agrobacterium* strains were grown on Luria Broth agar plates containing antibiotics in a 30°C incubator for 48 h. The bacteria were scraped from the plate and dissolved in infiltration buffer (10 mM $MgCl_2$, 10 mM MES pH5.6 and 200 μM acetosyringone). Cells were pelleted by centrifugation for 10 min and resuspended in fresh buffer. For GV3101, the bacterial suspension was diluted to a final OD_{600} of 0.3 and for 1D4912 to OD_{600} of 0.15. Tubes were set to tumble on a nutator for 4-6 h in the dark before infiltration. After syringe infiltration of plants using a needle-less syringe, they were moved to a growth chamber (24°C day and 20°C night temperature). Plants were kept on a dark shelf and surrounded by shade cloth to minimize light exposure. For analysis of protein expression, samples were taken 48 h later. In tomato, cell death was fully developed by day 3 and in *N. benthamiana* cell death started to appear 72 h after infiltration and was complete by day 5.

Cloning

All enzymes and reagents were acquired from New England Biolabs. The *Pto* genes from LA2677 were cloned into the entry vector pJLSmart (Mathieu et al., 2014; Mathieu et al., 2007) followed by LR recombination into pGWB417 (Nakagawa et al., 2007). The corresponding yeast-two hybrid constructs were generated by restriction cloning. Details of the cloning protocols used were as described previously (Mathieu et al., 2014). Details of vectors and constructs used in this work are provided in the supplemental information (Table S2).

2.6 Acknowledgments

We thank Dr. Johannes Mathieu for technical advice and mentoring of C.M.K., Dr. Patricia Manosalva and Simon Schwizer for technical advice, Dr. Alan Collmer, Dr. Susan McCouch, Dr. Lawrence Smart and Dr. Patrick Boyle for helpful comments and Dr. Johannes Mathieu and Dr. Sarah Hind for critical review of the manuscript. We thank the C. M. Rick Tomato Genetics Resource Center at UC-Davis for providing tomato seeds. This research was supported, in part, by National Science Foundation grant IOS-1025642 (GBM).

2.7 References

Abramovitch, R.B., Kim, Y.-J., Chen, S., Dickman, M.B., and Martin, G.B. (2003). *Pseudomonas* type III effector AvrPtoB induces plant disease susceptibility by inhibition of host programmed cell death. EMBO J 22:60-69.

Bernal, A.J., Pan, Q., Pollack, J., Rose, L., Kozik, A., Willits, N., Luo, Y., Guittet, M., Kochetkova, E., and Michelmore, R.W. (2005). Functional analysis of the plant disease resistance gene *Pto* using DNA shuffling. J Biol Chem 280:23073-23083.

Bombarely, A., Rosli, H.G., Vrebalov, J., Moffett, P., Mueller, L.A., and Martin, G.B. (2012). A draft genome sequence of *Nicotiana benthamiana* to enhance molecular plant-microbe biology research. Mol Plant-Microbe Interact 25:1523-1530.

Burch-Smith, T.M., Anderson, J.C., Martin, G.B., and Dinesh-Kumar, S.P. (2004). Applications and advantages of virus-induced gene silencing for gene function studies in plants. Plant J. 39:734-746.

Chang, J.H., Tai, Y.S., Bernal, A.J., Lavelle, D.T., Staskawicz, B.J., and Michelmore, R.W. (2002). Functional analyses of the *Pto* resistance gene family in tomato and the identification of a minor resistance determinant in a susceptible haplotype. Mol Plant-Microbe Interact 15:281-291.

Chang, J.H., Tobias, C.M., Staskawicz, B.J., and Michelmore, R.W. (2001). Functional studies of the bacterial avirulence protein AvrPto by mutational analysis. Mol Plant-Microbe Interact 14:451-459.

Cheng, W., Munkvold, K.R., Gao, H., Mathieu, J., Schwizer, S., Wang, S., Yan, Y.B., Wang, J., Martin, G.B., and Chai, J. (2011). Structural analysis of *Pseudomonas syringae* AvrPtoB bound to host BAK1 reveals two similar kinase-interacting domains in a type III effector. Cell Host Microbe 10:616-626.

Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nurnberger, T., Jones, J.D., Felix, G., and Boller, T. (2007). A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. Nature 448:497-500.

Devarenne, T.P., Ekengren, S.K., Pedley, K.F., and Martin, G.B. (2006). Adi3 is a Pdk1-interacting AGC kinase that negatively regulates plant cell death. EMBO J 25:255-265.

Dong, J., Xiao, F., Fan, F., Gu, L., Cang, H., Martin, G.B., and Chai, J. (2009). Crystal structure of the complex between *Pseudomonas* effector AvrPtoB and the tomato Pto kinase reveals both a shared and a unique interface compared with AvrPto-Pto. Plant Cell 21:1846-1859.

Doyle, J.J., and Doyle, J.L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bulletin 19:11-15.

Ekengren, S.K., Liu, Y., Schiff, M., Dinesh-Kumar, S.P., and Martin, G.B. (2003). Two MAPK cascades, NPR1, and TGA transcription factors play a role in Pto-mediated disease resistance in tomato. Plant J. 36:905-917.

Gimenez-Ibanez, S., Ntoukakis, V., and Rathjen, J.P. (2009). The LysM receptor kinase CERK1 mediates bacterial perception in *Arabidopsis*. Plant Signal Behav 4:539-541.

Gohre, V., Spallek, T., Haweker, H., Mersmann, S., Mentzel, T., Boller, T., de Torres, M., Mansfield, J.W., and Robatzek, S. (2008). Plant pattern-recognition receptor FLS2 is directed for degradation by the bacterial ubiquitin ligase AvrPtoB. Curr Biol 18:1824-1832.

Golemis, E.A., Serebriiskii, I., Jr., F.R.L., Kolonin, M.G., Gyuris, J., and Brent, R. (2008). Interaction trap/two-hybrid system to identify interacting proteins. In: Curr. Protoc. in Mol. Biol.--Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K., eds. New York: John Wiley and Sons Inc. 20.21:20.21.21–20.21.35.

Gomez-Gomez, L., and Boller, T. (2000). FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. Molecular cell 5:1003-1011.

Grube, R.C., Radwanski, E.R., and Jahn, M. (2000). Comparative genetics of disease resistance within the *Solanaceae*. Genetics 155:873-887.

Hall, B.G. (2013). Building phylogenetic trees from molecular data with MEGA. Mol Biol Evol 30: 1229-1235.

He, P., Shan, L., Lin, N.C., Martin, G.B., Kemmerling, B., Nurnberger, T., and Sheen, J. (2006). Specific bacterial suppressors of MAMP signaling upstream of MAPKKK in *Arabidopsis* innate immunity. Cell 125:563-575.

Lin, N.C., and Martin, G.B. (2005). An *avrPto/avrPtoB* mutant of *Pseudomonas syringae* pv. *tomato* DC3000 does not elicit Pto-mediated resistance and is less virulent on tomato. Mol Plant-Microbe Interact 18:43-51.

Lin, N.C., and Martin, G.B. (2007). Pto- and Prf-mediated recognition of AvrPto and AvrPtoB restricts the ability of diverse *Pseudomonas syringae* pathovars to infect tomato. Mol. Plant Microbe Interact. 20:806-815.

Martin, G.B. (2012). Suppression and activation of the plant immune system by *Pseudomonas syringae* effectors AvrPto and AvrPtoB. In: Effectors in Plant-Microbe Interactions--Martin, F., and Kamoun, S., eds. Oxford, UK.: Wiley-Blackwell. 123-154.

Martin, G.B., Brommonschenkel, S.H., Chunwongse, J., Frary, A., Ganal, M.W., Spivey, R., Wu, T., Earle, E.D., and Tanksley, S.D. (1993). Map-based cloning of a protein kinase gene conferring disease resistance in tomato. Science 262:1432-1436.

Mathieu, J., Schwizer, S., and Martin, G.B. (2014). Pto kinase binds two domains of AvrPtoB and its proximity to the effector E3 ligase determines if it evades degradation and activates plant immunity. PLoS Pathog 10:e1004227.

Mathieu, J., Warthmann, N., Kuttner, F., and Schmid, M. (2007). Export of FT protein from phloem companion cells is sufficient for floral induction in *Arabidopsis*. Curr Biol 17:1055–1060.

Mucyn, T.S., Clemente, A., Andriotis, V.M., Balmuth, A.L., Oldroyd, G.E., Staskawicz, B.J., and Rathjen, J.P. (2006). The tomato NBARC-LRR protein Prf interacts with Pto kinase in vivo to regulate specific plant immunity. Plant Cell 18:2792-2806.

Mucyn, T.S., Wu, A.J., Balmuth, A.L., Arasteh, J.M., and Rathjen, J.P. (2009). Regulation of tomato Prf by Pto-like protein kinases. Mol Plant-Microbe Interact 22:391-401.

Mueller, K., Chinchilla, D., Albert, M., Jehle, A.K., Kalbacher, H., Boller, T., and Felix, G. (2012). Contamination risks in work with synthetic peptides: flg22 as an example of a pirate in commercial peptide preparations. Plant Cell 24:3193-3197.

Nakagawa, T., Suzuki, T., Murata, S., Nakamura, S., Hino, T., Maeo, K., Tabata, R., Kawai, T., Tanaka, K., Niwa, Y., et al. (2007). Improved Gateway binary vectors: high-performance vectors for creation of fusion constructs in transgenic analysis of plants. Biosci Biotechnol Biochem 71:2095-2100.

Nguyen, H.P., Chakravarthy, S., Velásquez, A.C., McLane, H.S., Zeng, L., Park, D.-W., Collmer, A., and Martin, G.B. (2010). Methods to study PAMP-triggered immunity using tomato and *Nicotiana benthamiana*. Mol Plant-Microbe Interact 23:991-999.

Ntoukakis, V., Balmuth, A.L., Mucyn, T.S., Gutierrez, J.R., Alexandra, M.E.J., and Rathjen, J.P. (2013). The tomato Prf complex Is a molecular trap for bacterial effectors based on Pto transphosphorylation. PLoS Pathog 9.

Pedley, K.F., and Martin, G.B. (2003). Molecular basis of Pto-mediated resistance to bacterial speck disease in tomato. Annu Rev Phytopathol 41:215-243.

Peralta, I.E., Spooner, D.M., and Knapp, S. (2008). Systematic botany monographs *Solanum* (*Solanaceae*). United States of America: Amer Society of Plant Taxonomists.

Qi, D., Dubiella, U., Kim, S.H., Sloss, D.I., Dowen, R.H., Dixon, J.E., and Innes, R.W. (2014). Recognition of the protein kinase AVRPPHB SUSCEPTIBLE1 by the disease resistance protein RESISTANCE TO PSEUDOMONAS SYRINGAE5 is dependent on S-acylation and an exposed loop in AVRPPHB SUSCEPTIBLE1. Plant Physiol. 164:340–351.

Riely, B.K., and Martin, G.B. (2001). Ancient origin of pathogen recognition specificity conferred by the tomato disease resistance gene Pto. Proc Natl Acad Sci USA 98:2059-2064.

Robatzek, S., Bittel, P., Chinchilla, D., Kochner, P., Felix, G., Shiu, S.H., and Boller, T. (2007). Molecular identification and characterization of the tomato flagellin receptor LeFLS2, an orthologue of Arabidopsis FLS2 exhibiting characteristically different perception specificities. Plant Mol Biol 64:539-547.

Ronald, P.C., Salmerson, J.M., Carland, F., M., and Staskawicz, B.J. (1992). The cloned avirulence gene *avrPto* induces disease resistance in tomato cultivars containing the *Pto* resistance gene. J Bacteriol 174:1604-1611.

Rose, L.E., Langley, C.H., Bernal, A.J., and Michelmore, R.W. (2005). Natural variation in the Pto pathogen resistance gene within species of wild tomato (Lycopersicon). I. Functional analysis of Pto alleles. Genetics 171:345-357.

Rosebrock, T.R., Zeng, L., Brady, J.J., Abramovitch, R.B., Xiao, F., and Martin, G.B. (2007). A bacterial E3 ubiquitin ligase targets a host protein kinase to disrupt plant immunity. Nature 448:370-374.

Rosli, H.G., Zheng, Y., Pombo, M.A., Zhong, S., Bombarely, A., Fei, Z., Collmer, A., and Martin, G.B. (2013). Transcriptomics-based screen for genes induced by flagellin and repressed by pathogen effectors identifies a cell wall-associated kinase involved in plant immunity. Genome biology 14:R139.

Salmeron, J.M., Barker, S.J., Carland, F.M., Mehta, A.Y., and Staskawicz, B.J. (1994). Tomato mutants altered in bacterial disease resistance provide evidence for a new controlling pathogen recognition. Plant Cell 6:511-520.

Salmeron, J.M., Oldroyd, G.E., Rommens, C.M., Scofield, S.R., Kim, H.S., Lavelle, D.T., Dahlbeck, D., and Staskawicz, B.J. (1996). Tomato *Prf is* a member of the leucine-rich repeat class of plant disease resistance genes and lies embedded within the *Pto* kinase gene cluster. Cell 86:123-133.

Shan, L., He, P., Li, J., Heese, A., Peck, S.C., Nurnberger, T., Martin, G.B., and Sheen, J. (2008). Bacterial effectors target the common signaling partner BAK1 to disrupt multiple MAMP receptor-signaling complexes and impede plant immunity. Cell Host Microbe 4:17-27.

Shan, L., He, P., Zhou, J.M., and Tang, X. (2000a). A cluster of mutations disrupt the avirulence but not the virulence function of AvrPto. Mol Plant-Microbe Interact 13:592-598.

Shan, L., Thara, V.K., Martin, G.B., Zhou, J.M., and Tang, X. (2000b). The *Pseudomonas* AvrPto protein is differentially recognized by tomato and tobacco and is localized to the plant plasma membrane. Plant Cell 12:2323-2338.

Tamura, K. (1992). Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G + C-content biases. Mol Biol Evol 9:678-687.

Tamura, K., Stecher, G., D., P., Filipski, A., and Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol Bio Evol:2725–2729.

Velasquez, A.C., Chakravarthy, S., and Martin, G.B. (2009). Virus-induced gene silencing (VIGS) in *Nicotiana benthamiana* and tomato. J Vis Exp 28:e1292.

Vleeshouwers, V.G., Martens, A., van Dooijeweert, W., Colon, L.T., Govers, F., and Kamoun, S. (2001). Ancient diversification of the Pto kinase family preceded speciation in *Solanum*. Mol Plant-Microbe Interact 14:996-1005.

Wroblewski, T., Tomczak, A., and Michelmore, R. (2005). Optimization of *Agrobacterium*-mediated trasient assays of gene expression in lettuce, tomato and Arabidopsis. Plant Biotechnol J 3:259-273.

Wu, A.J., Andriotis, V.M., Durrant, M.C., and Rathjen, J.P. (2004). A patch of surface-exposed residues mediates negative regulation of immune signaling by tomato Pto kinase. Plant Cell 16:2809-2821.

Wulf, J., Pascuzzi, P.E., Fahmy, A., Martin, G.B., and Nicholson, L.K. (2004). The solution structure of type III effector protein AvrPto reveals conformational and dynamic features important for plant pathogenesis. Structure 12:1257-1268.

Xiang, T., Zong, N., Zou, Y., Wu, Y., Zhang, J., Xing, W., Li, Y., Tang, X., Zhu, L., Chai, J., et al. (2008). *Pseudomonas syringae* effector AvrPto blocks innate immunity by targeting receptor kinases. Curr Biol 18:74-80.

Xiao, F., He, P., Abramovitch, R.B., Dawson, J.E., Nicholson, L.K., Sheen, J., and Martin, G.B. (2007). The N-terminal region of *Pseudomonas* type III effector

AvrPtoB elicits Pto-dependent immunity and has two distinct virulence determinants. Plant J 52:595-614.

Xing, W., Zou, Y., Liu, Q., Liu, J., Luo, X., Huang, Q., Chen, S., Zhu, L., Bi, R., Hao, Q., et al. (2007). The structural basis for activation of plant immunity by bacterial effector protein AvrPto. Nature 449:243-247.

Zeng, L., Velasquez, A.C., Munkvold, K.R., Zhang, J., and Martin, G.B. (2012). A tomato LysM receptor-like kinase promotes immunity and its kinase activity is inhibited by AvrPtoB. Plant J 69:92-103.

Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E.J., Jones, J.D., Felix, G., and Boller, T. (2004). Bacterial disease resistance in *Arabidopsis t*hrough flagellin perception. Nature 428:764-767.

CHAPTER 3

MOLECULAR CHARACTERIZATION OF NEW YORK PSEUDOMONAS SYRINGAE PV. TOMATO ISOLATES REVEALS FEATURES INTERMEDIATE BETWEEN RACE 0 AND RACE 1 STRAINS[2]

3.1 Abstract

Bacterial speck, a disease caused by *Pseudomonas syringae* pv. *tomato* (*Pst*), is a severe problem for fresh market tomato growers in New York (NY) and elsewhere. Race 1 strains are the most common *Pst* strains worldwide; they are highly virulent even on tomatoes carrying the *Pto* resistance gene and have displaced race 0 strains, which are avirulent on tomatoes with a functional *Pto* pathway. Pto is able to recognize AvrPto and AvrPtoB, two virulence factors (effectors) expressed by race 0, but absent in race 1 strains.

We collected *Pst* isolates from infected tomato plants across NY in 2015 and characterized them for their virulence on tomatoes, as well as for the presence of specific effectors. These new isolates have *avrPtoB* but, as reported for several race 1 strains, protein expression for this effector was not detectable. In contrast to race 1 strains, the NY isolates have a functional *avrPto* gene which is transcribed and translated. Virulence assays revealed that inside leaves the strains reached population sizes intermediate between typical race 0 and race 1 strains, suggesting that AvrPto is being recognized, but its recognition is masked during later stages of infection. Collectively, our data suggest that Pto confers at least partial resistance against current NY isolates, indicating that

[2] This chapter will be submitted in modified form to Molecular Plant Pathology and was written by Christine M. Kraus, Carolina Mazo, Christine D. Smart and Gregory B. Martin.

introgression of this resistance gene into fresh market tomato varieties will be of benefit to fresh market tomato growers.

3.2 Introduction

Bacterial speck caused by *Pseudomonas syringae* pv. *tomato* (*Pst*) is a serious disease of commercial field-grown tomatoes throughout the tomato-growing regions of North America. The symptoms appear as small necrotic lesions on leaflets, stems and fruits and can greatly impact both the yield and marketability of the crop. Disease can spread quickly, especially when aided by cool and wet summers that can occur in New York (NY) (Jones, 1991; Pedley and Martin, 2003). Bacterial speck is the most common bacterial disease of tomatoes in NY next to bacterial canker, caused by *Clavibacter michiganensis subsp. michiganensis* (Smart et al., 2015). In NY, large-scale processing tomato production is no longer practiced, but a significant amount of tomatoes are still grown locally for fresh consumption. Several resistant varieties of processing tomatoes are available, but few resistant fresh-market cultivars exist. Examples of *Pst* resistant fresh-market tomato cultivars, which express *Pto*, include ONT 7710, Rotam-4, in addition to fresh-market plums such as Peto 882 (Bosch et al., 1990; Buonaurio et al., 1996; Pitblado and MacNeill, 1983).

Pto-mediated resistance in tomato involves the cytoplasmic kinase Pto (for resistance against *P. syringae* pv. *tomato*) and the nucleotide binding leucine rich repeat (NB-LRR) protein Prf (Lin and Martin, 2007; Martin, 2012; Salmeron et al., 1996). Pto can recognize two unrelated *Pst* virulence effectors, AvrPto and AvrPtoB, leading in each case to the activation of effector triggered immunity (ETI) (Abramovitch et al., 2003; Kim et al., 2002; Martin, 2012; Mathieu et al., 2014; Rosebrock et al., 2007; Shan et al., 2000b). This recognition event culminates in a form of programmed cell death at the site of infection and suppression of pathogen growth. Pto-mediated resistance provided

effective disease control against bacterial speck for several decades, but more recent studies have shown that *Pst* populations worldwide have shifted towards virulent race 1 strains, which are able to evade Pto-mediated immunity (Cai et al., 2011).

Pst race 1 was first detected in 1986 in Canada and in 1993 in the USA and has since become the predominant race found in field grown tomatoes (Arredondo and Davis, 2000; Lawton and MacNeill, 1986). Race 1 strains can evade Pto recognition in three ways: absence of AvrPto and AvrPtoB, expression of protein variants that cannot be recognized by Pto, or loss of effector protein expression (Kunkeaw et al., 2010; Lin et al., 2006). In all cases, race 1 strains cause disease symptoms and grow to high population levels in tomato cultivars carrying Pto resistance. Pathogens exposed to resistant cultivars are under strong selective pressure to modify the recognized effectors and evade ETI. Boom and bust cycles usually occur when a resistant cultivar with single, major *R* gene is introduced into an agroecosystem to control a plant disease, such as in the primary tomato production states like California. Conceivably, the pathogen population exposed to this single *R* gene is under tremendous selective pressure to evolve. This constant pressure imposed by large swathes of a resistant cultivar quickly fixes a new allele in the population, shifting the population towards the new more virulent strain (McDonald and Linde, 2002). However, in predominantly fresh market producing states like NY, this pressure should be very low due to the absence of the *R* gene in significant portions of the tomato population.

The objectives of this study were to characterize *Pst* strains isolated throughout NY and assess whether Pto-mediated resistance would still be effective, even in the context of the recent humid and cool summers favorable to the disease. Our data will be useful to tomato breeders deciding whether an introgression of *Pto* into fresh market tomato varieties would benefit tomato growers in NY and beyond. Here we report that *Pto*, even under lab conditions favoring the pathogen, still confers at least partial resistance against

Pst isolates collected during a severe outbreak of bacterial speck in NY in 2015, most likely due to the presence of AvrPto in these isolates.

3.3 Results

Analysis of diagnostic effector genes indicates the 2015 NY isolates are race 1 *Pst* strains

Leaves infected with *Pst* were collected from infested tomato fields in six different NY counties (**Figure 3.1A**). *Pst* isolates were single-colony purified and checked for fluorescence under ultraviolet light as a first classification step (data not shown). Isolates that were fluorescent were further examined by PCR using primers designed to specifically amplify sequences from effectors that can discriminate between group I (associated with tomato) and other *Pst* groups (avirulent on tomato), as well as other tomato bacterial pathogens such as *Xanthomonas perforans* and *Clavibacter michiganensis* subsp *michiganensis*. We also employed primers to amplify effector sequences that allow the distinction between race 0 and race 1 strains. All primers used were previously described by Jones *et al*. *(Jones* et al., 2015*).* Results indicate that all 2015 isolates belong to the group I *Pst,* as demonstrated by the presence of *hopR*. Furthermore, we were able to detect *avrA* and *hopW1*, which are present in other race 1 strains such as T1 and NY-T1 (**Figure 3.1B and Table 3.1**). *HopN1* was amplified exclusively in the race 0 control DC3000 (**Figure 3.1B and Table 3.1**). These results indicate that, based on PCR diagnostic tests, all 2015 isolates group with the more virulent race 1 strains. From the six strains, NY-15114 isolated in Essex County and NY-15125 isolated in Tompkins County were chosen as representative isolates for further molecular characterization. NY-15125 was selected because it was isolated in a field that is only 20 min away from Cornell campus, representing the local *Pst* population. Working with this strain allowed us to also conduct field experiments in 2016 without

introducing a new *Pst* population into the area. NY-15114 was chosen because it was collected far away from NY-15125 and might show the biggest differences in pathogenicity as well as at the genomic and proteomic levels.

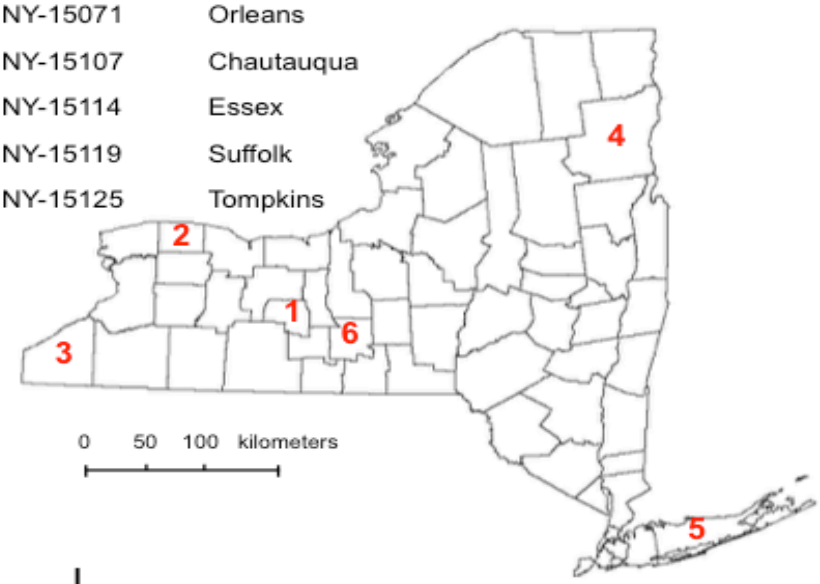
***Pst* strains isolated in NY are non-pathogenic on *Arabidopsis*, similar to other known race 1 *Pst* strains**

To date, only *Pst* DC3000 and *Pseudomonas syringae* pv. *maculicula* strains are known to be capable of infecting *Arabidopsis thaliana* accession Columbia (Col-0) (Almeida et al., 2009; Yan et al., 2008). We tested NY-15114 and NY-15125 strains for their ability to infect, proliferate in, and cause symptoms on *Arabidopsis* Col-0 plants. *Arabidopsis* leaves were syringe infiltrated with a bacterial suspension of 4×10^5 cfu/ml and symptom development was monitored for up to one-week post infiltration. We found that both strains were similar to T1 in being unable to cause disease on *Arabidopsis* leaves (**Figure 3.2A**).

To confirm our visual evaluation, tissue was sampled at day 0 (two hours after infiltration) and day 3 to measure the size of the bacterial populations. As expected, the NY isolates and T1 grew poorly in *Arabidopsis*. In contrast, DC3000 caused severe symptoms and had multiplied significantly by day 3 (**Figure 3.2B**), confirming that most *P. s*. pv. *tomato* strains are non-pathogenic in *Arabidopsis*.

A

	Strain	County collected
1	NY-15070	Yates
2	NY-15071	Orleans
3	NY-15107	Chautauqua
4	NY-15114	Essex
5	NY-15119	Suffolk
6	NY-15125	Tompkins



B

Specific to:	*Pst*	Race 0	Race 1	
	hopR	*hopN1*	*avrA*	*hopW1*
DC3000	+	+	-	-
NY-15070	+	-	+	+
NY-15071	+	-	+	+
NY-15107	+	-	+	+
NY-15114	+	-	+	+
NY-15119	+	-	+	+
NYS15125	+	-	+	+
T1	+	-	+	+
NY-T1	+	-	+	+

Figure 3.1. Diagnostic PCR analysis to detect effectors specific for race 0 or race 1 strains group the 2015 NY isolates with race 1 strains. (A) Map depicting the counties in which *Pst* samples were isolated from infected tomato plants. (B) Table summarizing the results of genotyping PCRs using specific primers against predicted virulence effectors that allow discrimination between race 0 and race 1 isolates of *Pst*.

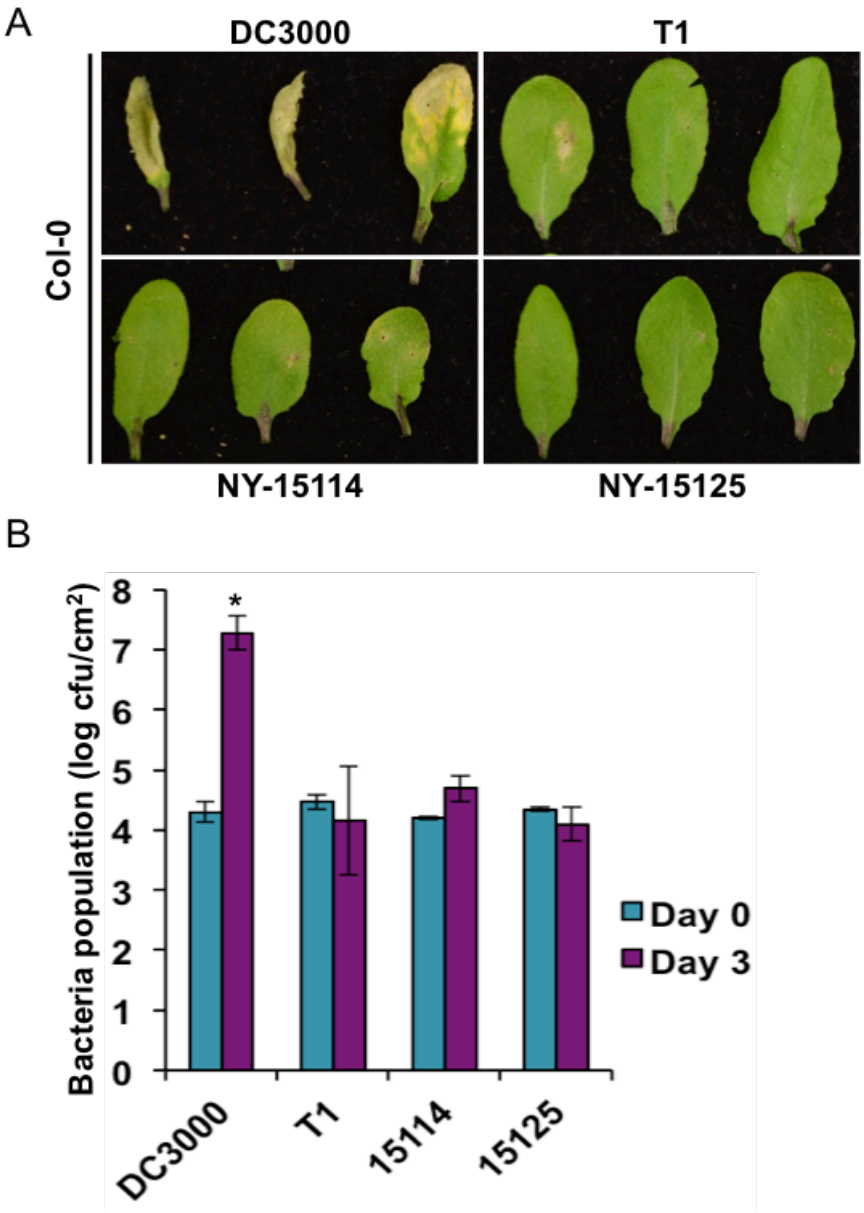


Figure 3.2. The 2015 NY isolates do not cause disease on *Arabidopsis thaliana* Col-0. (A) *Arabidopsis* Col-0 leaves were syringe infiltrated with 5 x 10^4 CFU/ml per strain. All photographs were taken 7 days after inoculation. (B) Leaf samples were taken at day 0 and day 3 after inoculation and bacterial populations were determined. Data is presented as mean ± SD. The asterisk indicates a significant difference ($P < 0.05$) between day 0 and day 3 based on a Welch's test (unequal variance t-test) for normal distributed data or the Wilcoxon test for nonparametric data. These experiments were performed three times, and similar results were obtained.

Pst strains collected in NY can partially overcome Pto-mediated resistance

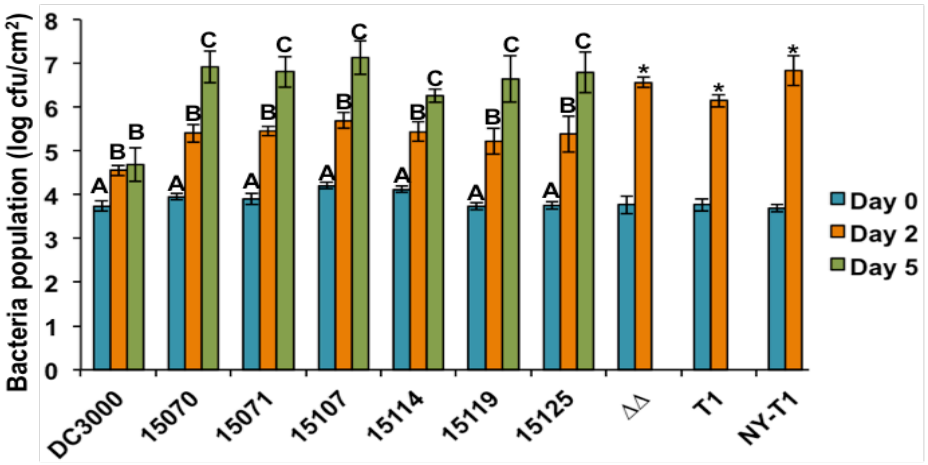
To test whether the Pto-mediated resistance pathway would be effective in inhibiting the growth of the NY isolates, we vacuum-infiltrated four week old Rio Grande-PtoR plants (RG-PtoR) expressing Pto/Prf from *Solanum pimpinellifolium* (*Spim*) and RG-prf3 plants (RG-PtoR null for *Prf*) with the different 2015 NY strains. Strains DC3000 (race 0), DC3000Δ*avrPto*Δ*avrPtoB*, T1 and NY-T1 (race 1) were used as controls. To monitor for the ability of the new isolates to grow in the different tomato genotypes, leaf tissue samples were taken immediately (day 0) and at day two after infiltration to determine the size of the bacterial populations. Our results show that all NY isolates grew by day two to populations intermediate between DC3000, a strain that cannot infect tomatoes with a functional *Pto* pathway, and the very virulent *Pst* strains such as T1, NY-T1 and DC3000Δ*avrPto*Δ*avrPtoB* (**Figure 3.3A**). Due to this novel intermediate phenotype, we decided to include an additional data point three days later (day 5). To our surprise, all 2015 NY *Pst* strains were able to continue growing to population levels high enough that by day 6, bacterial speck symptoms were visible throughout the plant leaves and stem (**Figure 3.3A** and **3.3B**). In contrast, no additional growth or symptom development was detected in plants infiltrated with DC3000 (**Figure 3A**). No samples were collected at day 5 for the race 1 strains, as the tissue was already too necrotic. As expected, bacterial titers for all strains reached high levels in RG-prf3 tomato plants, causing disease symptoms as early as three days post inoculation (**Figure 3.3C**). The results described above demonstrate that all six 2015 NY isolates show identical virulence profiles on RG-PtoR and RG-PtoR *prf-3* plants. Under lab conditions they appear to partially suppress Pto-mediated recognition and their populations reach sufficient numbers to cause speck symptoms, even on a resistant Pto-expressing tomato variety. However, in comparison to race 1 strains, the infection is milder and does not kill the plant.

2015 NY strains have both *avrPto* and *avrPtoB,* but only AvrPto protein is detectable

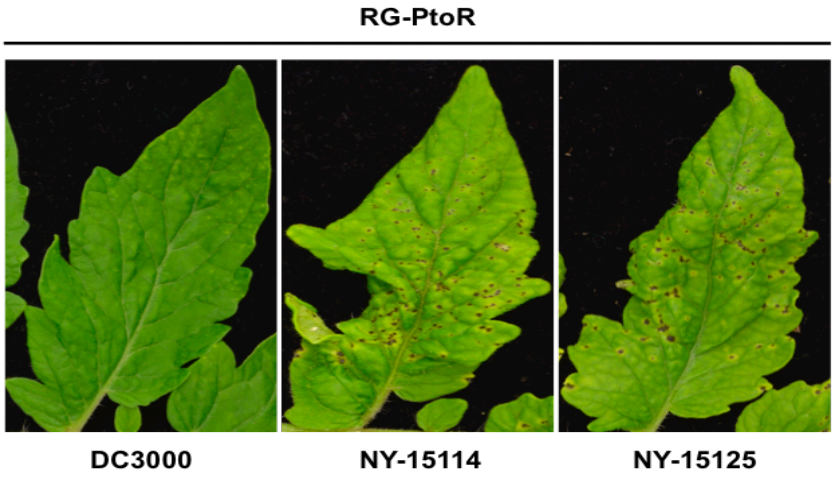
Since the virulence of NY strains on RG-PtoR tomato plants was less than that of other race 1 strains, we decided to test them for the presence of *avrPto* and *avrPtoB*. These two *Pst* effectors are recognized by Pto, triggering a hypersensitive response. Using sets of specific primers to amplify *avrPto* and a fragment of *avrPtoB* that can differentiate between DC3000 and T1 *avrPtoB*, we were able to demonstrate that the NY-15114 and NY-15125 genomes contain the genes for both effectors (**Figure 3.4A** and **Table 3.1**). The *avrPto* sequence of both strains is identical to *avrPto* in JL1065, and this allele differs in just four amino acids from the $avrPto_{DC3000}$ allele (**Figure 3.5)**. We only obtained an amplicon for *avrPtoB* from the NY strains when using the T1 specific *avrPtoB* primers. We additionally amplified and sequenced the complete *avrPtoB* gene from both strains, and the results show that the NY *avrPtoB* allele is 100% identical to the $avrPtoB_{T1}$ allele (data not shown).

To determine whether these two effector genes are transcribed, we grew the *Pst* strains in *hrp*-inducing minimal medium (HrpMM) to mimic plant infection, thereby inducing effector gene expression. RNA was then extracted and subjected to cDNA synthesis using random hexamer primers. The primers previously tested on DNA were used for a reverse transcriptase polymerase chain reaction (RT-PCR). Both genes are highly expressed under inducing conditions in NY-15114 and NY-15125, to levels comparable to other tested strains (**Figure 3.4B** and **Table 3.1**). For all PCRs, primers amplifying the *16S RNA* were used as positive controls (**Figure 3.4A, 3.4B** and **Table 3.1**).

A


Bacteria population (log cfu/cm²)
0
1
2
3
4
5
6
7
8
A
B
C
*
DC3000
15070
15071
15107
15114
15119
15125
ΔΔ
T1
NY-T1
Day 0
Day 2
Day 5

B


RG-PtoR
DC3000
NY-15114
NY-15125

C

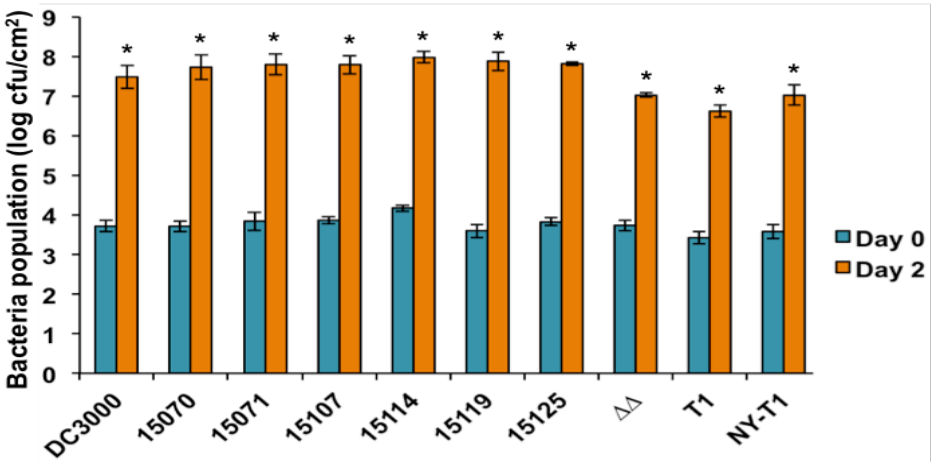

Bacteria population (log cfu/cm²)
0
1
2
3
4
5
6
7
8
9
*
DC3000
15070
15071
15107
15114
15119
15125
ΔΔ
T1
NY-T1
Day 0
Day 2

Figure 3.3 (*Previous page*). *Pst* strains isolated in NY grow to intermediate population levels on tomato plants with a functional Pto pathway, as compared to race 0 and the more virulent race 1 strains. (A) RG-PtoR or (C) RG-prf3 plants were vacuum infiltrated with the indicated *Pst* strains at a final concentration of 5 x 10^4 CFU/ml and bacterial populations were measured at day 0, 2 and when possible at day 5 post inoculation. Results are shown as the mean of 3 samples including standard deviation. Different letters indicate significant differences (p value <0.05). In cases of equal variance a one-way ANOVA followed by Tukey's HSD post hoc test was performed; in cases of unequal variance, the Brown-Forsythe test followed by a Games-Howell post hoc test was used. For pairwise comparison data, the asterisk indicates a significant difference (P <0.05) between day 0 and day 2 based on a Wilcoxon test (unequal variance t-test). (B) Photographs were taken 7 days after infiltration of representative leaves of vacuum infiltrated RG-PtoR plants showing symptoms.

It has been previously shown that *Pst* strain T1 and other race 1 *Pst* isolates collected in California such as A9, 19, 838-8, 838-16, 22 and 23 post-transcriptionally suppress accumulation of AvrPtoB protein by an unknown mechanism, thereby evading Pto mediated recognition of this effector by the host (Kunkeaw et al., 2010; Lin et al., 2006). Since the *avrPtoB* sequences in the 2015 NY *Pst* strains are identical to those of T1 and NY-T1 strains, we tested whether this is also the case in NY-15114 and NY-15125. Additionally, we hypothesized that AvrPto protein is expressed, identical to JL1065 and DC3000, since all four strains share an almost identical gene sequence. To test for protein expression, we grew the *Pst* strains in HrpMM and protein accumulation was detected using antibodies raised specifically against AvrPtoB or AvrPto. We were able to detect AvrPtoB protein with our anti-AvrPtoB antibody in the DC3000 samples only. However, endogenous AvrPto protein was detected in the two 2015 NY *Pst* samples, similar to completely avirulent DC3000. Thus we conclude that NY-15114 and NY-15125 strains suppress AvrPtoB protein accumulation, identical to T1 and NY-T1. However, race 1 strains usually lack or have a mutated allele of *avrPto*, but the NY strains not only transcribe, but also translate *avrPto* to a functional protein during infection (**Figure 3.4C**).

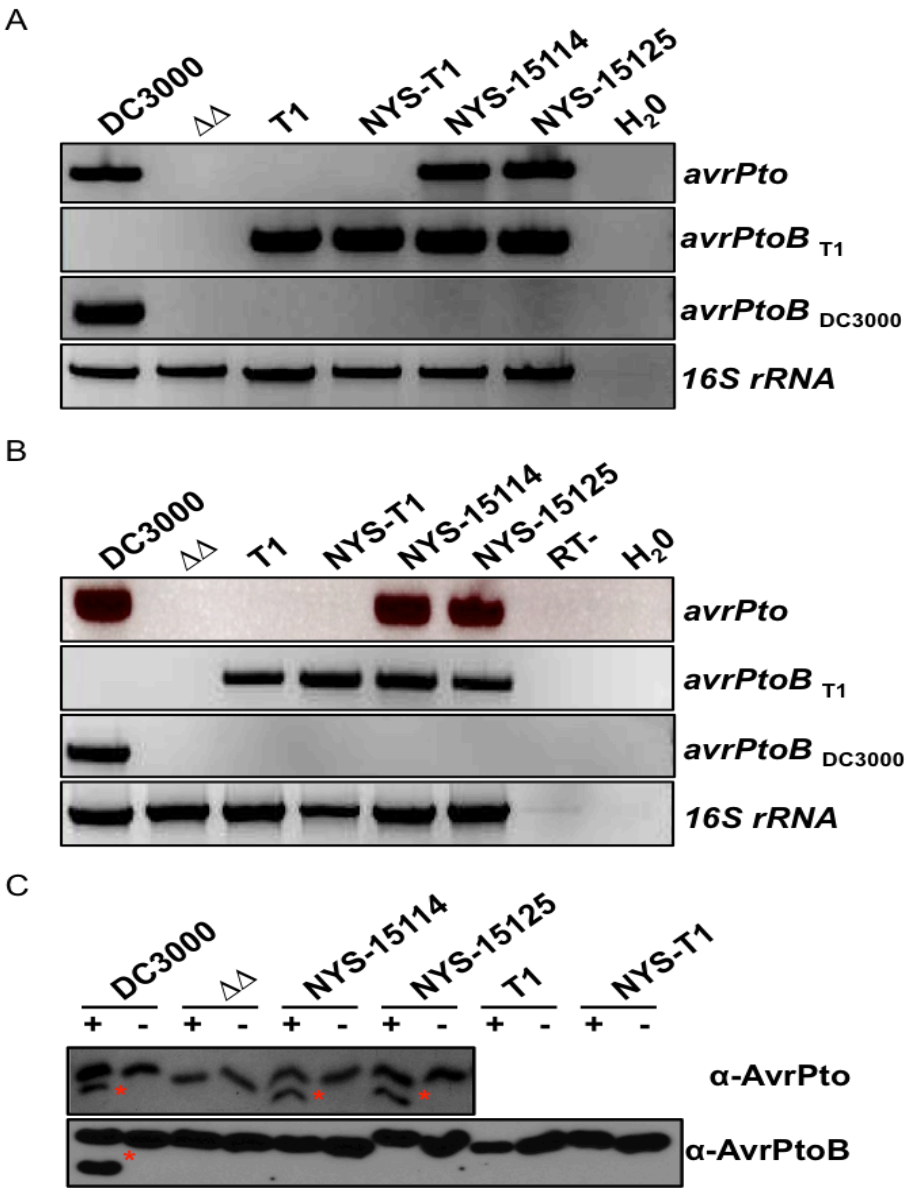


Figure 3.4. 2015 NY isolates induce *avrPto* and *avrPtoB* gene expression in an *hrp*-dependent manner, but only AvrPto protein accumulates in the cell. (A) Colony PCR shows the presence of *avrPto* and *avrPtoB* genes in all tested strains. (B) RT-PCR analysis to detect effectors transcripts. cDNA was synthesized from RNA isolated from bacteria grown in HrpMM. Primers amplifying 16S rRNA were used as an internal control for (A) and (B). (C) Detection of AvrPto and AvrPtoB protein accumulation by Western blotting using endogenous antibody raised against each protein. Bacterial cells were grown in HrpMM overnight and lysed in Laemmli Sample Buffer for protein extraction. A red asterix indicates the protein with the correct molecular weight for AvrPto and AvrPtoB. A non-specific band with a higher molecular weight shows equal loading in each lane.

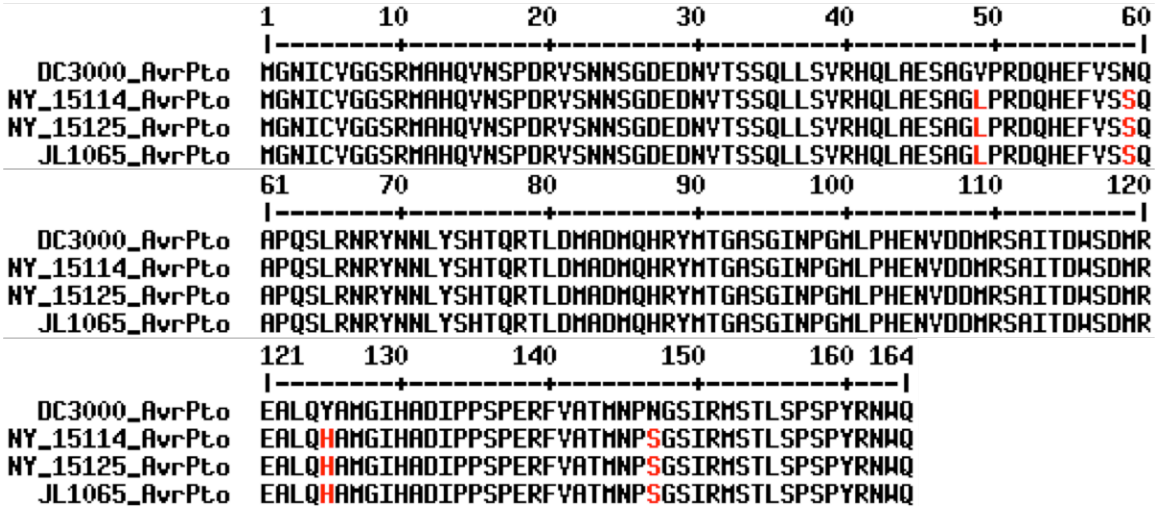


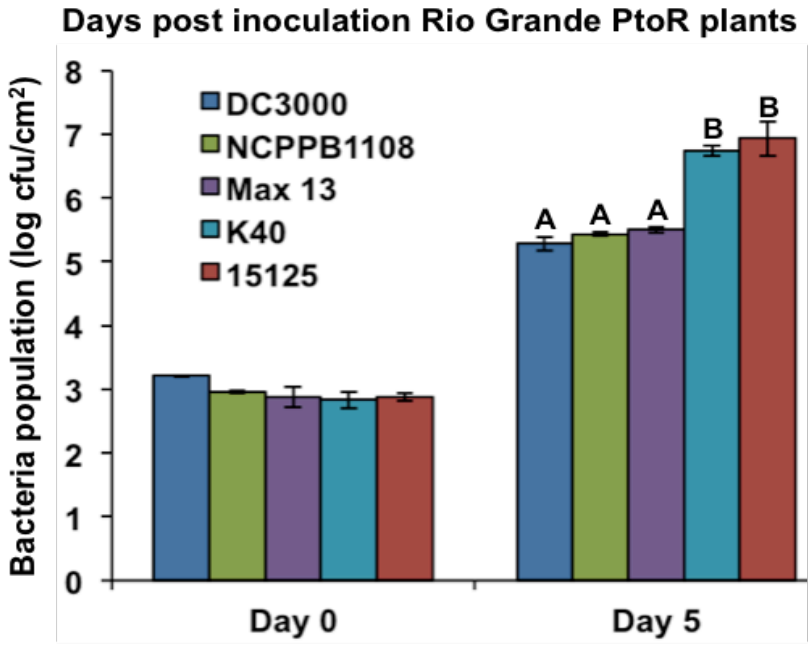
Figure 3.5. Amino acid sequence alignment comparing AvrPto from NY-isolates to DC3000 and JL1065 with the four amino acid differences highlighted in red.

Pst strain K40 shows the same phenotype on RG-PtoR plants as the NY isolates

Since 2003, when the first *Pseudomonas syringae* pv. *tomato* genome, DC3000, was published, several other strains have been sequenced and their genomes are publicly available (Buell et al., 2003) (http://www.pseudomonas-syringae.org). Using this resource, we looked for *Pst* strains that have a functional *avrPto* gene, but do not express AvrPtoB protein (speculating that if the sequence is identical to the T1 *avrPtoB* allele, it would not be expressed). JL1065 and PT23 were ruled out because Lin and colleagues have shown that these two strains express and secrete AvrPtoB (Lin et al., 2006). Max13, K40 and NCPPB1108 *Pst* strains were tested for their ability to grow on RG-PtoR and RG-prf3 tomato plants and compared to NY-15125 and DC3000. Samples were taken immediately (day 0) and at day 5 after infiltration of RG-PtoR plants and at day 2 for RG-prf3 plants. Max13 and NCPPB1108 grew to the same low titers as DC3000 on tomato plants with a functional Pto pathway (**Figure 3.6A**), demonstrating that the Pto pathway can fully recognize these strains and inhibit *Pst* growth. However, K40, a strain that was isolated in Virginia in 2005, showed the same intermediate growth phenotype

and late symptom development by day 5 as the 2015 NY isolates (**Figure 3.6A**). All tested strains grew to high levels on susceptible RG-prf3 plants, confirming their ability to infect tomato plants and grow to high titers when not recognized by Pto (**Figure 3.6B**).

A



B

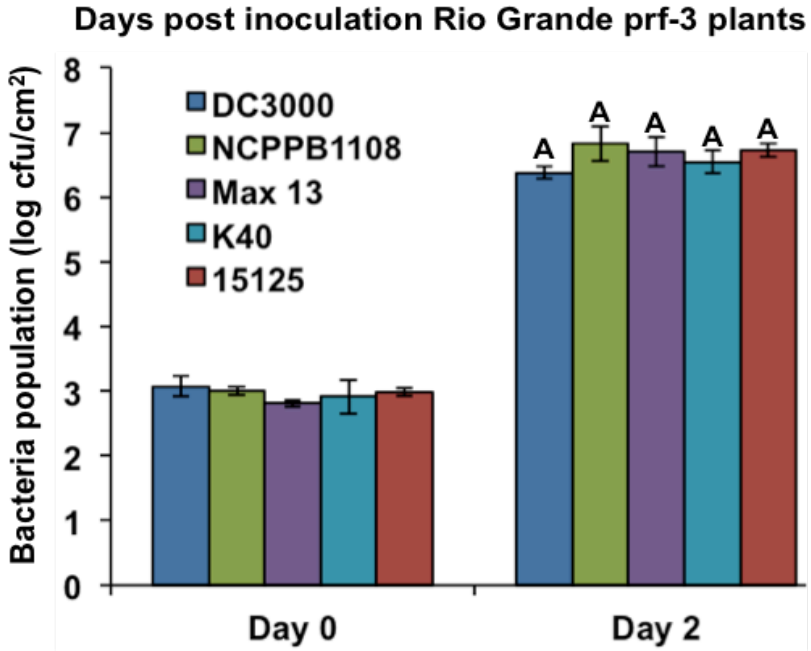


Figure 3.6 (*Previous page*). The K40 strain shows the same intermediate growth phenotype as the 2015 NY *Pst* isolates. Different sequenced strains with a functional AvrPto, but lacking AvrPtoB expression, were vacuum infiltrated into (A) RG-PtoR or (B) RG-prf3 plants at 5 x 10^4 CFU/ml. Bacterial populations were measured at 0 and 5 days for RG-PtoR and at 0 and 2 days for RG-prf3 plants after infiltration. The error bars indicate standard deviations for three replicates. Different letters indicate significant differences (p value <0.05). The experiments were performed twice with similar results.

Table 3.1. Primers used in this study

Primer name	Gene	Sequence (5' → 3')
oTK187	*hopR1*	GAGATGGAACATGGCATCAG
oTK189	*hopR1*	AGGTGAACAGTGTCGTCTC
oTK183	*hopN1*	AATGGAAGCGAGTGTCTGC
oTK186	*hopN1*	GATTCTGGTCTTGATGTATTGCG
oTK175	*avrA*	CGATCTCTGTCGAACAATGC
oTK177	*avrA*	GAAGACCTTGGTTCTTTCGG
oTK180	*hopW1*	GAACAGCAGACACTCAAAGG
oTK181	*hopW1*	CCTGTGTCCAATTTGTCCTC
oPB01	*avrPto*	ATGGGAAATATATGTGTCGG
oPB02	*avrPto*	TCATTGCCAGTTACGGTACG
oTK212	$avrPtoB_{DC3000}$	TATCGTTCAGCAATTGGTCAGTG
oTK213	$avrPtoB_{DC3000}$	CCCCGGGTTCAGGTTAA
oTK214	$avrPtoB_{T1}$	GCCACGCGATAGCTCTTCCTTCTC
oTK215	$avrPtoB_{T1}$	AACAACCGCCTGCCGCTCGTAAC
oTK216	*16S rRNA*	GCGGCAGGCCTAACACAT
oTK127	*16S rRNA*	GTTCCCCTACGGCTACCTT

3.4 Discussion

The aim of this work was to characterize the molecular basis of *Pst* virulence within a sampling of isolates from 2015, a period of widespread bacterial speck disease outbreaks in NY. The observation that the global *Pst* population has shifted towards the more virulent race 1 group serves to highlight the importance of knowing the exact identity of the dominant bacterial population (Cai et al., 2011). Our second goal was to generate information to allow us to give a recommendation on whether introgression of *Pto* into the fresh market tomato cultivars grown in this area would be worthwhile.

Using primers designed to amplify effector sequences that can differentiate between race 0 and race 1 strains, our data suggest that all NY isolates belong to the virulent race 1 group (Jones et al., 2015). This shows the necessity of deeper molecular analysis even for well-studied host-pathogen systems. Solely relying on effector profiling to differentiate between race 0 and 1, we would have over-estimated the virulence potential of these isolates. By investigating the RNA and protein level of AvrPto and AvrPtoB, we were able to reveal that, contrary to what would have been predicted based on this race 1 classification, both effectors were present as functional genes at the transcriptional level, and AvrPto protein was expressed at levels sufficient to activate resistance in Pto-expressing tomatoes. The combination of DNA sequences, virulence and Western blot assays enabled us to identify the actual virulence potential of these isolates. This reliable identification of the virulence of strains present in the field is of crucial importance to breeders and growers in order to make the appropriate disease management decisions.

The NY isolates reached intermediate population levels in RG-PtoR plants in environmentally-controlled growth chambers as compared to typical race 0 and 1 control strains. Importantly, the bacterial populations reached levels sufficiently high to cause symptoms on leaves and stems by day 6, but not so high as to kill the plant, as seen with

T1 or NY-T1. This uniformity in genetic features and virulence is quite interesting considering that samples were collected from distinct geographic areas in NY, from the Canadian border to Long Island. A possible explanation for this uniformity could be a single pathogen source, derived from infected seeds or seedlings. Examples for such occurrences have been documented in the past (McCarter et al., 1983). To confirm this hypothesis, a survey to determine the sources of affected growers would be required. Long distance movement of *Pst* in the environment is also a possibility; it has been demonstrated that these bacteria can be isolated from the environment, for example from the rain and snow, indicating that they spread readily (Monteil et al., 2013; Morris et al., 2008). NY-15114 isolated in Tompkins county did not originate in a tomato grower field, but was instead isolated from a Cornell University research plot, and the seeds used had been collected from tomato lineages grown in a research greenhouse over many generations and not sourced from an outside supplier. As no outbreak had occurred in the greenhouse, an infection of plants *in situ* is the most likely explanation for this infection, indicating that pathogen spread through the environment is at the very least partially responsible for this recent outbreak, potentially exacerbated by mild, rainy summers like the one in 2015.

We further looked for the presence, as well as transcription and translation, of *avrPto* and *avrPtoB*. DNA sequencing of these genes revealed that *avrPtoB* in the NY isolates is identical to the $avrPtoB_{T1}$ allele. We were able to detect RNA using qRT-PCR, but no protein accumulation using endogenous AvrPtoB antibodies. $avrPtoB_{JL1065}$ and $avrPtoB_{PT23}$ differ in only 3 amino acids from the $avrPtoB_{T1}$ allele and in those strains AvrPtoB is expressed, secreted and also recognized by Pto. This indicates that the absence of AvrPtoB protein in T1-like strains is not due to intrinsic protein instability of the T1 allele, but more likely active repression/inhibition of translation of AvrPtoB by T1 and NY-T1. Suppression of AvrPtoB protein translation appears to be a strategy widely

shared by *Pst* strains, but the mechanism underlying this phenomenon is still unknown (Kunkeaw et al., 2010; Lin et al., 2006). In contrast to T1 and NY-T1, all NY-isolates possess a functional *avrPto* identical to the JL1065 allele that can be recognized by Pto (Ronald et al., 1992). The recognition of either AvrPto or AvrPtoB by Pto is sufficient to trigger an ETI response and halt infection (Kim et al., 2002). Why these strains modulate AvrPtoB expression while having AvrPto which can still be recognized by the host is a conundrum that we cannot explain. Nonetheless, AvrPto expression enhances *Pst* pathogenicity in tomato plants lacking the *Pto* gene boosting bacterial growth (Nguyen et al., 2010; Shan et al., 2000a) and most tomato cultivars grown in NY do not have the *Pto* resistance gene.

Growth of NY isolates is delayed in RG-PtoR plants, although these strains are fully virulent RG-prf3 plants. This observation suggests that Pto or a Pto family member is recognizing an effector in the NY isolates, and AvrPto is the obvious candidate. One way to confirm that recognition of AvrPto is the underlying mechanism of RG-PtoR resistance against NY isolates would be the generation of a NY strain with a deletion of the *avrPto* gene. However, despite much effort, we have been unable to delete the *avrPto* gene from these strains. We employed variations of several different transformation protocols (electroporation and biparental mating, with variations of OD_{600} and washing buffers) without any success. The poor transformability is an issue that we are continuing to try to resolve. The NY strains are able to reach high population levels in RG-prf3 plants, and the only difference between this cultivar and RG-PtoR is a mutation in a gene important for the Pto pathway (Salmeron et al., 1994), indicating that recognition of either AvrPtoB or AvrPto is the most likely reason for the observed resistance. Combined with the absence of detectable AvrPtoB protein in these strains, AvrPto is the most likely avirulence factor in these strains.

We took advantage of the large number of publicly available *Pst* genome sequences, as well as previous papers, to mine for strains that do not express AvrPtoB, but have a functional *avrPto* gene. Out of the three other strains that we tested for virulence on RG-PtoR and RG-prf3 plants, only K40 behaved similar to NY-15114 and NY-15125. Max13 and NCPPB1108 grew poorly on RG-PtoR, but to high levels on RG-prf3 plants. K40 was isolated in 2005 in Virginia, the third biggest fresh market tomato producer in the US with a minimal processing tomato market (Cai et al., 2011) (USDA; https://www.ers.usda.gov/topics/crops/vegetables-pulses/tomatoes.aspx). As a result Virginia most likely has a low prevalence of *Pto* in their tomato populations. It is conceivable that the presence of AvrPto in K40 confers a fitness advantage.

The early suppression of Pto-mediated resistance during infection with NY strains in the presence of AvrPto is quite interesting. We did not detect a quantitative difference in AvrPto protein expression between DC3000 and the two NY isolates. This leads us to hypothesize the existence of another potentially novel effector in these strains and K40, that has the more general function of suppressing ETI later in the pathway. Other *Pst* effectors, in addition to known effectors from *Xanthomonas*, can interact with and suppress MAP kinase proteins (MPK), Mitogen-activated protein kinase kinase proteins (MKK), plant promoters and transcription factors (TF) involved in downstream signaling (Büttner, 2016). For example, HopF2 from *Pst* DC3000 targets MKK5 *in vitro* and inhibits the PTI response (Wang et al., 2010). *Pst* HopAI1 phosphorylates MPK3, MPK4 and MPK6 in a way that prevents re-phosphorylation (Zhang et al., 2007; Zhang et al., 2012). AvrRps4 from *Pseudomonas syringae* pv. *pisi* interacts with WRKY domain-containing proteins and thus interferes with WRKY transcription factor-dependent defenses (Sarris et al., 2015). Other effectors such as XopD from *Xanthomonas campestris* are delivered to the nucleus and interact with TF such as AtMYB30 in *Arabidopsis* to repress transcription of defense- and senescence-related plant genes

(Canonne et al., 2011; Raffaele and Rivas, 2013). Furthermore, it has been shown that many effectors suppress ETI triggered responses by masking the avirulence activity of another effector (Guo et al., 2009; Jackson et al., 1999; Wei et al., 2015).

Overall, our data suggest that introgression of *Pto* into NY-grown tomato cultivars is still a viable strategy to manage bacterial speck in NY. Under lab conditions *Pto* suppresses bacterial growth of the NY isolates to a level that the plant, while showing some bacterial speck symptoms, does not succumb to the disease. In 2016, we conducted a field trial with resistant and susceptible tomatoes that we spray-inoculated with NY-15125. While 2016 was a very dry summer in NY, rains towards the end of the summer spread the pathogen, and we were able to observe bacterial speck on leaves, stems and fruit only on the susceptible varieties, while *Pto*-expressing plants remained disease-free (data not shown). We will continue testing *Pto* resistance in the field, but our preliminary data indicate that under natural conditions *Pto* recognizes NY *Pst* strains and that introducing this *R* gene into fresh market varieties will be beneficial to tomato growers in the state.

3.5 Materials and Methods

Isolation of *Pst* strains from the field

The *Pst* isolates utilized in the present study were isolated from diseased tissue collected in NY during the 2015 outbreaks using KBM semi-selective media. All *Pst* isolates were stored in 20% glycerol + 60 mM sucrose at –80°C.

Plant material

Tomatoes were grown in a greenhouse with 16 h light and a temperature of 24ºC / 8 h dark and a temperature of 22ºC and 65% humidity. Sowing and transplanting were performed as described previously (Kraus et al., 2016). *Arabidopsis thaliana* plants were

grown in soil in an environmentally controlled chamber under fluorescent lighting (150 μmol m^{-2} s^{-1}) with a 16 h light / 8 h dark cycle at 25°C.

Virulence assays in tomato

All strains used were grown in KB medium and prepared for vacuum infiltration using a protocol described previously (Kraus et al., 2016). Plants were vacuum infiltrated at a final concentration of 5 x 10^4 CFU/ml. Samples for day 0 were taken 2 hpost infiltration and scored again for disease symptoms and bacterial growth at day 2, 5. Pictures were taken 7 days after infiltration. Three discs were taken per plant per time point. Samples for the different time points were taken from the same plants, but different leaflets. Results are shown as the mean of four biological replicates, including standard deviation.

Virulence Assay in *Arabidopsis thaliana*

Pst strains grown on KB medium were diluted in 10 mM $MgCl_2$ to a final concentration of 5 x 10^4 CFU/ml. Several leaves per plant were pressure-infiltrated with a needleless syringe. The development of disease symptoms was monitored for up to 1 week after infiltrations. Leaf tissue samples were taken 2 h after infiltration (day 0) and 3 days later to measure bacterial proliferation. Three discs were taken per plant for three biological replicates per time point. Samples for day 0 and 3 were taken from different plants. Results are shown as the mean of three biological replicates, including standard deviation.

Effector genotyping by liquid colony PCR

Pst strains were grown in liquid KB medium overnight. The next day 100 µl aliquots were lysed for 10 min at 90ºC followed by a 5 min full speed centrifugation. One to two µl of the supernatant were used as template to perform a standard GoTaq PCR protocol. Primers used in this study are listed in Table 3.1**.**

RNA extraction and cDNA Synthesis for Reverse transcriptase PCR (RT–PCR)

Bacterial cultures were grown in *hrp*-inducing minimal medium (50 mM phosphate buffer, pH 5.7, 7.6 mM $(NH_4)_2SO_4$, 1.7 mM $MgCl_2$, 1.7 mM NaCl, 10 mM fructose) to an final concentration of 2 x 10^8 CFU/ml. RNA was extracted following the RNAprotect® Bacteria Reagent Handbook (Qiagen) protocol. Isolated RNA was treated with Turbo DNase from Ambion according to manufacturer's instructions. 600 ng of RNA were used for cDNA synthesis with random hexamer primers with a Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). The same primers were used as previously for the colony PCR (Table S1).

***Pst* protein expression**

Pst strains at an OD_{600} = 0.4 were grown overnight in 5 ml *hrp*-inducing liquid minimal or KB media containing appropriate antibiotics at room temperature at 220 rpm. The next day, the OD_{600} was set to a final concentration of 0.5. One ml of each bacterial pellet was centrifuged, resuspended in water and centrifuged again. Washed bacterial pellets were boiled for 10 min 100 µl of Laemmli buffer and 5 µl were used for immunoblot analysis.

Immunoblot assay

SDS-PAGE and subsequent transfer to PVDF membranes for western blotting was performed according to standard procedures (Harlow and Lane 1988). AvrPtoB antibody was affinity purified from antiserum by PVDF transfer before use for detection. Specifically, AvrPtoB protein was expressed in *E. coli* and resolved on 8% SDS-PAGE. After transfer, the PVDF membrane was stained with Ponceau S and the strip containing the antigen protein was cut out and rinsed with TBS-T until the membrane was no longer pink. After blocking the membrane with 5% milk/TBS-T for 1 h at room temperature with gentle rocking the membrane was cut into small pieces and inserted into a 15ml centrifuge tube containing 2 ml of diluted serum with 8 ml of TBS. The centrifuge tube was incubated overnight at 4ºC. The next day the blot was washed three times with TBS-

T and bound antibody was eluted with 2 ml of 0.1 M Acidic Glycine pH 2.4 by vortexing for 1 min, incubation for 2 min on a rotator and vortexing again for 1 min. After transferring the Acidic Glycine Buffer to a tube, 200 µl of 1M Tris pH 8.0 was added for neutralization. Elution and neutralization steps were repeated a total of three times. AvrPtoB antibody was concentrated using Centrifugal Filter Units (Millipore) following manufacturer's instructions. For the immunoblot assay, purified AvrPtoB antibody was used at a concentration of 1:1,000. For AvrPto recognition, the antisera containing the polyclonal AvrPto antibody was directly used at a concentration of 1:20,000. Secondary goat anti-rabbit IgG HRP conjugate at a dilution of 1:20,000 was used for detection (Promega).

3.6 Acknowledgments

We thank Dr. Johannes Mathieu and Dr. Ruth Fahey for critical review of the manuscript. We also thank NY tomato growers for providing leaf samples with bacterial speck. No conflict of interest declared.

3.7 References

Abramovitch, R.B., Kim, Y.-J., Chen, S., Dickman, M.B., and Martin, G.B. (2003). *Pseudomonas* type III effector AvrPtoB induces plant disease susceptibility by inhibition of host programmed cell death. EMBO J 22:60-69.

Almeida, N.F., Yan, S., Lindeberg, M., Studholme, D.J., Schneider, D.J., Condon, B., Liu, H., Viana, C.J., Warren, A., Evans, C., et al. (2009). A draft genome sequence of *Pseudomonas syringae* pv. *tomato* T1 reveals a type III effector repertoire significantly divergent from that of *Pseudomonas syringae* pv. *tomato* DC3000. Mol Plant-Microbe Interact 22:52-62.

Arredondo, C.R., and Davis, R.M. (2000). First report of *Pseudomonas syringae* pv. *tomato* race 1 on tomato in California. Plant Disease 84:371.

Bosch, S.E., Boelema, B.H., Serfontein, J.J., and Swanepoel, A.E. (1990). 'Rotam 4', a multiple disease-resistant fresh-market tomato. Hortscience 25:1313–1314.

Buell, C.R., Joardar, V., Lindeberg, M., Selengut, J., Paulsen, I.T., Gwinn, M.L., Dodson, R.J., Deboy, R.T., Durkin, A.S., Kolonay, J.F., et al. (2003). The complete genome sequence of the *Arabidopsis* and tomato pathogen *Pseudomonas syringae* pv. *tomato* DC3000. Proc Natl Acad Sci USA 100:10181-10186.

Buonaurio, R., Stravato, V.M., and Cappelli, C. (1996). Occurrence of *Pseudomonas syringae* pv. *Tomato* race 1 in Italy on *Pto* gene-bearing tomato plants. J Plant Phytopath 144:437-440.

Büttner, D. (2016). Behind the lines-actions of bacterial type III effector proteins in plant cells. FEMS Microbiol Rev 40: 894–937.

Cai, R., Lewis, J., Yan, S., Liu, H., Clarke, C.R., Campanile, F., Almeida, N.F., Studholme, D.J., Lindeberg, M., Schneider, D., et al. (2011). The plant pathogen *Pseudomonas syringae* pv. *tomato* is genetically monomorphic and under strong selection to evade tomato immunity. PLoS Pathog 7:e1002130.

Canonne, J., Marino, D., Jauneau, A., Pouzet, C., Brière, C., Roby, D., and Rivas, S. (2011). The *Xanthomonas* type III effector XopD targets the *Arabidopsis* transcription factor MYB30 to suppress plant defense. Plant Cell 23:3498-3511.

Guo, M., Tian, F., Wamboldt, Y., and Alfano, J.R. (2009). The majority of the type III effector inventory of *Pseudomonas syringae* pv. *tomato* DC3000 can suppress plant immunity. Mol Plant-Microbe Interact 22:1069-1080.

Jackson, R.W., Athanassopoulos, E., Tsiamis, G., Mansfield, J.W., Sesma, A., Arnold, D.L., Gibbon, M.J., Murillo, J., Taylor, J.D., and Vivian, A. (1999). Identification of a pathogenicity island, which contains genes for virulence and avirulence, on a large native plasmid in the bean pathogen *Pseudomonas syringae* pathovar *phaseolicola*. Proc Natl Acad Sci USA 96:10875-10880.

Jones, J.B. (1991). Bacterial speck. In: Compendium of tomato diseases--Jones, J.B., Jones, J.P., Stall, R.E., and Zitter, T.A., eds. St. Paul, MN: APS Press. 26-27.

Jones, L.A., Saha, S., Collmer, A., Smart, C.D., and Lindeberg, M. (2015). Genome-assisted development of a diagnostic protocol for distinguishing high virulence *Pseudomonas syringae* pv. *tomato* strains. Plant Disease 99:527-534.

Kim, Y.J., Lin, N.-C., and Martin, G.B. (2002). Two distinct *Pseudomonas* effector proteins interact with the Pto kinase and activate plant immunity. Cell 109:589-598.

Kraus, C.M., Munkvold, K.R., and Martin, G.B. (2016). Natural variation in tomato reveals differences in the recognition of AvrPto and AvrPtoB effectors from *Pseudomonas syringae*. Mol Plant 9:639–649.

Kunkeaw, S., Tan, S., and Coaker, G. (2010). Molecular and evolutionary analyses of *Pseudomonas syringae* pv. *tomato* race 1. Mol Plant-Microbe Interact 23:415-424.

Lawton, M.B., and MacNeill, B.H. (1986). Occurrence of race 1 of *Pseudomonas syringae* pv. *tomato* on field tomato in southwestern Ontario. Journal Plant Pathology 8:85–88.

Lin, N.C., Abramovitch, R.B., Kim, Y.J., and Martin, G.B. (2006). Diverse AvrPtoB homologs from several *Pseudomonas syringae* pathovars elicit Pto-dependent resistance and have similar virulence activities. Appl Environ Microbiol 72:702-712.

Lin, N.C., and Martin, G.B. (2007). Pto- and Prf-mediated recognition of AvrPto and AvrPtoB restricts the ability of diverse *Pseudomonas syringae* pathovars to infect tomato. Mol. Plant Microbe Interact. 20:806-815.

Martin, G.B. (2012). Suppression and activation of the plant immune system by *Pseudomonas syringae* effectors AvrPto and AvrPtoB. In: Effectors in Plant-Microbe Interactions--Martin, F., and Kamoun, S., eds. Oxford, UK.: Wiley-Blackwell. 123-154.

Mathieu, J., Schwizer, S., and Martin, G.B. (2014). Pto kinase binds two domains of AvrPtoB and its proximity to the effector E3 ligase determines if it evades degradation and activates plant immunity. PLoS Pathog 10:e1004227.

McCarter, S.M., Jones, J.B., Gitaitis, R.D., and Smitley, D.R. (1983). Survival of *Pseudomonas syringae* pv. *tomato* in association with tomato seeds, soil, host tissue, and epiphytic weed hosts in Georgia. Phytopathology 73:1393–1398.

McDonald, B.A., and Linde, C. (2002). Pathogen population genetics, evolutionary potential, and durable resistance. Plant Path 41:523-527.

Monteil, C.L., Cai, R., Liu, H., Llontop, M.E., Leman, S., Studholme, D.J., Morris, C.E., and Vinatzer, B.A. (2013). Nonagricultural reservoirs contribute to emergence and evolution of *Pseudomonas syringae* crop pathogens. New Phytol 199:800-811.

Morris, C.E., Sands, D.C., Vinatzer, B.A., Glaux, C., Guilbaud, C., Buffière, A., Yan, S., Dominguez, H., and Thompson, B.M. (2008). The life history of the plant pathogen *Pseudomonas syringae* is linked to the water cycle. The ISME journal 2:321–334.

Nguyen, H.P., Yeam, I., Angot, A., and Martin, G.B. (2010). Two virulence determinants of type III effector AvrPto are functionally conserved in diverse *Pseudomonas syringae* pathovars. New Phytol 187:969-982.

Pedley, K.F., and Martin, G.B. (2003). Molecular basis of Pto-mediated resistance to bacterial speck disease in tomato. Annu Rev Phytopathol 41:215-243.

Pitblado, R.E., and MacNeill, B.H. (1983). Genetic basis of resistance to *Pseudomas syringae* pv. *tomato* in field tomatoes. Can J Plant Pathol 5:251-255.

Raffaele, S., and Rivas, S. (2013). Regulate and be regulated: integration of defense and other signals by the AtMYB30 transcription factor. Front Plant Sci.

Ronald, P.C., Salmerson, J.M., Carland, F., M., and Staskawicz, B.J. (1992). The cloned avirulence gene *avrPto* induces disease resistance in tomato cultivars containing the *Pto* resistance gene. J Bacteriol 174:1604-1611.

Rosebrock, T.R., Zeng, L., Brady, J.J., Abramovitch, R.B., Xiao, F., and Martin, G.B. (2007). A bacterial E3 ubiquitin ligase targets a host protein kinase to disrupt plant immunity. Nature 448:370-374.

Salmeron, J.M., Barker, S.J., Carland, F.M., Mehta, A.Y., and Staskawicz, B.J. (1994). Tomato mutants altered in bacterial disease resistance provide evidence for a new locus controlling pathogen recognition. Plant Cell 6:511-520.

Salmeron, J.M., Oldroyd, G.E., Rommens, C.M., Scofield, S.R., Kim, H.S., Lavelle, D.T., Dahlbeck, D., and Staskawicz, B.J. (1996). Tomato *Prf is* a member of the leucine-rich repeat class of plant disease resistance genes and lies embedded within the *Pto* kinase gene cluster. Cell 86:123-133.

Sarris, P.F., Duxbury, Z., Huh, S.U., Ma, Y., Segonzac, C., Sklenar, J., Derbyshire, P., Cevik, V., Rallapalli, G., Saucet, S.B., et al. (2015). A plant immune receptor detects pathogen effectors that target WRKY transcription factors. Cell 161:1089–1100.

Shan, L., He, P., Zhou, J.M., and Tang, X. (2000a). A cluster of mutations disrupt the avirulence but not the virulence function of AvrPto. Mol Plant-Microbe Interact 13:592-598.

Shan, L., Thara, V.K., Martin, G.B., Zhou, J.M., and Tang, X. (2000b). The *Pseudomonas* AvrPto protein is differentially recognized by tomato and tobacco and is localized to the plant plasma membrane. Plant Cell 12:2323-2338.

Smart, C.D., Lange, H.W., and Tancos, M.A. (2015). Tomato Bacterial Diseases of 2014. In: Proceedings from the Empire State Producers Expo; Syracuse, NY: New York State Vegetable Growers Association, http://www.hort.cornell.edu/expo/2015proceedings.php.

Wang, Y., Li, J., Hou, S., Wang, X., Li, Y., Ren, D., Chen, S., Tang, X., and Zhou, Y. (2010). A *Pseudomonas syringae* ADP-ribosyltransferase inhibits *Arabidopsis* mitogen-activated protein kinase kinases. Plant Cell 22:2033–2044.

Wei, H., Chakravarthy, S., Mathieu, J., Helmann, T., Stodghill, P., Swingle, B., Martin, G.B., and Collmer, A. (2015). *Pseudomonas syringae* pv. *tomato* DC3000 Type III secretion effector polymutants reveal an interplay between HopAD1 and AvrPtoB. Cell Host Microbe 17:752-762.

Yan, S., Liu, H., Mohr, T.J., Jenrette, J., Chiodini, R., Zaccardelli, M., Setubal, J.C., and Vinatzer, B.A. (2008). Role of recombination in the evolution of the model plant pathogen *Pseudomonas syringae* pv. *tomato* DC3000, a very atypical tomato strain. Appl Environ Microbiol 74:3171-3181.

Zhang, J., Shao, F., Li, Y., Cui, H., Chen, L., Li, H., Zou, Y., Long, C., Lan, L., Chai, J., et al. (2007). A *Pseudomonas syringae effector* inactivates MAPKs to suppress PAMP-induced immunity in plants. Cell Host Microbe 1:175-185.

Zhang, Z., Wu, Y., Gao, M., Zhang, J., Kong, Q., Liu, Y., Ba, H., Zhou, J., and Zhang, Y. (2012). Disruption of PAMP-induced MAP kinase cascade by a *Pseudomonas syringae* effector activates plant immunity mediated by the NB-LRR protein SUMM2. Cell Host Microbe 11:253-263.

CHAPTER 4

PERSPECTIVES AND FUTURE DIRECTIONS

4.1 Future directions

Despite having been studied intensively, the *Pseudomonas syringae* pv. *tomato* (*Pst*) - tomato pathosystem continues to reveal new information about plant immunity responses and bacterial pathogenicity. Throughout my dissertation research, I focused on further characterizing this pathosystem by using natural variation present in wild tomato accessions and *Pst* field isolates.

The importance of a negatively charged Pto activation domain for response to AvrPto

In chapter 2, I demonstrated that the natural variation present in wild tomato accessions can be used to investigate the molecular mechanisms involved in host recognition of pathogens. As demonstrated before, our screen of over 100 wild tomato accessions for their ability to recognize *Pst* delivering AvrPto or AvrPtoB confirmed that most tomato accessions are susceptible to *Pst* (Rosebrock et al., 2007). *Solanum chmielewskii* (*Schm*) accessions are confined to a small geographical area in the Peruvian Andes. Interestingly, most of the *Schm* accessions tested (16 out of 19) were resistant to DC3000 only when AvrPtoB was present. Subsequent experiments involving the individual replacement of specific residues in Pto from *Schm* LA2677 or *S. pimpinellifoium* identified a single residue at position 193 as essential for Pto to respond to AvrPto. The discovery that D193 plays an central role in the response of Pto to AvrPto was surprising because, based on the crystal structure of the AvrPto-Pto complex, this residue is not located within or near the AvrPto-specific interface or the shared interface involving the P + 1 loop, the two domains of Pto that have been shown to be involved in interaction with AvrPto and

regulation of the immune response that follows (Dong et al., 2009; Wu et al., 2004; Xing et al., 2007).

These data provide the first evidence for a difference in downstream signaling after recognition of AvrPto and AvrPtoB; previously known differences in the interaction between Pto and the two effectors were limited to differences in the physical interaction through the interaction loops between the effectors and the kinase.

However, our current results do not shed light on the molecular mechanism underlying this differential signaling. Amino acid D193 is part of the activation domain of Pto, a domain known to be important for Pto downstream signaling (Ntoukakis et al., 2013). Substitutions of the phosphorylated amino acids S198 or T199 to alanine have been shown to inhibit the elicitation of the AvrPto–Pto-mediated hypersensitive response (Sessa et al., 2000). However, these two individual substitutions did not interfere with ability of the plant to trigger a response to AvrPtoB (Kraus, C.M. and Martin, G.B., unpublished data). D193 is not phosphorylated, but under neutral pH contributes a negative charge to the domain, similar to phosphorylated S198 and T199. We are in the process of individually substituting the remaining negatively charged amino acids in the activation loop of Pto and testing the effects on the plant response to AvrPto and AvrPtoB. To date, I have found three independent negatively charged amino acids present in the activation domain that, if substituted, limit Pto to eliciting an HR only in response to AvrPtoB recognition. Although preliminary, my hypothesis is that the sum of negative charges in this domain, rather than a specific amino acid residue, is the factor responsible for the activation of Pto by AvrPto.

The Myristoylated Alanine-Rich C Kinase Substrates (MARCKS) are proteins implicated in cell motility, phagocytosis, membrane traffic and mitogenesis. MARCKS have the ability to cycle between the membrane and the cytosol, depending on their

phosphorylation status. The non-phosphorylated proteins are attached to the cell membrane through a myristoylation motif and electrostatic interactions with phospholipids. However, phosphorylation of MARCKS introduces negative charges, which neutralize this electrostatic interaction, culminating in the displacement of MARCKS from the membrane. The mechanism by which this protein cycles on and off the membrane has been termed the myristoyl-electrostatic switch (Seykora et al., 1996). Pto also has a myristoylation motif; however its importance for Pto defense function is still unclear (Balmuth and Rathjen, 2007; de Vries et al., 2006; Loh et al., 1998). *In vivo* localization of 35S:Pto-GFP transformed with *Agrobacterium* into *N. benthamiana* cell-suspension cultures, as well as subcellular fractionation of *N. benthamiana* leaf tissue transiently expressing wild-type Pto-3HA show accumulation of Pto in the cytoplasm (de Vries et al., 2006). Pto localization was tested without the presence of an effector, which could impact its localization. The *Pst* effectors AvrPto and AvrPtoB localize differently in the plant cell after infection; AvrPto is plasma-membrane bound through its myristoylation motif (Shan et al., 2000), whereas AvrPtoB is suggested to be a soluble protein present in the plant cell cytoplasm after infection (de Vries et al., 2006; Kim et al., 2002). This hypothesis still needs to be tested, but it is intriguing to think that Pto could change its localization depending on its phosphorylation status to detect the presence of AvrPto or AvrPtoB.

There is also always the possibility that AvrPto and AvrPtoB modify Pto differently and Prf or other co-proteins recognize this modification in different ways. We do not have any evidence of this since Pto and Prf have been shown to always be in a complex even after effector recognition (Mucyn et al., 2006) and to address this possibility we would need a co-crystal structure of Pto, Prf and the effectors.

The use of natural variation to study the ancestral *Pto* gene

The natural variation present in wild tomato accessions has been intensively used to breed specific new qualities into commercial tomato varieties (Grandillo et al., 2011). This variation can also be used to reconstruct ancestral genes and use them as the appropriate background to study functional diversification. To identify key amino acid changes that can explain differential function of different family members can be difficult, since many amino acid changes can be detrimental, rendering the protein nonfunctional, or because the new function can be due to the change of several amino acids in the protein instead of being attributable to one specific substitution. Furthermore, functionally irrelevant sequence differences might additionally have accumulated over time, and specific mutations might have epistatic effects causing a single change to have different effects in different protein family members (Harms and Thornton, 2010). In conclusion, characterization of protein family members by single amino acid swapping can be tedious, time consuming and will not always provide informative results.

To overcome these limitations, one potential solution is to determine the ancestral gene from which all different family members diversified and use this new background, in which the sequence changes actually occurred, to study the importance of specific residues for their different functions (Harms and Thornton, 2010). It has been proposed that Pto functions as a PRR kinase decoy to trap AvrPto and AvrPtoB to activate ETI (Martin, 2012; van der Hoorn and Kamoun, 2008; Zipfel, 2014). However, Pto and the PRRs kinase sequences are very diverse at the amino acid level (with only 30-40% identity), making it unlikely that *Pto* evolved from any AvrPto- and AvrPtoB-targeted well-characterized PRR. (Schwizer, S. and Martin, G.B., unpublished data; (Gimenez-Ibanez et al., 2009; Gohre et al., 2008; Shan et al., 2008; Xiang et al., 2008)).

Mal1 and Mal2 were identified as highly expressed membrane bound receptor-like kinases that show high similarity to Pto. Silencing of the *N. benthamiana* orthologs

resulted in compromised induction of immune response and enhanced susceptibility to *Pst.* The Mal1 and Mal 2 kinase domains interact with AvrPtoB lacking the E3 ligase domain in a yeast two-hybrid system. (Schwizer, S. and Martin, G.B., unpublished data). A phylogenetic tree including the *Pto* orthologs of several wild tomato accessions, tomato cultivar Heinz, *Solanum lycopersicoides,* Potato, Eggplant, Iochroma and pepper showed that *Mal1* is present in all tested *Solanum* species and situated *Mal1* as the closest ancestral gene to the *Pto* orthologs, making this gene the most likely candidate from which *Pto* might have evolved (Kraus, C.M., Strickler, S., Martin G.B., unpublished data). Further bioinformatical and biological analysis needs to be performed to further support the hypothesis that Mal1 is the ancestral progenitor of the Pto family.

Molecular characterization of *Pst* isolates present in New York State

In chapter 3, I characterize *Pst* isolates collected from infected field tomatoes throughout New York (NY) in 2015 and demonstrate that they all have similar virulence activity. Under laboratory conditions favoring the pathogen, all NY strains grew to bacterial population levels intermediate between the avirulent strain DC3000 and the aggressive *Pst* strains T1 and NY-T1 by day 2, and continued growing to levels that cause bacterial speck symptoms on *Pto*-expressing tomato variety RG-PtoR by day 6. We hypothesize this intermediate growth is due to Pto recognition of AvrPto from the 2015 NY strains, but that this recognition is later suppressed, potentially by another effector. Nonetheless, to be certain that AvrPto is the effector recognized thus slowing down bacterial growth during the first days of infection, we still need to create an *avrPto* knockout in at least one 2015 NY strain. Although I tried different variations of mating and electroporation methods, I have not yet succeeded. All 2015 NY isolates, as well as T1 and NY-T1 grown on KB or LB media accumulate an excess amount of exopolysaccharide, which might be detrimental for bacterial transformation via transconjugation or electroporation. Further electroporation attempts trying to transform NY-15114 and

NY-15125 with overexpression plasmids were also unsuccessful, demonstrating that probably the transfer of the vector into the cell and not the homologous recombination is failing. Transformation by mating/electroporation to delete (Kvitko and Collmer, 2011) or to overexpress genes has been used in DC3000 for a long time with high rates of success. Further attempts to improve the *Pst* transformation protocol by growing the *Pst* KB plates supplemented with glutamine to reduce the accumulation of exopolysaccharide are being tested (Martin et al., 1988). If successful, growth curves will be used to test whether a 2015 NY isolate *avrPto* mutant can now grow to population levels as high as T1 or NY-T1.

Primers designed to detect effectors that differentiate race 0 from race 1 *Pst* strains (Jones et al., 2015) did not reveal any genetic differences between the 2015 isolates. However, further sequencing by Pacbio and Illumina of two isolates, NY-14115 and NY-15125, has revealed genomic differences even at the effector repertoire level between the isolates and also differences with respect to DC3000, T1 and NY-T1 (Kraus, C.M., Saha, S., Lindeberg, M., Martin G.B., unpublished data). We are in the process of assembling the genome sequences and determining putative effectors that could be suppressing the recognition of AvrPto by Pto. Effectors that can interfere with the activity of other effectors have been shown in the past to be a way of suppressing ETI (Guo et al., 2009; Jackson et al., 1999; Wei et al., 2015). Furthermore, with the improvement of the *Pst* transformation protocol we will be able to delete specific candidate effectors to study the resultant increase or decrease in virulence in tomatoes expressing *Pto*. At the same time, we will be able to transform DC3000 with the candidates (or DC3000Δ*avrPtoB* since we do not know if the novel effector will also suppress ETI activation by AvrPtoB) and test their ability to overcome Pto recognition partially or completely. It will be interesting to learn more about the new effector targets in the plant. Of course there is always the possibility that the partial inhibition of AvrPto recognition is not due to a novel effector.

Western blot analysis using antibodies raised against AvrPto did not show a difference in total AvrPto protein amounts between DC3000, NY-25114 and NY-15125. This result does not rule out differences in the amounts of AvrPto protein that get translocated into the plant cell. Future quantitative translocation experiments should answer this specific question. Another explanation could be that AvrPto from the NY fields cannot be recognized as well by Pto due to amino acid sequence differences. Although the AvrPto sequence of the NY strains is identical to $AvrPto_{JL1065}$ (and only differs in 4 amino acids from DC3000), JL1065 also has a functional *avrPtoB* gene, with protein being secreted and recognized by Pto (Kim et al., 2002; Ronald et al., 1992). Complementation of a NY isolate carrying an *avrPto* deletion with either its endogenous *avrPto* or *avrPto* from DC3000 will give us a decisive answer to this question. Recognition of $AvrPto_{DC3000}$ delivered by a NY strain would demonstrate that indeed the four amino acid differences are the reason for the partial subversion of Pto recognition. A fourth explanation, which would be as interesting as finding a new effector, would be that there is a posttranslational modification of AvrPto that is interfering with the full recognition of this effector by Pto. If we continue seeing the same intermediate growth phenotype in the line complemented with $AvrPto_{DC3000}$ we should be able to pinpoint it to either a new effector which suppresses ETI activation downstream of Pto or a novel posttranslational modification of the effector. Hopefully, our future gain- and loss-of-function experiments will shed light on possible mechanisms of this intermediate phenotype.

As mentioned before, the 2015 isolates are able to overcome Pto recognition under laboratory conditions favoring the pathogens. Although 2016 was an unusually dry summer in Ithaca, we did not observe any bacterial speck on Pto-expressing tomato plants grown in field plots inoculated with NY-15125. By the end of the summer, tomatoes lacking Pto exhibited speck symptoms on leaves, stems and fruits. We will continue with the screening; however, preliminary data indicate that, under field

conditions, the introgression of Pto might be sufficient to protect tomatoes from the *Pst* populations currently present in NY. A continued survey of the *Pst* population will be needed to assess any genetic changes that might occur. This would be of particular interest if more tomato varieties carrying the *Pto* locus will be grown in the area, because that would increase the pressure on the *Pst* population to overcome this recognition (McDonald and Linde, 2002).

4.2 References

Balmuth, A., and Rathjen, J.P. (2007). Genetic and molecular requirements for function of the Pto/Prf effector recognition complex in tomato and *Nicotiana benthamiana*. Plant J 51:978-990.

de Vries, J.S., Andriotis, V.M., Wu, A.J., and Rathjen, J.P. (2006). Tomato Pto encodes a functional N-myristoylation motif that is required for signal transduction in *Nicotiana benthamiana*. Plant J 45:31-45.

Dong, J., Xiao, F., Fan, F., Gu, L., Cang, H., Martin, G.B., and Chai, J. (2009). Crystal structure of the complex between *Pseudomonas* effector AvrPtoB and the tomato Pto kinase reveals both a shared and a unique interface compared with AvrPto-Pto. Plant Cell 21:1846-1859.

Gimenez-Ibanez, S., Ntoukakis, V., and Rathjen, J.P. (2009). The LysM receptor kinase CERK1 mediates bacterial perception in *Arabidopsis*. Plant Signal Behav 4:539-541.

Gohre, V., Spallek, T., Haweker, H., Mersmann, S., Mentzel, T., Boller, T., de Torres, M., Mansfield, J.W., and Robatzek, S. (2008). Plant pattern-recognition receptor FLS2 is directed for degradation by the bacterial ubiquitin ligase AvrPtoB. Curr Biol 18:1824-1832.

Grandillo, S., Chetelat, R.T., Knapp, S., Spooner, D.M., Peralta, I.E., Cammareri, M., Perez, P., Termolino, P., Chiusano, M.L., Ercolano, M.R., et al. (2011). From *Solanum sect. Lycopersicon*. In: Wild crop relatives: genomic and breeding resources. Vegetables (1st edition)--Kole, C., ed. Berlin, Heidelberg, New York: Springer. 129-215.

Guo, M., Tian, F., Wamboldt, Y., and Alfano, J.R. (2009). The majority of the type III effector inventory of *Pseudomonas syringae* pv. *tomato* DC3000 can suppress plant immunity. Mol Plant-Microbe Interact 22:1069-1080.

Harms, M.J., and Thornton, J.W. (2010). Analyzing protein structure and function using ancestral gene reconstruction. Curr Opin Struct Biol 20:360–366.

Jackson, R.W., Athanassopoulos, E., Tsiamis, G., Mansfield, J.W., Sesma, A., Arnold, D.L., Gibbon, M.J., Murillo, J., Taylor, J.D., and Vivian, A. (1999). Identification of a pathogenicity island, which contains genes for virulence and avirulence, on a large native plasmid in the bean pathogen *Pseudomonas syringae* pathovar *phaseolicola*. Proc Natl Acad Sci USA 96:10875-10880.

Jones, L.A., Saha, S., Collmer, A., Smart, C.D., and Lindeberg, M. (2015). Genome-assisted development of a diagnostic protocol for distinguishing high virulence *Pseudomonas syringae* pv. *tomato* strains. Plant Disease 99:527-534.

Kim, Y.J., Lin, N.C., and Martin, G.B. (2002). Two distinct *Pseudomonas* effector proteins interact with the Pto kinase and activate plant immunity. Cell 109:589-598.

Kvitko, B.H., and Collmer, A. (2011). Construction of *Pseudomonas syringae* pv. *tomato* DC3000 mutant and polymutant strains. Methods Mol Bio 712.

Loh, Y.T., Zhou, J., and Martin, G.B. (1998). The myristylation motif of Pto is not required for disease resistance. Mol Plant-Microbe Interact 11:572-576.

Martin, G.B. (2012). Suppression and activation of the plant immune system by *Pseudomonas syringae* effectors AvrPto and AvrPtoB. In: Effectors in Plant-Microbe Interactions--Martin, F., and Kamoun, S., eds. Oxford, UK.: Wiley-Blackwell. 123-154.

Martin, G.B., Chapman, K., and Chelm, B.K. (1988). Role of the *Bradyrhizobium japonicum ntrC* gene product in differential regulation of the glutamine synthetase II gene (*glnII*). J Bacteriol 170:5452-5459.

McDonald, B.A., and Linde, C. (2002). Pathogen population genetics, evolutionary potential, and durable resistance. Plant Path 41:523-527.

Mucyn, T.S., Clemente, A., Andriotis, V.M., Balmuth, A.L., Oldroyd, G.E., Staskawicz, B.J., and Rathjen, J.P. (2006). The tomato NBARC-LRR protein Prf interacts with Pto kinase in vivo to regulate specific plant immunity. Plant Cell 18:2792-2806.

Ntoukakis, V., Balmuth, A.L., Mucyn, T.S., Gutierrez, J.R., Jones, A.M., and Rathjen, J.P. (2013). The tomato Prf complex is a molecular trap for bacterial effectors based on Pto transphosphorylation. PLoS Pathog 9:e1003123.

Ronald, P.C., Salmeron, J.M., Carland, F.M., and Staskawicz, B.J. (1992). The cloned avirulence gene *avrPto* induces disease resistance in tomato cultivars containing the *Pto* resistance gene. J Bacteriol 174:1604-1611.

Rosebrock, T.R., Zeng, L., Brady, J.J., Abramovitch, R.B., Xiao, F., and Martin, G.B. (2007). A bacterial E3 ubiquitin ligase targets a host protein kinase to disrupt plant immunity. Nature 448:370-374.

Sessa, G., D'Ascenzo, M., and Martin, G.B. (2000). Thr38 and Ser198 are Pto autophosphorylation sites required for the AvrPto-Pto-mediated hypersensitive response. EMBO J 19:2257-2269.

Seykora, J.T., Myat, M.M., Allen, L.H., Ravetch, J.V., and Aderem, A. (1996). Molecular determinants of the myristoyl-electrostatic switch of MARCKS. J Biol Chem 271:18797-18802.

Shan, L., He, P., Li, J., Heese, A., Peck, S.C., Nurnberger, T., Martin, G.B., and Sheen, J. (2008). Bacterial effectors target the common signaling partner BAK1 to disrupt multiple MAMP receptor-signaling complexes and impede plant immunity. Cell Host Microbe 4:17-27.

Shan, L., Thara, V.K., Martin, G.B., Zhou, J.M., and Tang, X. (2000). The *Pseudomonas* AvrPto protein is differentially recognized by tomato and tobacco and is localized to the plant plasma membrane. Plant Cell 12:2323-2338.

van der Hoorn, R.A., and Kamoun, S. (2008). From guard to decoy: a new model for perception of plant pathogen effectors. Plant Cell 20:2009-2017.

Wei, H., Chakravarthy, S., Mathieu, J., Helmann, T., Stodghill, P., Swingle, B., Martin, G.B., and Collmer, A. (2015). *Pseudomonas syringae* pv. *tomato* DC3000 Type III secretion effector polymutants reveal an interplay between HopAD1 and AvrPtoB. Cell Host Microbe 17:752-762.

Wu, A.J., Andriotis, V.M., Durrant, M.C., and Rathjen, J.P. (2004). A patch of surface-exposed residues mediates negative regulation of immune signaling by tomato Pto kinase. Plant Cell 16:2809-2821.

Xiang, T., Zong, N., Zou, Y., Wu, Y., Zhang, J., Xing, W., Li, Y., Tang, X., Zhu, L., Chai, J., et al. (2008). *Pseudomonas syringae* effector AvrPto blocks innate immunity by targeting receptor kinases. Curr Biol 18:74-80.

Xing, W., Zou, Y., Liu, Q., Liu, J., Luo, X., Huang, Q., Chen, S., Zhu, L., Bi, R., Hao, Q., et al. (2007). The structural basis for activation of plant immunity by bacterial effector protein AvrPto. Nature 449:243-247.

Zipfel, C. (2014). Plant pattern-recognition receptors. Trends in immunology 35:345-351.