Title

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Mapping and Identification of the *RXopJ4* Resistance Gene and the Search for New Sources of Durable Resistance to Bacterial Spot Disease of Tomato

by

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University of California, Berkeley

Committee in charge:

Professor Brian J. Staskawicz, Chair
Professor Sarah C. Hake
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Abstract

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Bacterial spot of tomato (*Solanum lycopersicum*) is a devastating disease that severely limits yields in important tomato-growing regions, including the southeastern United States, where the predominant bacterial spot pathogen species is *Xanthomonas perforans*. Attempts to control the disease with antibiotics and copper-based pesticides have led to the selection of bacterial strains that are resistant to these treatments. Therefore, we turn to genetic sources of resistance as a sustainable path to reduce crop losses to bacterial spot disease.

This work describes the fine mapping and identification of the *RXopJ4* disease resistance locus from the wild tomato relative *Solanum pennellii* LA716. *RXopJ4* resistance depends on recognition of the *X. perforans* type III effector protein XopJ4. We developed a collection of fourteen molecular markers to map on a segregating F_2 population from a cross between the susceptible parent *S. lycopersicum* FL8000 and the resistant parent *RXopJ4* 8000 OC_7. In the F_2 population, a 190-kb segment on chromosome 6 cosegregated with resistance. This fine mapping enabled the identification of three *RXopJ4* candidate genes, all of which encode putative intracellular serine-threonine protein kinases. Transient coexpression of the XopJ4 effector with each kinase revealed a promising *RXopJ4* candidate gene that triggered a hypersensitive response (HR) in *Nicotiana benthamiana*. Mutations in both XopJ4 and RXopJ4 identified conserved residues required for recognition and the induction of a hypersensitive cell death phenotype. Homozygous transgenic tomato plants containing the *RXopJ4* candidate gene have been constructed and will soon be evaluated for disease resistance.

In addition, we undertook a genomic survey of fourteen *X. perforans* field isolates from all five fresh market tomato production zones of Florida, revealing a preliminary set of core type III effectors common to all isolates. We used this set of core effectors to inform a search for new sources of resistance to bacterial spot disease and identified accessions of *Nicotiana* and *Solanum americanum* that recognize core *X. perforans* effectors. Finally, we performed disease assays on 224 wild tomato accessions and found nine potential sources of bacterial spot disease resistance.

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Introduction

In nature, a subset of living organisms depends on the suffering of others for its very existence. This is a troubling reality for humankind, but also a rich source of fascinating questions for biologists. Fortunately, scientific interest in these questions has propelled innovations to diminish the suffering caused by pathogenic microorganisms. During the twentieth century we learned to protect our own bodies using vaccines and antibiotics, and to safeguard our crops from disease by finding genetic sources of resistance.

Yet long before we discovered viruses, bacteria, or pathogenic fungi and oomycetes, we felt their effects acutely. Notably, the Black Death killed about one-third of Europe's population in The Middle Ages. The bacterium responsible for this devastation was not identified until 1894, by Alexandre Yersin, and was later named *Yersinia pestis* (Stenseth et al., 2008). The late nineteenth century saw the dawn of the field of microbiology, as Robert Koch demonstrated that anthrax, cholera, and tuberculosis were all caused by microorganisms, and formulated his revolutionary postulates. These postulates stated that the pathogen must be isolated from the diseased organism in pure culture, must cause the disease when introduced into a healthy host, and then be re-isolated from the experimental host (Fredericks and Relman, 1996).

In 1885, J.C. Arthur was the first to apply Koch's postulates to a plant disease, demonstrating that fire blight of apple and pear trees was caused by the bacterium *Erwinia amylovora* (Mansfield et al., 2012). In the twentieth century, the re-discovery of Mendel's laws of inheritance led to the development of modern plant breeding. From the outset, disease resistance was a key trait sought by breeders; early efforts to improve wheat cultivars included resistance to yellow rust caused by the fungus *Puccinia glumarum* (Biffen, 1905). Around the same time, the plant pathologist Elvin C. Stakman observed that pathogen resistance was often accompanied by a rapid localized cell death known as the hypersensitive response (HR) (Mur et al., 2008). In the 1940s, Harold Henry Flor systematically analyzed the interactions among various strains of flax rust and flax cultivars. His studies on the inheritance of resistance in the plant and the elicitation of resistance by the pathogen led to a new understanding of plant disease resistance, now referred to as the gene-for-gene concept (Flor, 1971).

Still, it was not until the development of molecular genetic tools that we were able to uncover the host-pathogen interaction underlying gene-for-gene resistance. In the 1980s, Peter Lindgren, Richard Peet, and Nickolas Panopoulos used transposon mutagenesis to discover strains of the bean pathogen *Pseudomonas syringae* pv. *phaseolicola* that were impaired in their ability to elicit HR in resistant plants, but were also unable to cause disease in susceptible plants. The mutations lay in a genomic cluster dubbed the "hypersensitive response and pathogenicity", or *hrp*, gene cluster (Lindgren et al., 1986; Tampakaki et al., 2010). Later, the sequence of the *hrp* genes from *Xanthomonas campestris* pv. *vesicatoria* revealed their similarity to genes required for the secretion of virulence factors by animal

pathogens such as *Yersinia pestis* (Fenselau et al., 1992). The *hrp* genes are now known to encode a type III secretion system (T3SS), or injectosome, which is common to many species of Gram-negative bacterial pathogens and symbionts of both plants and animals. The T3SS, a specialized protein complex related to components of the bacterial flagellum, injects type III secreted effector proteins (T3SEs) directly into eukaryotic host cells, thereby modulating host cell activities to achieve pathogenicity (Cornelis, 2006).

Plants possess a basal immune system that depends on the recognition of conserved microbial molecules, called pathogen-associated molecular patterns (PAMPs), such as flagellin and elongation factor Tu. PAMPs are perceived by plant pattern recognition receptors (PRRs), which activate signaling pathways that result in PAMP-triggered immunity (PTI). Phytopathogenic bacteria deliver a variety of T3SEs into plant hosts to interfere with PTI through distinct but complementary mechanisms. In light of this, we now understand that gene-for-gene resistance is based on the specific recognition of a T3SE by a plant resistance (R) protein, a second layer of plant defense known as effector-triggered immunity (ETI) (Jones and Dangl, 2006).

Bacteria in the genus *Xanthomonas*, besides playing an important role in the elucidation of type III secretion by phytopathogenic bacteria, also cause significant agricultural diseases, including bacterial blight of rice and citrus canker (Ryan et al., 2011). This work focuses on bacterial spot disease of tomato (*Solanum lycopersicum*), a major problem in tomato-growing regions with high humidity and frequent rainfall. Bacterial spot affects both tomato and pepper plants and is caused by four species of *Xanthomonas—X. euvesicatoria*, *X. vesicatoria*, *X. perforans*, and *X. gardneri* (Jones et al., 2004). In the United States, *X. perforans* is the predominant tomato pathogen in the southeast (Jones, Bouzar, et al., 1998), while *X. gardneri* is an emerging problem in the Midwest (S. A. Miller, personal communication). Although genetic resistance to bacterial spot disease has been bred into commercial pepper lines, neither gene-for-gene nor quantitative forms of resistance exist among currently grown commercial tomato varieties. Growers have attempted to control the disease using copper-based pesticides and antibiotics, but the bacteria have developed resistance to both these treatments (Stall et al., 2009).

Natural effector-triggered immunity found in related solanaceaous plant species may be the best hope for durable resistance to bacterial spot disease. The *Bs2* gene from the wild pepper species *Capsicum chacoense* has shown promise in field trials of transgenic tomato plants (Horvath et al., 2012). However, the use of *Bs2*-containing pepper varieties has already led to the emergence of pathogen strains with mutations in the recognized effector gene, *avrBs2*, that are able to overcome the resistance (Kearney and Staskawicz, 1990; Gassmann et al., 2000). Therefore, a durable strategy must rely on multiple sources of gene-for-gene resistance in addition to quantitative resistance. Importantly, the recent ability to sequence whole genomes of pathogen strains isolated from the field will enable us to determine which pathogen effectors are conserved over space and time, and are thus likely to be the most useful targets for gene-for-gene resistance.

The primary emphasis of my dissertation work has been the precise identification and analysis of a second gene-for-gene resistance to bacterial spot disease, *RXopJ4* and *xopJ4*. In parallel, I have begun to use genome sequence data to identify conserved effectors in *X. perforans* and search for new sources of gene-for-gene resistance to target these effectors. Chapter 1 describes the fine mapping of the *RXopJ4* disease resistance gene from the wild accession *Solanum pennellii* LA716. This work made use of a previously developed collection of introgression lines of *S. pennellii* in *S. lycopersicum* (Eshed and Zamir, 1994), as well as new genome and transcript sequence data for the development of precise polymerase chain reaction (PCR)-based molecular markers. Mapping these markers on a segregating population of 910 F_2 plants allowed the delineation of the *RXopJ4* resistance locus to 190 kb on the long arm of chromosome 6. Chapter 2 presents the examination of three *RXopJ4* candidate genes using an *Agrobacterium*-mediated transient expression assay in *Nicotiana benthamiana*. One of the three candidate genes produced a XopJ4-dependent HR and encodes a putative intracellular serine-threonine protein kinase. Mutational analysis of the RXopJ4 candidate and the XopJ4 effector revealed conserved residues required for recognition. Phenotypic analysis of transgenic tomato plants containing the *RXopJ4* candidate gene is underway.

Chapter 3 illustrates the identification of a preliminary set of core type III effectors among field isolates of *X. perforans*. Markerless gene deletions of core effectors were constructed and may be used to study both the individual and collective virulence contributions of effectors. Several core effectors were also adapted for use as probes in *Agrobacterium*-mediated transient expression assays on various solanaceous hosts, enabling the discovery of new gene-for-gene interactions. Consonant with this work, Chapter 4 catalogs the screening of wild *Solanum* germplasm for resistance to *X. perforans* using disease assays. With the invaluable assistance of the undergraduates Lily Liu and Joshua Chiu, we were able to find nine candidate resistant wild accessions, in some cases quantifying the resistance and showing its dependence on type III effector recognition. Through the identification and analysis of a known resistance gene, in addition to an informed quest for new sources of resistance, these investigations have established a resource for further understanding of plant defense mechanisms and the development of durable resistance to bacterial spot disease of tomato.

Chapter 1:
Fine genetic mapping of *RXopJ4*, a bacterial spot disease resistance locus from *Solanum pennellii* LA716

Introduction

Bacterial spot is among the most significant diseases limiting tomato production throughout tropical and subtropical regions (Jones et al., 2005). Attempts to control the disease have included seed decontamination as well as the application of bactericides such as streptomycin and copper-based compounds. However, streptomycin has been ineffective since the 1960s, and copper resistance has also become nearly ubiquitous among pathogen strains in the field (Obradovic et al., 2008; Stall et al., 2009). While new strategies employing systemic acquired resistance and biocontrol have shown promise (Obradovic et al., 2008), most recent efforts are focused on genetic resistance as a sustainable path to the control of bacterial spot disease.

The causative agent of bacterial spot of both tomato and pepper was once considered a single species, *Xanthomonas campestris* pv. *vesicatoria*. Jones and colleagues (Jones et al., 2004) used DNA:DNA hybridization to reveal four distinct species: *X. euvesicatoria*, *X. vesicatoria*, *X. gardneri*, and *X. perforans*, which differ in their distribution, metabolic properties, and effector repertoires (Potnis et al., 2011). In the southeastern United States, where nearly half of all fresh market tomatoes in the USA are grown (Glades Crop Care, 1999) and where bacterial spot disease is a major problem due to warm, humid conditions, *X. euvesicatoria* was long the predominant species. Since the mid-1990s, however, it has been replaced by *X. perforans* (Jones, Stall, et al., 1998); the competitive advantage of *Xp* is likely due to its production of bacteriocins that antagonize *Xe* strains (Hert et al., 2005). The virulence of xanthomonads and other bacterial pathogens depends on a type III secretion system, which delivers an arsenal of effector proteins directly into plant cells. These effectors function to suppress components of the plant immune system. Some plants, however, have evolved resistance proteins that can directly or indirectly recognize pathogen effectors and activate a more robust defense response known as effector-triggered immunity; this recognition is specific, in that a given resistance protein is triggered by only one or two particular effectors (Jones and Dangl, 2006).

Cultivated tomato (*Solanum lycopersicum*) lacks effective genetic resistance to bacterial spot disease, and breeding efforts have employed related wild species as sources of resistance. These have included both quantitative resistance, which is present in the accession *S. lycopersicum* var. *cerasiforme* PI 114490 (Hutton et al., 2010), and effector-triggered immunity conferred by single dominant loci, such as *Xv3*, found in the breeding line *S. lycopersicum* Hawaii 7981 and in at least two *S. pimpinellifolium* accessions (Wang et al., 2011). Unfortunately, *Xv3* resistance cannot be effectively used in the field in Florida, since many pathogen strains isolated over

the past decade do not contain the recognized *avrXv3* effector gene. This may be due to selective pressure conferred by grape tomatoes, which likely originated from *S. pimpinellifolium* (Stall et al., 2009). The *avrBs2* effector gene, however, is highly conserved among diverse xanthomonads (Kearney and Staskawicz, 1990) and has been shown to play a role in virulence on tomato (Zhao et al., 2011). The *Bs2* resistance gene from the pepper species *Capsicum chacoense* is effective against bacterial spot in transgenic tomato plants (Tai et al., 1999; Horvath et al., 2012).

A durable disease resistance strategy will ideally combine quantitative sources of resistance with effector-triggered immunity. A key approach to achieving durability will be the use of multiple resistance genes recognizing conserved pathogen effectors that play a role in virulence, in order to minimize the possibility for pathogen strains to undergo mutation of the recognized effector with no cost to fitness. Previous work has shown that the *Xp* effector XopJ4 [formerly designated AvrXv4 (Potnis et al., 2011)], a putative SUMO protease, plays a role in pathogen virulence (Roden, Eardley, et al., 2004). A preliminary survey of *Xp* field isolates from throughout the state of Florida (See Chapter 3) suggests that the *xopJ4* effector gene is conserved in the pathogen, making it a promising target for durable resistance. The XopJ4 effector is recognized by a dominant resistance locus, *RXopJ4*, in the wild accession *Solanum pennellii* LA716. The locus was previously referred to as *Xv4* and was reported to map to a ~20-cM segment on chromosome 3 (Astua-Monge et al., 2000). Since the taxonomy of the *Xanthomonas* genus continues to evolve, and there is overlap among type III effector repertoires of different species, we have chosen to base the name of this resistance locus, *RXopJ4*, on the recognized pathogen effector (XopJ4), rather than on the pathogen species.

In this study, we sought to further refine the mapping of the *RXopJ4* locus to identify cosegregating markers that would aid in the cloning of the resistance gene or genes. Initially, using a collection of introgression lines of *S. pennellii* in *S. lycopersicum*, we determined that the resistance locus lies within a 4.2-Mb region on the long arm of chromosome 6. We developed or adapted fourteen molecular markers across this region and used these for fine mapping on an F_2 population. Analysis of recombinant F_3 plants showed that a 190-kb genomic region cosegregates with resistance. Our markers can be used for the detection of resistant recombinants and further fine mapping, and for the identification of candidate genes with the eventual goal of durable resistance to bacterial spot disease of tomato.

Results

Phenotypic analysis of XopJ4-dependent hypersensitive response

In order to determine the chromosomal location of the *RXopJ4* resistance locus from *S. pennellii*, we employed a collection of fifty introgression lines (ILs) from the cross between *S. pennellii* LA716 and *S. lycopersicum* M82 (Eshed and Zamir, 1994). Each line contains a homozygous introgression of *S. pennellii* over one segment of one chromosome, while the rest of its genome is *S. lycopersicum*; introgressed segments

are overlapping. Each IL was inoculated with the compatible strain *Xe* 69-1 containing the empty vector pLAFR6 or pLAFR6 *xopJ4* (Figure 1-1). IL 6-2 and its sub-line IL 6-2-2 exhibited a XopJ4-dependent hypersensitive response (HR), a rapid cell death associated with pathogen resistance (Jones and Dangl, 2006). However, IL 6-1, which contains a *S. pennellii* introgression that partially overlaps with those of ILs 6-2 and 6-2-2 (Figure 1-2), did not exhibit HR. Furthermore, ILs 3-3 and 3-4, which Astua-Monge et al. (2000) identified as containing the *RXopJ4* locus, did not exhibit XopJ4-dependent HR. The XopJ4-dependent HR of ILs 6-2 and 6-2-2, but not IL 6-1, indicated that the *RXopJ4* locus was within a 4.2-Mb region on the long arm of chromosome 6 (Figure 1-2).

Cosegregation of HR and resistance with *Sp* introgression in an F_2 mapping population

The ILs 6-2 and 6-2-2 proved undesirable as resistant parents of an F_2 mapping population due to a linkage drag that conferred poor fertility and germination, small leaves, and necrosis of leaves upon exposure to cold or low light intensity, as well as an autogenous necrosis as the plants grew older. Instead, a resistant seventh outcross line from crosses between various commercial tomato lines and *Sp* LA716 was used (See Materials and Methods). This line displayed a XopJ4-dependent HR (Figure 1-1) but was missing part of the *S. pennellii* introgression present in ILs 6-1, 6-2, and 6-2-2 (Figures 1-2, 1-3) and, importantly, also lacked the necrotic phenotype of ILs 6-2 and 6-2-2. A mapping F_2 population was generated from a cross between FL8000 and this outcrossed line (designated *RXopJ4* 8000 OC_7). To assess the segregation of HR in this mapping population, we inoculated a collection of seventeen F_2 plants with *Xe* 69-1 containing pLAFR6 or pLAFR6 *xopJ4*, and found that XopJ4-dependent HR segregated as a single locus (Figure 1-4). Plants homozygous for *Sp* LA716 at the *RXopJ4* locus exhibited strong HR, while plants homozygous for FL8000 did not exhibit HR. Heterozygous plants occasionally displayed a strong HR, but most produced weak or no HR; only some heterozygous plants were disease-resistant (data not shown), suggesting that *RXopJ4* resistance is semi-dominant.

To verify the disease resistance of the *RXopJ4* 8000 OC_7 parent line, we conducted a bacterial growth assay on this line, the susceptible parent FL8000, and *Sp* LA716. In order to assess the XopJ4 dependence of the resistance, we inoculated each line with the bacterial strains *Xp* 4B-WT, 4BΔ*xopJ4*, and 4BΔ*xopJ4*/comp. *Sp* LA716 and *RXopJ4* 8000 OC_7 restricted the growth of *Xp* 4B-WT (Figure 1-5). This resistance was dependent on XopJ4 recognition, since these lines were susceptible to the 4BΔ*xopJ4* deletion strain; complementation of this strain restored recognition by the resistant lines. The *Sl* FL8000 line, however, was susceptible to *Xp* 4B-WT, 4BΔ*xopJ4*, and 4BΔ*xopJ4*/comp strains. The overall lower levels of bacterial growth seen in *Sp* LA716 are likely due to factors other than the *RXopJ4* resistance.

Mapping of the *RXopJ4* resistance locus

Initially, F_2 plants from a cross between *Sl* FL7060 and IL 6-2-2 were scored at the genetic markers TG352, CT83, TG164, and CT204. This analysis revealed that the marker positions were not as previously mapped; for instance, among 347 F_2 individuals, no recombinants were found between TG352 and TG164, which are 12.9 cM apart on the Tomato-EXPEN 1992 map (Figure 1-6a). This could be partially explained by a suppressed homeologous recombination rate between FL7060 and the relatively short *Sp* introgression in IL 6-2-2 (Canady et al., 2006), but could also be due to the difficulty of accurately genotyping with RFLP markers. To remedy the discrepancy between the 1992 linkage map and the observed recombination rate, and to avoid the possible suppressed recombination resulting from a short *Sp* introgression, we mapped five CAPS markers—TG118, CT83, C2_At1g21640, TG352, and TG164—on a *Sl* M82 x *Sp* LA716 F_2 population. This revealed, in particular, a location for the marker TG352 that differed from the EXPEN-1992 and 2000 linkage maps (Figure 1-6b). At the same time, a fully assembled version of the tomato genome sequence became available (Bombarely et al., 2011; Sato et al., 2012). This provided a more accurate picture of the distances between markers, and also showed that TG164 and TG352 were in the opposite order from the M82 x *Sp* LA716 linkage map—this order was based on a single recombinant (Figure 1-6b,c).

Subsequently, the markers CT83, TG352, and J385 were scored on 910 individuals from an FL8000 x *RXopJ4* 8000 OC_7 F_2 population; 27 recombinant plants were identified—a recombination rate of ~3% across the 4.8 Mb spanned by these three markers. All F_2 plants were recombinant only on one copy of chromosome 6, and thus were heterozygous on one side of the recombination breakpoint. Since, as previously discussed, the resistance phenotype was inconsistent in heterozygotes, the F_3 progeny of these plants were collected and homozygous recombinants were scored for both HR and disease resistance. The genotypes and phenotypes of key recombinant individuals are shown in Figure 1-3. The left boundary of the *RXopJ4* resistance locus is defined by the recombinants 5C3 and 18B10, which have a crossover from the *Sp* genotype to the *Sl* genotype between J350 and 06g060670, and are susceptible. The right boundary is defined by the recombinant 60B2, which has a crossover from the *Sp* to the *Sl* genotype between J351 and J352 and is resistant. The *Sp* introgressions in these recombinants indicate that the *RXopJ4* locus lies within a 190-kb region between the markers J350 and J352.

Discussion

In the present study, we have defined the *RXopJ4* resistance locus from *S. pennellii* LA716 to a 190-kb segment on the long arm of chromosome 6. We first narrowed the resistance to a 4.2-Mb region within the *Sp* introgressions of ILs 6-2 and 6-2-2. Then, we used a combination of previous linkage maps, our own linkage map of the

RXopJ4 region, and new molecular markers to map the resistance on a segregating FL8000 x *RXopJ4* 8000 OC_7 F_2 population; this enabled fine mapping to a region of 190 kb. Although this resistance locus was originally reported to map to chromosome 3 (Astua-Monge et al., 2000), upon re-characterization of the *S. pennellii* ILs, we were unable to reproduce this result (Figure 1-1).

The emergence of an annotated genome sequence for *S. lycopersicum* and preliminary genome and mRNA sequence data for *S. pennellii* greatly facilitated the development of precise molecular markers for fine mapping. Recently, the mapping of both a bacterial spot resistance quantitative trait locus (QTL) from *S. lycopersicum* var. *cerasiforme* PI 114490 (Hutton et al., 2010) and a major resistance gene, *Rx4,* from *S. pimpinellifolium* PI 128216 (Pei et al., 2011), was also aided by genome sequence data. The sequencing of more *Solanum* accessions and the identification of SNPs from cultivated varieties will continue to accelerate the process of marker development and thus the mapping of disease resistance and other desirable traits in the future.

Linkage drag is a common problem resulting from the introgression of disease resistance into crop plants by conventional breeding, and has been characterized by Lewis and colleagues (2007) in backcrossed tobacco lines containing the *N* gene, which confers resistance to tobacco mosaic virus. Notably, transgenic lines expressing the *N* gene exhibited significant increases in yield and cash return compared to backcrossed resistant lines. Similarly, the *S. pennellii* LA716 introgression lines 6-2 and 6-2-2 contain the *RXopJ4* resistance locus, but suffer from a linkage drag that confers low fruit yield, small fruit, and an autogenous leaf necrosis. A seventh outcross resistant line showed improved traits and was suitable for mapping of the *RXopJ4* locus, but may still have a disadvantage in the field when compared to the parent line FL8000. Transgenic approaches to disease resistance may mitigate the effects of linkage drag seen in backcrossed lines; alternatively, because of barriers to the acceptance of transgenic crops, backcrossed resistant lines can be developed that contain a minimal genomic region from the wild accession.

Another common feature of disease resistance genes is incomplete dominance. This has been observed for the *Xv3* and *Rx4* resistance genes, which were identified in different accessions but map to the same region of chromosome 11. Based on allelism tests that failed to identify susceptible F_2 plants from a cross between *Xv3* and *Rx4* resistant parents, the genes are either closely linked or are alleles of the same gene (Wang et al., 2011). While *Xv3* heterozygous plants developed HR more slowly than homozygous resistant plants (Wang et al., 2011), *Rx4* heterozygotes showed similar disease ratings to homozygous plants under field conditions (Robbins et al., 2009). *RXopJ4* heterozygotes exhibited slow, weak, or nonexistent HR, and also showed inconsistent phenotypes in disease assays. This could be problematic in the field, since hybrids are usually grown for their increased vigor. However, the heterozygous resistance phenotype may be improved in transgenic plants or in a different genetic background; moreover, the additive effect of multiple resistance genes in a single line may outweigh any reduced resistance

due to heterozygosity.

Once the *RXopJ4* gene has been identified, it can be combined with *Bs2,* perhaps in an elite line containing the bacterial spot resistance QTL identified by Hutton et al. (2010), to produce a line that we expect will possess durable resistance to *X. perforans* in the southeastern United States and other regions where similar strains predominate. Next-generation sequencing now makes it possible to survey pathogen strains from the field for their type III effector repertoires and other virulence components. This approach will aid the development of durable resistance, as the search for new sources of resistance can be targeted to resistance genes that recognize the most conserved pathogen effectors (Bart et al., 2012). Surveying pathogen populations from different geographic areas could also guide strategies for both broadly applicable and region-specific disease resistance. Genetic resistance to bacterial spot disease has heretofore met with limited success in the field. Now, though, the use of genome sequence data for both pathogen and host will enable targeted resistance through precise marker-assisted selection and gene identification. We anticipate that these developments will help to accelerate the process of breeding and gene discovery to produce durably resistant varieties.

Materials and Methods

Plant materials and DNA extraction

Solanum pennellii LA716 was obtained from Charles M. Rick at UC Davis. Jay W. Scott at the Gulf Coast Research and Education Center of the University of Florida provided the *S. lycopersicum* line FL8000, and the M82 line was received from Roger T. Chetelat at UC Davis. *S. pennellii* introgression lines were obtained from the C.M. Rick Tomato Genetics Resource Center at UC Davis, except IL 6-1, which was provided by Zachary Lippman at Cold Spring Harbor Laboratory. The *RXopJ4* 8000 OC_7 resistant parent was produced from an initial cross between *Sp* LA716 and *Sl* H7998 (Astua-Monge et al., 2000), followed by five generations of outcrossing resistant F_2 plants to *Sl* FL216 (a seventh backcross line from the cross between *Sl* FL7060 and *S. pimpinellifolium* PI 128216 containing *Xv3* resistance), and then two outcrosses to FL8000, which also contains *Xv3* resistance. The eighth F_1 generation and F_2 mapping population were generated from another outcross to FL8000; 910 plants from this F_2 population were used for fine mapping of the *RXopJ4* resistance locus. Seeds were sown directly in soil and allowed to germinate and grow for 2 weeks in a growth chamber at 24°C with 12 h light/dark cycle. Seedlings were then transferred to a greenhouse with supplemental lighting.

For DNA extraction, ~60 mg young leaf tissue was collected per plant. The extraction protocol was adapted from one provided by Lauren Headland at UC Davis. Briefly, tissue was frozen at -80 °C overnight. Tissue was then disrupted in a BeadBeater (BioSpec Products, Bartlesville, OK, USA) with a 3-mm glass bead and extraction buffer containing 200 mM Tris–HCl (pH 7.5), 250 mM NaCl, 25 mM EDTA,

and 14 μg/ml RNase A. Samples were then treated with SDS followed by extraction with phenol:chloroform:isoamyl alcohol (25:24:1). After phase extraction, DNA was precipitated with isopropanol, washed with 70% ethanol, and resuspended in 1 mM Tris pH 8.

Bacterial strains, inoculum preparation and inoculation

Bacterial strains used in this study are described in Table 1-1. The *Xp* 4BΔ*xopJ4* strain was constructed using the suicide vector pLVC18 containing an upstream fragment spanning 1,054 bp of the xopJ4 promoter plus 33 bp of its open reading frame (ORF), and a 1,108-bp downstream fragment beginning at 7 bp past the stop codon; a BamHI restriction site was introduced between the two fragments. A double homologous recombination event resulted in deletion of the *xopJ4* ORF, which was confirmed by PCR and Sanger sequencing. The 4BΔ*xopJ4* deletion strain was complemented by selecting for a single crossover with pLVC18 containing 846 bp of the *xopJ4* promoter plus the ORF.

For inoculation, bacteria were grown on nutrient yeast glycerol agar (NYGA) supplemented, as appropriate, with 100 μg/ml rifampicin, 25 μg/ml kanamycin, and 10 μg/ml tetracycline, and were incubated at 28°C for 48–72 h. Cells were then washed from agar plates with 1 mM $MgCl_2$ and concentration was adjusted as necessary. Inoculation was performed by leaf infiltration using a 1-ml needleless syringe. For HR, two sections of each leaflet were infiltrated with suspensions of *Xe* 69-1 containing pLAFR6 or pLAFR6 *xopJ4*, both at 3 x 10^8 cfu/ml (OD_{600} = 0.3). For disease assays, whole leaflets were infiltrated with *Xp* 4B-WT, 4BΔ*xopJ4*, or 4BΔ*xopJ4*/comp at ~10^4 cfu/ml. For disease assays, inoculated plants were placed in a growth chamber at 24°C with 12 h light/dark cycle. For HR assays, inoculated plants were placed either in a growth chamber or in a greenhouse. HR was assessed 24–48 h after inoculation, while disease was assessed 10–14 days after inoculation. For the growth assay, four to six 0.8-cm^2 punches were taken from leaves of each genotype at each time point and homogenized in 1 mM $MgCl_2$ in a BeadBeater; appropriate dilutions were plated on NYGA supplemented with 50 μg/ml rifampicin and 50 μg/ml cycloheximide (with 12.5 μg/ml kanamycin for the complemented mutant strain).

Marker development and PCR conditions

For mapping of the *RXopJ4* resistance locus, we used a total of 14 molecular markers spanning the ~27 cM or 7.3 Mb from TG118 to CT204 (Table 1-2; Figures 1-2, 1-3, 1-6) on the long arm of chromosome 6. Thirteen of these markers are cleaved amplified polymorphic sequence (CAPS) markers, for which a short (<1,000 bp) sequence is amplified by polymerase chain reaction (PCR) and then digested with a restriction enzyme to yield product sizes that differ between the two parent genotypes—in this case, *Sl* and *Sp* (Figure 1-7). One marker, SLM 6-15, is a simple sequence repeat (SSR) marker; it spans a region of tandem TA repeats that is 39

nucleotides longer in *Sl* than in *Sp* and can thus be visualized directly by agarose gel electrophoresis without restriction digestion (Figure 1-7).

The markers TG118, CT83, C2_At1g21640, TG164, TG352, and CT204 were derived from RFLP or CAPS markers used in previous mapping studies (Figure 1-6). Sequences, and in some cases CAPS protocols, for these markers were obtained from the Sol Genomics Network (Bombarely et al., 2011). Primers were optimized for PCR and CAPS visualization and are listed in Table 1-2. 06g060670 is derived from a predicted gene model in the International Tomato Annotation Group (ITAG) 2.3 genome annotation release (Sato et al., 2012). J350, J351, J352, J353, J366, and J385 markers were adapted from CAPS marker predictions based on preliminary *Sl* and *Sp* mRNA sequence information (manuscript in preparation). The SLM 6-15 SSR marker protocol was taken directly from Geethanjali et al. (2010). *S. pennellii* genome assemblies were provided by Anthony Bolger, Alisdair Fernie, and Björn Usadel at the Max Planck Institute for Molecular Plant Physiology, Golm, Germany.

Primers were designed using Amplify (Bill Engels, University of Wisconsin, USA) and CLC Main Workbench (CLC bio, Aarhus, Denmark) and were obtained from Integrated DNA Technologies (Coralville, IA, USA). PCR was conducted using Klentaq LA (DNA Polymerase Technology, St. Louis, MO, USA) according to the manufacturer's protocol. Initial denaturation was at 94°C for 5 min, followed by 35 cycles of 5s at 94°C, 30s at 50–60°C (Table 1-2), and 1 min at 68°C, and a final extension at 68°C for 7 min. Restriction digestion was carried out for 2 h at the appropriate temperature with enzymes and buffers purchased from New England Biolabs (Ipswich, MA, USA). PCR and restriction digestion were performed in a programmable thermal controller (PTC-100; MJ Research, Inc., Watertown, MA, USA). PCR/restriction products were separated on 3% (SLM 6-15 and J351) or 2.5% agarose gels (all other markers) and stained with ethidium bromide (SLM 6-15 and J351) or GelRed (Phenix Research Products, Candler, NC, USA) (all other markers; GelRed stain was included in the loading dye).

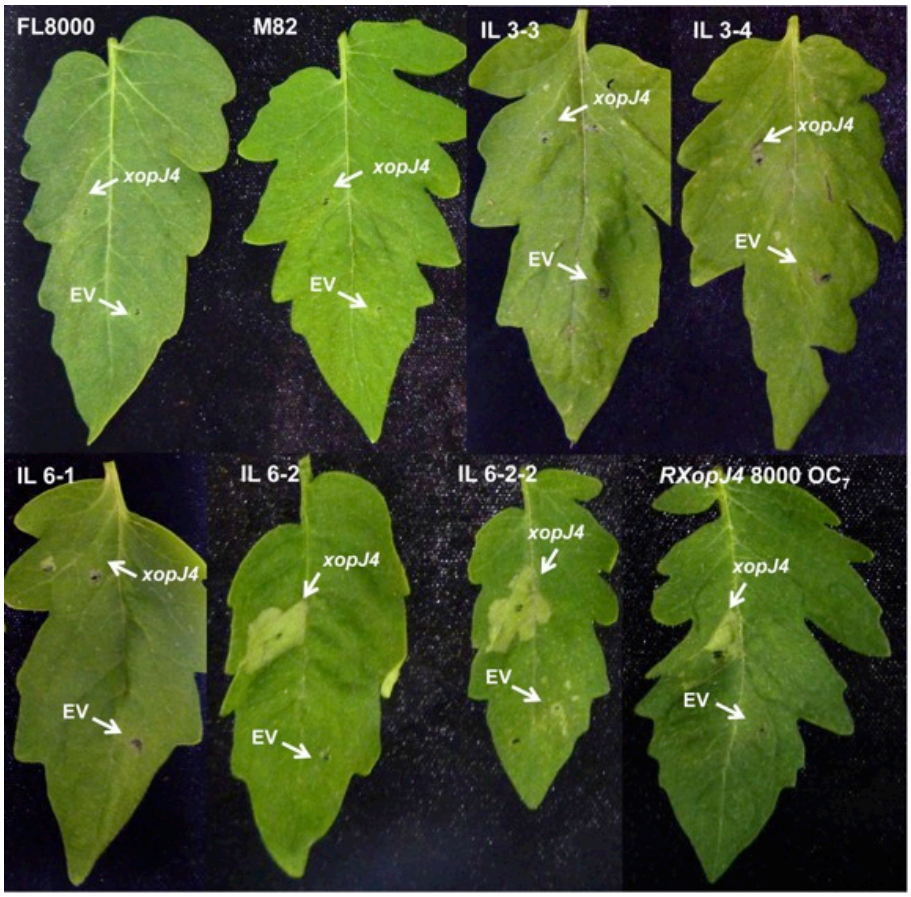


Figure 1-1. The effector XopJ4 elicits a hypersensitive response in the *S. pennellii* introgression lines (ILs) 6-2 and 6-2-2 and *RXopJ4* 8000 OC_7. Plants were inoculated with the compatible strain *Xe* 69-1 carrying pLAFR6 empty vector (EV) or pLAFR6 *xopJ4* at a concentration of ~3 x 10^8 cfu/ml. Photographs were taken at 24 h post inoculation.

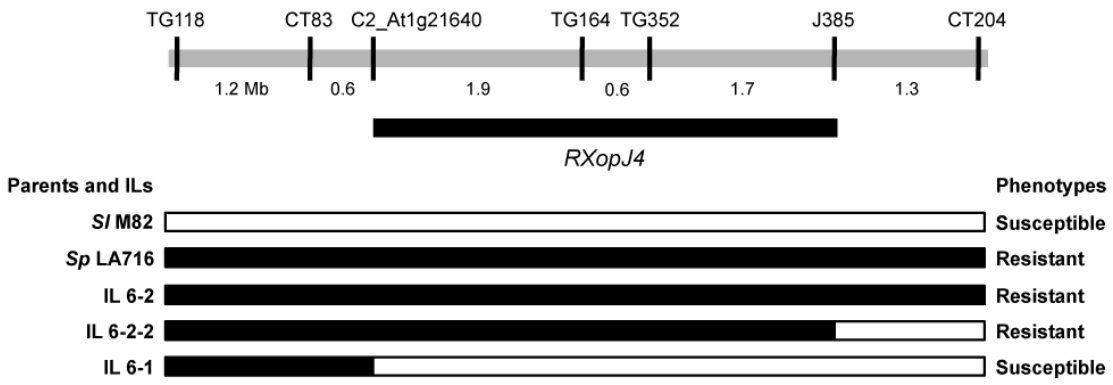


Figure 1-2. Initial mapping of the *RXopJ4* resistance locus using *S. pennellii* introgression lines (ILs), showing molecular marker genotypes and phenotypes of parent lines and ILs. White bars indicate the marker genotype of *S. lycopersicum* M82 and black bars indicate *S. pennellii* LA716. Distances between markers are not drawn to scale but are indicated in megabases (Mb). A black bar indicates the 4.2-Mb region containing the *RXopJ4* locus.

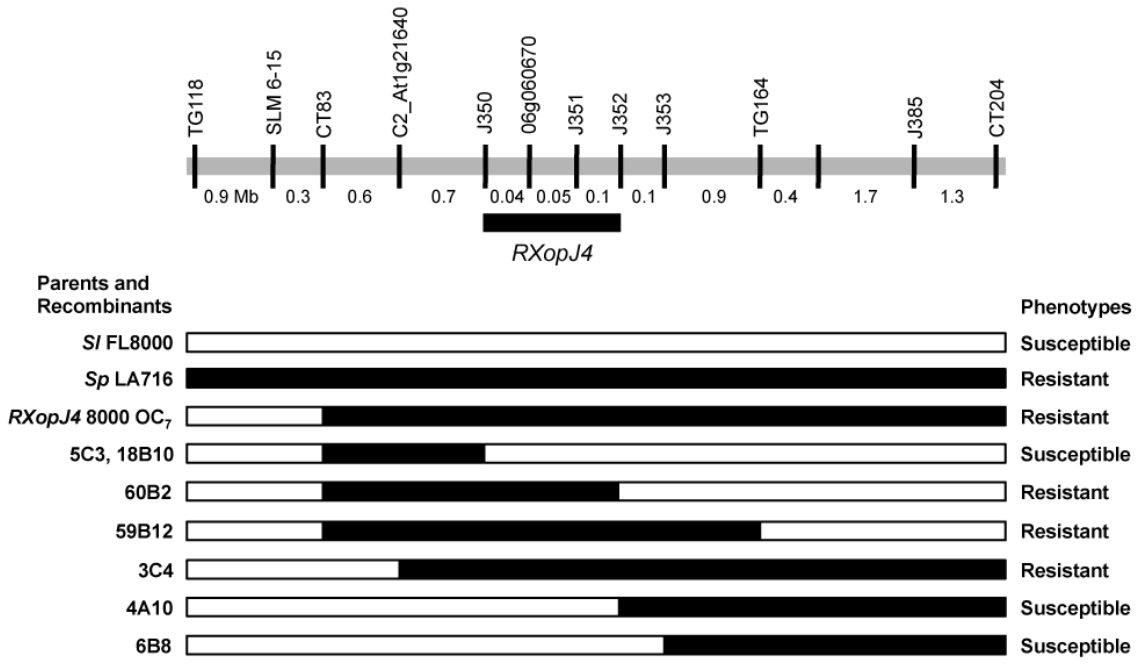


Figure 1-3. Fine mapping of the *RXopJ4* resistance locus using an FL8000 x *RXopJ4* 8000 OC_7 F_2 population, showing molecular marker genotypes and phenotypes of parent lines and recombinant individuals. White bars indicate the marker genotype of *S. lycopersicum* FL8000 homozygotes and black bars indicate *S. pennellii* LA716 homozygotes. Recombinant phenotypes were assessed in homozygous individuals from the F_3 generation. Distances between markers are not drawn to scale but are indicated in megabases (Mb). A black bar indicates the 190-kb region containing the *RXopJ4* locus.

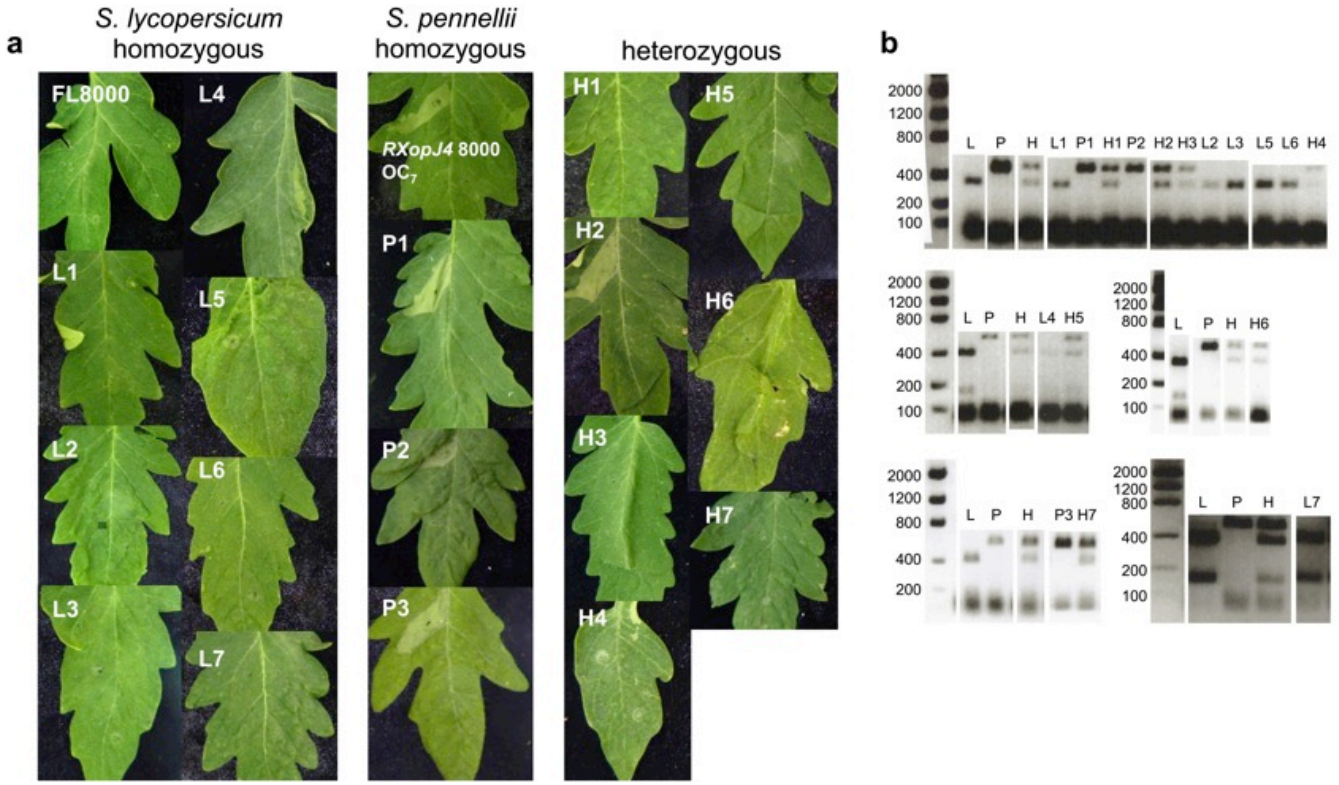


Figure 1-4. Cosegregation of XopJ4-dependent HR with the genetic marker C2_At1g21640 in 17 plants from an FL8000 x *RXopJ4* 8000 OC_7 F_2 population. **a** XopJ4-dependent HR. One leaflet of each plant was inoculated with the compatible strain *Xe* 69-1 carrying pLAFR6 empty vector (upper left) or pLAFR6 *xopJ4* (lower right) at a concentration of ~3 x 10^8 cfu/ml. Photographs were taken at 24 h post infiltration. FL8000 and *RXopJ4* 8000 OC_7 lines are shown as susceptible and resistant controls, respectively. **b** Plants shown in (a) scored at the genetic marker C2_At1g21640. Sizes of bands in ladder are indicated in base pairs. *S. lycopersicum* homozygous (L), *S. pennellii* homozygous (P), and heterozygous (H) controls are shown for each gel. Note that restriction products do not run exactly true to size due to the GelRed nucleic acid stain added to the loading dye; this allows for clearer band visualization than staining with ethidium bromide.

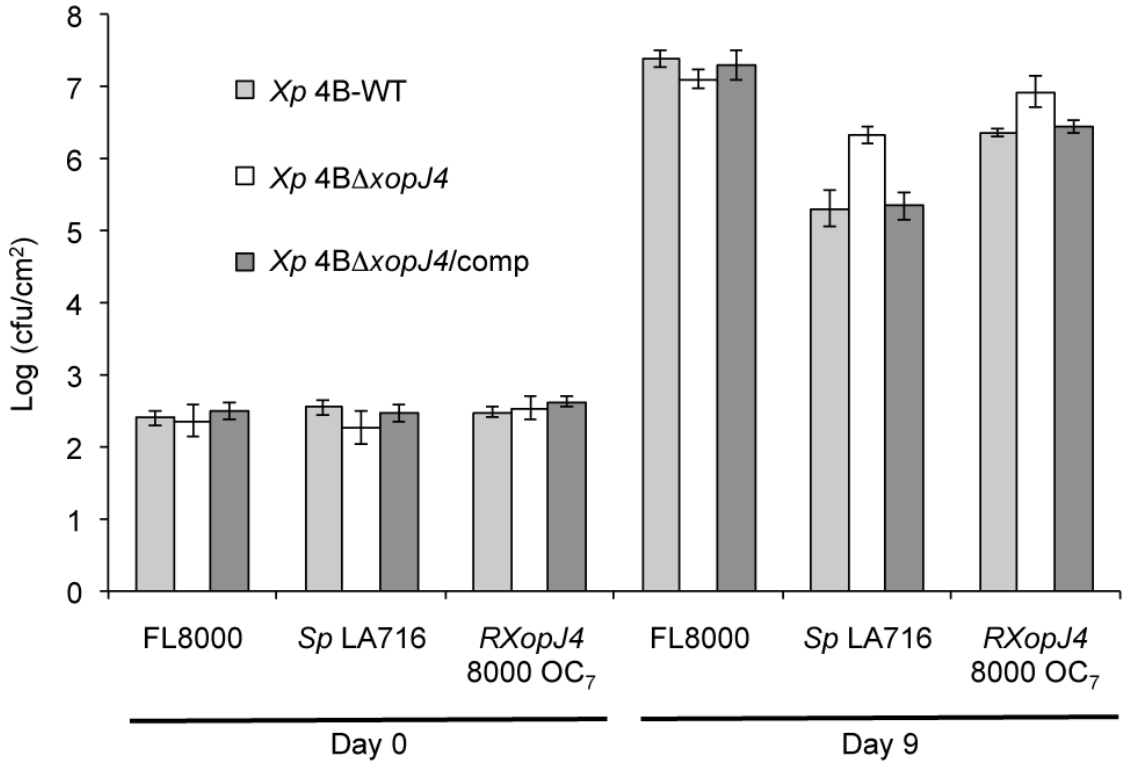


Figure 1-5. *Solanum pennellii* LA716 and *RXopJ4* 8000 OC_7 display XopJ4-dependent resistance to *X. perforans* infection. Whole leaflets of *Sl* FL8000, *Sp* LA716, or *RXopJ4* 8000 OC_7 plants were infiltrated with *Xp* 4B-WT (light gray bars), 4BΔ*xopJ4* (white bars), or 4BΔ*xopJ4*/comp (dark gray bars). Bacteria were syringe-infiltrated into leaves at 10^4 cfu/ml. Bacterial counts were determined on the day of infiltration (Day 0) and 9 days post infiltration (Day 9). Error bars indicate the standard deviation from the mean of 4–6 samples.

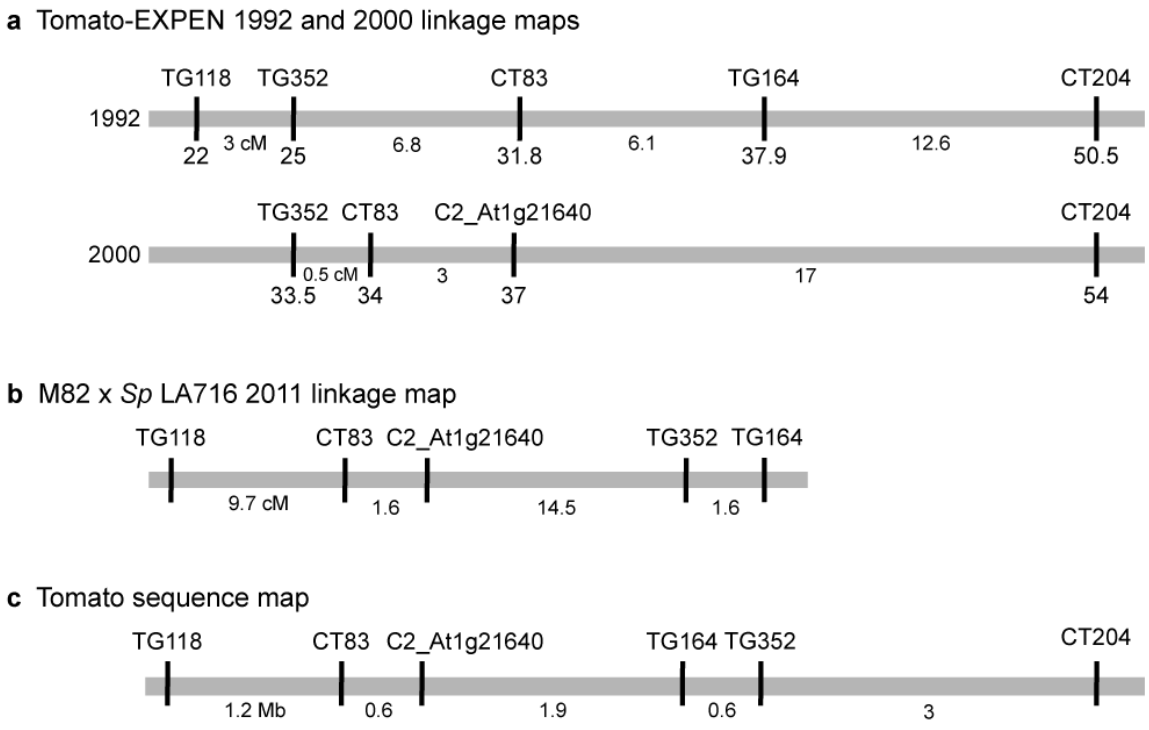


Figure 1-6. Maps of the *RXopJ4* region on tomato chromosome 6. Marker names are on the top with map positions and distances in centimorgans (cM, a, b) or megabases (Mb, c) between each marker on the bottom. Map distances are not to scale. **a** Linkage maps based on F_2 individuals from a cross between *S. lycopersicum* cv. VF36 and *S. pennellii* LA716. The Tomato-EXPEN 1992 map is based on RFLP markers and 67 F_2 individuals, while the 2000 map is based on RFLP and CAPS markers and 80 F_2 individuals; the 2000 map also includes conserved ortholog set (COS) markers such as C2_At1g21640 (Tanksley et al., 1992; Fulton et al., 2002). **b** Linkage map from this study based on CAPS markers and 62 F_2 individuals from a cross between *S. lycopersicum* cv. M82 and *S. pennellii* LA716. **c** Sequence map based on tomato genome sequence release SL2.40 (Sato et al., 2012).

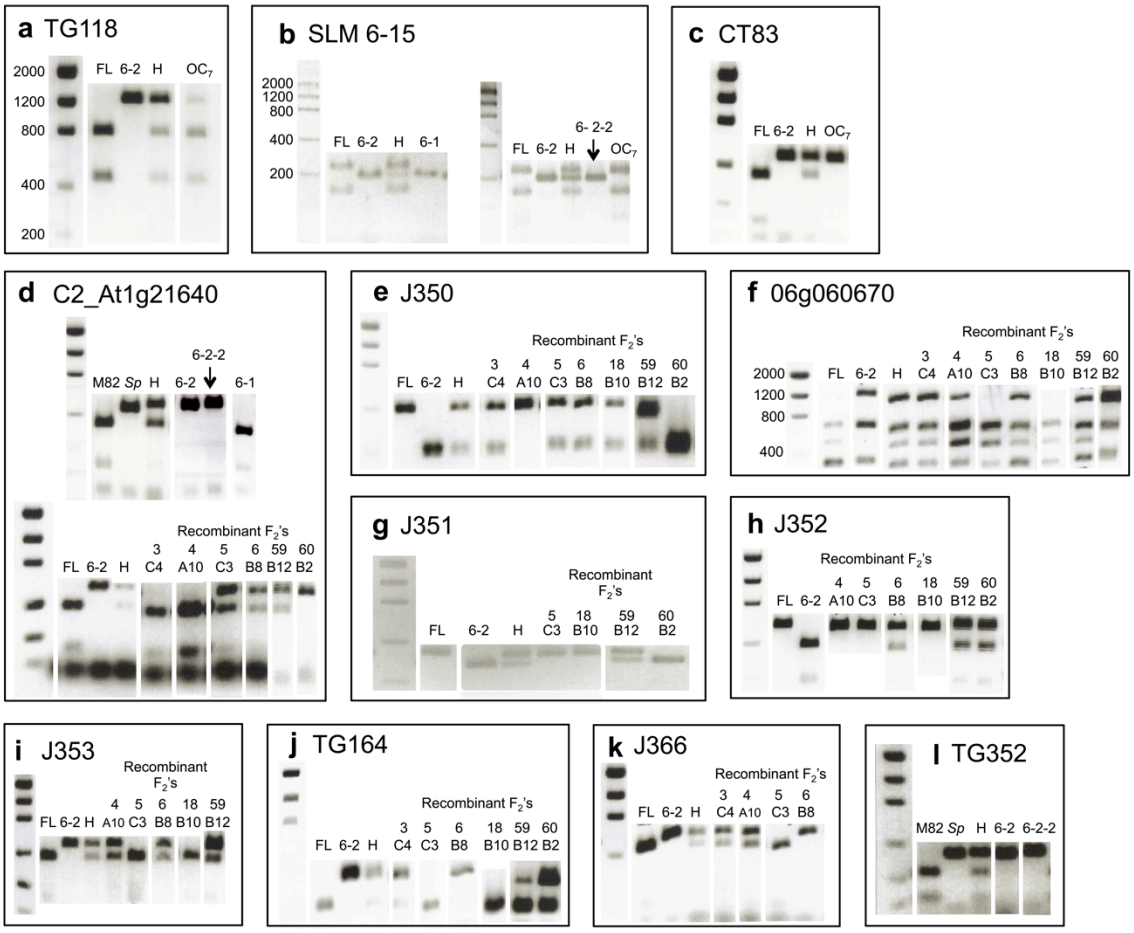


Figure 1-7. Agarose gel electrophoresis of CAPS (**a**, **c-l**) and SSR (**b**) genetic markers, highlighting scores of *Sl* FL8000 (FL) and M82; *Sp* LA716 (*Sp*); ILs 6-1, 6-2, and 6-2-2; *RXopJ4* 8000 OC_7; heterozygotes (H) from crosses M82 x *Sp* LA716 or FL8000 x *RXopJ4* 8000 OC_7; and key recombinant F_2 individuals from an FL8000 x *RXopJ4* 8000 OC_7 mapping population (3C4, 4A10, 5C3, 6B8, 18B10, 59B12, and 60B2). Band sizes in ladder are indicated in base pairs for (**a**), (**b**), and (**f**); all gels include the same ladder. Note that in (**a**), (**c-f**), and (**h-l**), restriction products do not run exactly true to size due to the GelRed nucleic acid stain added to the loading dye; this allows for clearer band visualization than staining with ethidium bromide. Gels in (**b**) and (**g**) were stained with ethidium bromide.

Table 1-1 Bacterial strains used in this study

Strain	Relevant characteristics
Xanthomonas euvesicatoria 69-1	Rif^R
X. euvesicatoria 69-1 (EV)	Rif^R, Tc^R; contains the empty vector pLAFR6
X. euvesicatoria 69-1 (*xopJ4*)	Rif^R, Tc^R; pLAFR6 with *xopJ4* subclone including native promoter
X. perforans 4B-WT	Rif^R
X. perforans 4BΔ*xopJ4*	Rif^R; *xopJ4* deletion mutant
X. perforans 4BΔ*xopJ4*/comp	Rif^R, Km^R; *xopJ4* deletion mutant complemented with pLVC18 *xopJ4* including native promoter

Rif rifampicin, *Tc* tetracycline, *Km* kanamycin

Table 1-2 Molecular marker information for mapping of the *RXopJ4* resistance locus in *Solanum pennellii* LA716

Marker name	Genome position[a]	Marker type	Primer sequences (5'-3')	Annealing temperature (°C)	Restriction enzyme	Product size (bp)		Reference
						Sl FL8000	*Sp* LA716	
TG118	32556111.. 32556711	CAPS	f: TACTGTCCATCCCTCATT r: GATATGGAAGTAGATGTGTC	50	SalI	330/271	601	Bombarely et al. 2011, This study
SLM 6-15	33442726.. 33442965	SSR	f: GGATTTCAGCTGCCTACTGAG r: TTCGGAGAACATAATAGGGGTTT	55	_	240	201	Geethanjali et al. 2010
CT83	33748762..33749158	CAPS	f: GACTGCAGAGGTTAACTGAC r: CGAGGCTTCCTCTCTGATG	60	ApoI	240/157	397	Bombarely et al. 2011, This study
C2_At1g21640	34364263..34364672	CAPS	f: AGAAAAGTCATCCATGGAAACAACAC r: TGGCCACAATGACACCATCACCTTG	55	ApoI	290/120	410	Bombarely et al. 2011, This study
J350	35036517..35036818	CAPS	f: CTGCTGGAATTGTAATTATTCACG r: TTGAGCATAAACAGTCCAAAACAT	55	HinfI	302	159/143	This study
06g060670	35078590..35079503	CAPS	f: CACCGGACACGAAGTATAAGACA r: CCAAACCACCAACCCAAA	53	HindIII	538/376	911	Bombarely et al. 2011, This study
J351	35127552..35127921	CAPS	f: CACCCTGAAAATCATCCAAAGCAC r: ACCCACACCCTCTATATAAA	55	HinfI	317/53	268/53/49	This study
J352	35241322..35241736	CAPS	f: AAAATTGGAGGGACAACAAATAAA r: AAGGAAGTTCAAGAGCTAAGGTCA	55	HincII	415	283/132	This study
J353	35359974..35360331	CAPS	f: GAGAGAAAATGGGATAACCTTCAA r: CACTTATTATCAGGAGGAGGAGGA	50	HinfI	268/90	358	This study
TG164	36270932..36271174	CAPS	f: GGTATAGTTTCATGCACATC r: CCTGTAGGTTTCTTTGATTC	50	ApoI	127/116	243	Bombarely et al. 2011, This study
J366	36676727..36677181	CAPS	f: CTTCACTTCTTGCCTGAGATGTTA r: AGATCAATTACCGATATCACCCAT	55	HinfI	360/95	455	This study
TG352	36837890..36838115	CAPS	f: GAGGGATGTCTCATGTAG r: CATATGTCCTGCTAAAAGAAG	50	Tsp509I	149/77	226	Bombarely et al. 2011, This study
J385	38589739..38590229	CAPS	f: GGCAAGTGAAGGTGTAAAAGTTCT r: GTTCAAAATTAAACAGAACGTCCC	55	HinfI	340/151	237/151/103	This study
CT204	39834480..39834861	CAPS	f: TCCCTCCTCAGATTTAGCTT r: TTGGTGCCCTCTTCTTTTT	50	BamHI	382	196/186	Bombarely et al. 2011, This study

[a] Coordinates on the SL2.40ch06 genome assembly (Sato et al. 2012)

Chapter 2:
Identification of a putative *RXopJ4* kinase gene and insights into the biochemical activities of XopJ4 and RXopJ4

Introduction

Bacterial spot disease, caused by four species of the genus *Xanthomonas*, is a major factor limiting tomato production worldwide and cannot be effectively controlled using pesticides or antibiotics (Stall et al., 2009). Xanthomonads subvert plant defenses by secreting an arsenal of type III effector proteins. These effector proteins are delivered into plant host cells, where they interfere with immune signaling pathways. This enables the bacteria to proliferate in the leaf apoplast, cause disease and eventually spread to new host plants. However, the arms race between plants and microbial pathogens has led to the evolution of effector-triggered immunity, classically known as gene-for-gene resistance (Flor, 1971). Resistance (*R*) genes encode proteins that are able to directly or indirectly recognize pathogen effector proteins and mount a robust defense response that restricts pathogen growth (Jones and Dangl, 2006).

Cultivated tomato (*Solanum lycopersicum*) does not contain *R* genes against bacterial spot disease. The *Bs2* gene from the pepper species *Capsicum chacoense*, however, has been deployed in the field to control bacterial spot disease of pepper and is also functional in transgenic tomato plants (Tai et al., 1999; Stall et al., 2009; Horvath et al., 2012). While *Bs2* resistance has been effective in the field over several seasons, previous work has shown that growing *Bs2*-containing pepper plants places selective pressure on pathogen populations. Various mutations of the recognized effector gene, *avrBs2*, have been identified in bacterial strains that have overcome *Bs2* resistance. While some of these mutations also resulted in reduced virulence, others appeared not to have a significant effect on bacterial growth in planta (Swords et al., 1996; Gassmann et al., 2000). Because *Xanthomonas* pathogens have the ability to undergo mutation of a recognized effector gene with minimal loss of virulence, the use of a single *R* gene in a crop plant is unlikely to be durable over the long term. A durable resistance strategy should employ multiple *R* genes recognizing conserved effectors that contribute to pathogen virulence.

To this end, we previously identified a disease resistance locus, *RXopJ4*, from the wild accession *Solanum pennellii* LA716 and mapped this locus to a 190-kb interval on the long arm of chromosome 6 (Chapter 1; Sharlach et al., 2012). *RXopJ4* resistance depends on recognition of the conserved pathogen effector XopJ4, which is required for full virulence of *Xanthomonas perforans* on tomato (Roden, Eardley, et al., 2004). The reasons for undertaking *RXopJ4* resistance gene identification are twofold: (1) to aid the development of a durable resistance strategy to bacterial spot disease and (2) to expand our understanding of plant disease resistance mechanisms.

Elucidating the mechanism of XopJ4 recognition is of particular interest, as XopJ4 is a member of the large and widely distributed YopJ superfamily of type III bacterial effectors, whose founding member is YopJ from *Yersinia pestis.* YopJ-like effectors are found in a variety of plant and animal pathogens, and several, including YopJ, HopZ1a from *Pseudomonas syringae*, and PopP2 from *Ralstonia solanacearum*, have demonstrated acetyltransferase activity (Lewis et al., 2011). Others may function as cysteine proteases, but both activities require a conserved catalytic triad common to all members of the YopJ superfamily (Orth, 2002).

HopZ1a and PopP2 are recognized by the *Arabidopsis thaliana* resistance proteins ZAR1 and RRS-1, respectively (Lewis et al., 2010; Tasset et al., 2010). ZAR1 is a classical NB-LRR resistance protein with a coiled-coil (CC) N-terminal domain, while RRS-1 is an atypical TIR-NB-LRR protein with a C-terminal WRKY domain. The activation of both R proteins requires the conserved catalytic cysteine residue of their cognate effectors. The virulence functions of HopZ1a, which include the inhibition of isoflavone biosynthesis in soybean (Zhou et al., 2011) and the disruption of the cytoskeleton in *Arabidopsis thaliana* (Lee et al., 2012), also require its catalytic cysteine and a conserved autoacetylated lysine residue. The mechanism of HopZ1a recognition by ZAR1 has not been fully elucidated. In contrast, the virulence targets of PopP2 have not been identified, but its recognition by RRS-1 has been shown to depend on the direct interaction of the two proteins in the nucleus and on the autoacetylation activity of PopP2, which, like HopZ1a and YopJ, requires conserved cysteine and lysine residues (Tasset et al., 2010). Recognition of PopP2 by RRS-1 also requires the expression of *RPS4*, a *P. syringae* resistance gene encoded at the same locus (Narusaka et al., 2009). The identification of the *RXopJ4* resistance gene may further illuminate the similarities and differences among plant recognition mechanisms of YopJ-like bacterial effector proteins.

In this work we have identified three candidate serine-threonine protein kinase genes within the *RXopJ4* locus and tested their ability to recognize XopJ4 using an *Agrobacterium*-mediated transient expression assay in *Nicotiana benthamiana*. One of the candidates, designated Sopen kinase 1, produced a hypersensitive response in the transient assay. We further demonstrated that upstream and downstream sequences of *Sopen kinase 1* (hereafter referred to as *RXopJ4*) in *S. pennellii* constitute an active native promoter and terminator, respectively. We also identified putative catalytic residues of both RXopJ4 and XopJ4 that were required for recognition in the transient assay. We generated primary transgenic tomato lines containing *RXopJ4* and plan to test homozygous T_1 plants for XopJ4-dependent resistance.

Results

Identification of candidate resistance genes at the *RXopJ4* locus

Previously we used molecular mapping to narrow the *RXopJ4* resistance locus to a 190-kb region on chromosome 6 of *S. pennellii* LA716 (Chapter 1; Sharlach et al., 2012). We then employed the International Tomato Annotation Group (ITAG) release 2.3 annotations of *S. lycopersicum* (Sato et al., 2012) and the gene prediction program Eukaryotic GeneMark.hmm (Lomsadze et al., 2005) to identify potential protein-coding genes within this region. This analysis revealed thirty-seven predicted genes in *S. lycopersicum* and twenty-nine in *S. pennellii*. BLAST searches were performed on all twenty-nine of the predicted proteins from *S. pennellii* and on those from *S. lycopersicum* that were not annotated by ITAG.

The vast majority of known plant disease resistance proteins (R proteins) that confer gene-for-gene resistance are intracellular nucleotide-binding site plus leucine-rich repeat (NB-LRR) proteins. Other R proteins, such as the tomato Cf proteins, are called receptor-like proteins (RLPs) and contain an extracellular LRR domain and a transmembrane domain (Dangl and Jones, 2001; Dodds and Rathjen, 2010). *Pto*, the first gene-for-gene resistance gene to be cloned, encodes a cytoplasmic serine-threonine protein kinase that interacts with an NB-LRR protein, Prf, to activate resistance (Martin et al., 1993; Mucyn et al., 2006).

Among the predicted genes at the *RXopJ4* locus in *S. pennellii*, none encode NB-LRRs or RLPs. However, three of these genes encode putative serine-threonine protein kinases. One of these is an ortholog of the annotated tomato gene Solyc06g060680; we have designated this Sopen06g060680. Sopen06g060680 encodes a 386-amino-acid protein, while the Solyc06g060680 protein is only 292 amino acids long; the catalytic domains of these two proteins are 77% identical. The other two putative kinase genes at the *RXopJ4* locus in *S. pennellii* do not have direct orthologs in *S. lycopersicum*; we have called these Sopen kinases 1 and 2. *S. lycopersicum* contains three additional putative kinase genes at this locus. Two of these, Solyc06g060690 and Solyc06g060700, were previously annotated by ITAG; the latter appears to be truncated, as it does not contain a complete catalytic kinase domain. Using GeneMark we identified a fourth kinase gene in *S. lycopersicum* and designated this Solyc kinase 4 (Figure 2-1).

Groups of closely related plant disease resistance genes commonly occur in clusters in the genome. In fact, this phenomenon was first identified in the tomato *Pto* locus (Martin et al., 1993) and has since been shown for NB-LRR *R* genes; duplications of disease resistance genes likely provide a framework for subsequent mutation and generation of new effector recognition specificities (Leister, 2004).

Transient expression of kinase 1 produces a XopJ4-dependent hypersensitive response in *Nicotiana benthamiana*

Agrobacterium-mediated transient expression of pathogen effectors and resistance proteins in *Nicotiana benthamiana* is a common method used to study recognition specificity in plant disease resistance. Coexpression of an effector and its cognate R protein produces a hypersensitive response (HR), which correlates with recognition leading to disease resistance (Rathjen et al., 1999). We used this transient expression method to test the three candidate kinase genes at the *RXopJ4* locus of *S. pennellii* for their ability to recognize the XopJ4 effector and induce an HR. As a control for the HR, we used coexpression the *Xanthomonas* effector AvrBs2 and the pepper R protein Bs2 (Tai et al., 1999). Only kinase 1 produced a XopJ4-dependent HR (Figure 2-2). Interestingly, kinase 1 also induced a slight HR when expressed alone, suggesting that the protein can signal a cell death response in *N. benthamiana* in the absence of the XopJ4 effector. A western blot of total protein from inoculated plant tissue (Figure 2-2c) revealed that kinase 2 was not expressed. Unlike Sopen06g060680 and kinase 1, the kinase 2 gene contains predicted introns. Perhaps its transcript was not properly spliced in *N. benthamiana* and this prevented its expression. Initial attempts to amplify the coding sequence of the kinase 2 gene from cDNA isolated from a resistant tomato line containing the *RXopJ4* locus were unsuccessful.

Nevertheless, the XopJ4-dependent HR we observed in the transient expression assay with kinase 1 led us to consider this the most likely *RXopJ4* candidate gene. Thus we cloned this gene along with its upstream and downstream sequences from *S. pennellii* to determine whether these functioned as a native promoter and terminator in the transient assay in *N. benthamiana*. Indeed, 2,061 base pairs upstream of the *Sopen kinase 1* open reading frame (ORF) led to protein expression and HR levels similar to those seen with the 35S promoter (Figure 2-3). An untagged version of the protein also functioned in the transient assay (Figure 2-3), as did a version containing 927 bp downstream of the kinase 1 ORF (Figure 2-4), replacing the octopine synthase terminator present in the pZP200n binary vector. The protein sequence of the 365-amino-acid putative RXopJ4 kinase is shown in Figure 2-5.

Putative catalytic residues in both XopJ4 and the candidate RXopJ4 kinase are required for the hypersensitive response

Several members of the YopJ family of bacterial type III effectors have been shown to function as acetyltransferases, while others function as SUMO proteases. Regardless of biochemical activity, all of these proteins require a conserved catalytic triad for their function. These residues are required for HR in response to XopJ4 (formerly known as AvrXv4) in *N. benthamiana* at a higher expression level, and possibly in a different genetic background, than in our assay (Roden, Eardley, et al., 2004; Chapter 3). We tested the putative catalytic mutant protein XopJ4 C219A in a

transient expression assay and showed that this residue is required for recognition of XopJ4 by the RXopJ4 kinase (Figure 2-6).

A conserved lysine residue has been shown to be autoacetylated and required for recognition of both PopP2 and HopZ1a (Tasset et al., 2010; Lee et al., 2012). We mutated this residue to arginine in XopJ4 but found that this did not affect recognition by the RXopJ4 kinase (Figure 2-4). This is consistent with preliminary results showing that XopJ4 autoacetylates in vitro when incubated with C-14-labeled acetyl-CoA, but cannot be detected with an antibody to acetylated lysine (M. B. Mudgett, personal communication). This suggests that XopJ4 may autoacetylate on serine and/or threonine residues (Mukherjee et al., 2007).

An alignment of the candidate RXopJ4 kinase with the other related kinases from the *RXopJ4* locus as well as the Pto kinase revealed a conserved threonine residue within the activation loop (Figure 2-7a). In Pto, this residue is required for direct interaction with and recognition of the AvrPto effector, as well as for full autophosphorylation (Xing et al., 2007). We mutated this residue to alanine in the candidate RXopJ4 kinase and observed an abrogation of the XopJ4-dependent HR in the *N. benthamiana* transient expression assay (Figure 2-7c). The T233A mutation also abrogated the XopJ4-independent slight HR observed when the protein was expressed alone. This suggests that this candidate RXopJ4 protein is an active kinase and that its kinase activity is required for XopJ4 recognition. The RXopJ4 candidate also contains the conserved aspartic acid residue of the "HRD" motif that is often required for proton transfer in serine-threonine protein kinases (Endicott et al., 2012).

Successful transformation of tomato with the candidate *RXopJ4* kinase gene

The Plant Transformation Core Research Facility at the University of Nebraska carried out transformations of the FL8000 tomato line with the following constructs: pZP200n 35S::*RXopJ4*-6xHA-TEV-3xFLAG, pZP200n Npro::*RXopJ4*-6xHA-TEV-3xFLAG, and Npro::*RXopJ4* (untagged). We used PCR to detect the *RXopJ4* transgene in primary transformants (Figure 2-8). We detected the transgene in 16 of 37 35S::*RXopJ4*-6xHA-TEV-3xFLAG lines, 14 of 20 Npro::*RXopJ4*-6xHA-TEV-3xFLAG lines, and 23 of 33 Npro::*RXopJ4* lines. In preliminary assays we did not identify disease resistance or XopJ4-dependent HR in these lines. However, this is not unexpected, since these hemizygous, primary transformants may not produce adequate quantities of protein or activate proper defense pathways to achieve HR and resistance. Furthermore, in the F_2 population used to map the *RXopJ4* locus, heterozygotes were not disease-resistant and showed weak, inconsistent HRs (Chapter 1; Sharlach et al., 2012). We plan to test disease and HR phenotypes, as well as *RXopJ4* transcript and protein levels, in homozygous transgenic plants of the T_1 generation.

Discussion

The present study has built on previous mapping work to tentatively identify the *RXopJ4* disease resistance gene from *S. pennellii* LA716. This gene encodes a putative cytoplasmic serine-threonine protein kinase and was initially identified by transient coexpression with the XopJ4 effector protein in *N. benthamiana*, which resulted in a robust hypersensitive response. This assay also enabled the identification of putative catalytic residues in both proteins that are required for recognition, in addition to the delineation of a native promoter and terminator sequence for the *RXopJ4* gene. Transgenic tomato lines containing the *RXopJ4* candidate kinase gene were generated and the transgene was detected in the majority of these lines by PCR amplification. Future work will include the characterization of disease resistance in homozygous T_1 plants.

To date, more than one hundred plant disease resistance genes have been identified (Sanseverino et al., 2012). Most have been delineated by classical map-based cloning methods and transgenic complementation using genomic clones. Our approach enabled a more informed strategy due to genome sequence information and experimental data from the transient expression assay. Present and future efforts to clone disease resistance genes will benefit from the use of next-generation sequencing methods on bulk segregants (Takagi et al., 2013). *Agrobacterium*-mediated transient expression or virus-induced gene silencing methods may be employed to evaluate candidate genes. Both single mutations and quantitative traits have already been mapped by whole-genome resequencing in the model plant species *Arabidopsis thaliana* and rice (*Oryza sativa*), but the increasing availability of genome sequence data for other species will broaden the applicability of these methods (Schneeberger and Weigel, 2011).

Models of effector recognition by plant disease resistance proteins include both direct and indirect recognition. Direct interactions have been demonstrated in a few cases, including the L and AvrL proteins of flax and flax rust (Dodds et al., 2006), Pi-ta and AVR-Pita of rice and rice blast (Jia et al., 2000), and, as previously discussed, RRS-1 and PopP2 (Deslandes et al., 2003). The RPP1 R protein of *Arabidopsis* is also likely to interact directly with the downy mildew effector ATR1 (Krasileva et al., 2010). However, indirect recognition seems to be a more common mechanism. This model is often referred to as the guard hypothesis, meaning that the R protein guards another host protein that is modified by the effector. This "pathogen-induced modified-self" molecular pattern activates the R protein to trigger an immune response (Jones and Dangl, 2006).

Either model may apply to the case of XopJ4 recognition by RXopJ4. In support of direct recognition, other YopJ-like effectors are known to acetylate host kinases. YopJ itself acetylates human MAPK kinases to prevent their phosphorylation and thus inhibit immune responses (Mukherjee et al., 2006). The *Xanthomonas* effector XopJ2 [also known as AvrBsT (Potnis et al., 2011)] interacts with pepper SNF1-related kinase 1 (SnRK1), which is involved in AvrBs1-dependent HR, and suppresses this HR (Szczesny et al., 2010) by an unknown mechanism.

Lewis and colleagues (2011) have suggested that disruption of kinase signaling may be a general mechanism employed by YopJ-like effectors to suppress host immunity. It is possible that the RXopJ4 kinase is a virulence target of XopJ4 and is involved in immune signaling; perhaps another protein, possibly an NB-LRR, guards the kinase and triggers resistance. If so, this NB-LRR must be conserved between *S. lycopersicum* and *S. pennellii*, as no NB-LRR genes are present at the *RXopJ4* locus of *S. pennellii*.

An alternate scenario is that the RXopJ4 kinase evolved to act as a decoy, mimicking other kinase(s) that are virulence targets of XopJ4, which may acetylate these kinases to inactivate signaling. The decoy model of effector recognition was developed based in part on evidence from the case of AvrPto and Pto. AvrPto is known to inhibit the receptor-like kinases FLS2 and EFR, which are involved in PAMP perception; Pto does not appear to play a role in basal immunity, but may be a decoy for AvrPto, guarded by the NB-LRR protein Prf (Van Der Hoorn and Kamoun, 2008). Preliminary evidence suggests that HopZ1a recognition by ZAR1 may follow an analogous pattern, in that a non-functional kinase is also required for recognition and forms a complex with this YopJ-like effector and its cognate CC-NB-LRR protein (J. D. Lewis, personal communication). Perhaps this is a common mechanism for plant perception of YopJ-like effectors. A third possibility is that RXopJ4 itself acts as a guard, perceiving the modification (likely acetylation) of another host protein by XopJ4.

Preliminary co-immunoprecipitation experiments have not detected a direct association between XopJ4 and the RXopJ4 kinase, but we cannot rule out this possibility. Study of the virulence targets of XopJ4 may also help to elucidate the nature of its recognition. In addition to protein-protein interaction studies, testing the dependence of RXopJ4 resistance on known signaling genes such as *SGT1*, *RAR1*, *NDR1*, and *EDS1* may suggest whether RXopJ4 resistance involves an NB-LRR protein (Nishimura and Dangl, 2010). Future experiments will also test the catalytic activities of both proteins and their dependence on the residues identified here. Mass spectrometry analysis may identify which residues are autoacetylated and autophosphorylated and also reveal potential transacetylation and transphosphorylation activities of these proteins.

While many further experiments will be necessary to understand the mechanism of XopJ4 recognition by RXopJ4, the *RXopJ4* resistance gene may soon be deployed in the field along with the *Bs2* gene—progress toward a durable resistance strategy against bacterial spot disease of tomato. We continue to analyze field isolates of *X. perforans* to understand the repertoire of conserved effectors and choose informed targets for durable resistance. As we have seen throughout the history of plant pathology, detailed mechanistic understanding often lags behind practical applications, and the current disease burden in the field demands the deployment of genetic resistance as soon as it is discovered.

Materials and Methods

Gene prediction

Gene predictions were performed using Eukaryotic GeneMark.hmm version bp 3.9e (Lomsadze et al., 2005) with a Hidden Markov Model trained on *Arabidopsis thaliana* gene models. For *S. pennellii*, we submitted 189,231 bp of genomic sequence corresponding to the coordinates 2322548..2511778 on scaffold 194 of the SpennV190 assembly provided by Anthony Bolger, Alisdair Fernie, and Björn Usadel at the Max Planck Institute for Molecular Plant Physiology, Golm, Germany. For *S. lycopersicum*, we submitted 209,901 bp of genomic sequence corresponding to the coordinates 35034599..35244499 on chromosome 6 of the tomato genome (Sato et al., 2012). These sequences spanned the region between the markers J350 and J352 used to map the *RXopJ4* locus (Chapter 1; Sharlach et al., 2012). The output from Eukaryotic GeneMark.hmm consisted of 29 predicted genes in *S. pennellii* and 37 in *S. lycopersicum*. These genes were annotated by a BLASTP search (Altschul et al., 1990) of predicted proteins against the nr database of NCBI. Those genes with multiple top hits identified as serine-threonine protein kinases were selected for further analyses.

Bacterial strains, plants, and growth conditions

All bacterial strains used in this study are listed in Table 2-1. *Escherichia coli* and *Agrobacterium tumefaciens* strains were grown on Luria agar supplemented with the appropriate antibiotics at 37°C and 28°C, respectively. Bacterial DNA transformation was conducted using chemically competent *E. coli* (New England Biolabs, Ipswich, MA). Plasmids were introduced into *A. tumefaciens* GV3101 by triparental mating with a helper *E. coli* strain containing the pRK600 plasmid. Electrocompetent *A. tumefaciens* C58C1 cells were prepared by Karl Schreiber and transformed using standard methods. *Nicotiana benthamiana* Nb-1 plants were grown in a controlled growth chamber at 24°C with 12 h light/dark cycle before infiltration and were moved to 24 h light conditions after infiltration.

Gene manipulation and plasmid construction

All plasmids used in this study are listed in Table 2-1; primers used for gene amplification and site-directed mutagenesis are listed in Table 2-2. Polymerase chain reaction (PCR) was used to amplify genes from *S. pennellii* LA716 or *X. perforans* 4B genomic DNA. PCR products were cloned into the pENTR™/D-TOPO® entry vector and introduced into Gateway®-compatible destination vectors using LR Clonase™. pENTR™/D-TOPO® and LR Clonase™ kits were purchased from Life Technologies (Grand Island, NY). Sanger sequencing was used to verify the sequence of DNA constructs. Point mutations were introduced using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA).

Cloning of the *RXopJ4* gene with its native promoter and terminator was achieved in stages. First, a genomic sequence beginning 2,061 bp upstream of the start codon and ending at position 914 of the ORF was amplified and cloned into the pENTR™/D-TOPO® entry vector. This plasmid was transformed into chemically competent *dam*$^-$ *E. coli* cells, purified, and sequentially digested with BclI (blocked by *dam* methylation), which cut at position 459 of the ORF, and AscI, which cut within the vector downstream of the insert. The same digests were performed on entry vectors containing the *RXopJ4* ORF untagged and with the 6xHA-TEV-3xFLAG tags. The fragments containing 460-1098 bp or 460-1095-tags were ligated in downstream of the rest of the *RXopJ4* gene. To add the native terminator, a genomic sequence beginning 648 bp upstream of the start codon and ending 927 bp downstream of the stop codon was amplified and cloned into an entry vector. Both this plasmid and the plasmid containing -2061-1098 bp of *RXopJ4* were digested with XbaI (cut at position 813 of the ORF) and AscI, and subsequent ligation followed a similar strategy.

Agrobacterium-mediated transient expression and protein immunoblotting

A. tumefaciens was grown on Luria agar supplemented, as appropriate, with 100 μg/ml rifampicin, 35 or 50 μg/ml kanamycin, and 50 μg/ml gentamycin or 5 μg/ml tetracycline. Plates were incubated at 28°C overnight. Cells were suspended in 1 ml of induction medium (10 mM $MgCl_2$, 10 mM MES, 150 mM acetosyringone). Bacterial concentrations were measured and adjusted with induction medium to OD_{600} = 0.1 (~1 x 10^8 cfu/ml). Resulting cultures were pre-induced for ~3 h at room temperature. For co-infiltrations, cultures carrying individual constructs were mixed at a 1:1 ratio. Young *N. benthamiana* leaves were infiltrated using a 1-ml needleless syringe. Plants were placed at room temperature under constant light for 40 to 50 h to observe the development of HR.

To detect protein expression, 0.8-cm^2 leaf disks were collected at ~24 h post infiltration. Samples were frozen in liquid nitrogen and ground in a BeadBeater (BioSpec Products, Bartlesville, OK) with a 3-mm glass bead. Protein was extracted with 75 μl of Laemmli buffer (0.24M Tris-HCl pH 6.8, 6% SDS, 30% glycerol, 16% β-mercaptoethanol, 0.006% bromophenol blue, 10M urea). Samples were boiled for 5 minutes and centrifuged at maximum speed for 10 minutes in a tabletop centrifuge at room temperature. Supernatants were transferred to fresh tubes before analysis by SDS-PAGE and immunoblotting.

Generation of transgenic tomato lines

Shirley Sato (Plant Transformation Core Research Facility, University of Nebraska) carried out transformations of the FL8000 tomato line with the following constructs: pZP200n 35S::*RXopJ4*-6xHA-TEV-3xFLAG, pZP200n Npro::*RXopJ4*-6xHA-TEV-3xFLAG, and Npro::*RXopJ4* (untagged). A detailed transformation protocol can be found at http://biotech.unl.edu/node/211. Briefly, tomato seeds were sterilized

prior to germination on medium. Explants were co-cultivated with *A. tumefaciens* C58C1 containing the indicated constructs. Explants were then transferred to shoot initiation medium containing kanamycin to select for transformants. This was followed by shoot elongation and rooting. Rooted plantlets were transferred to soil and placed in a growth chamber at 24^{o}C with 12 h light/dark cycle for several weeks before being transferred to a greenhouse with supplemental lighting.

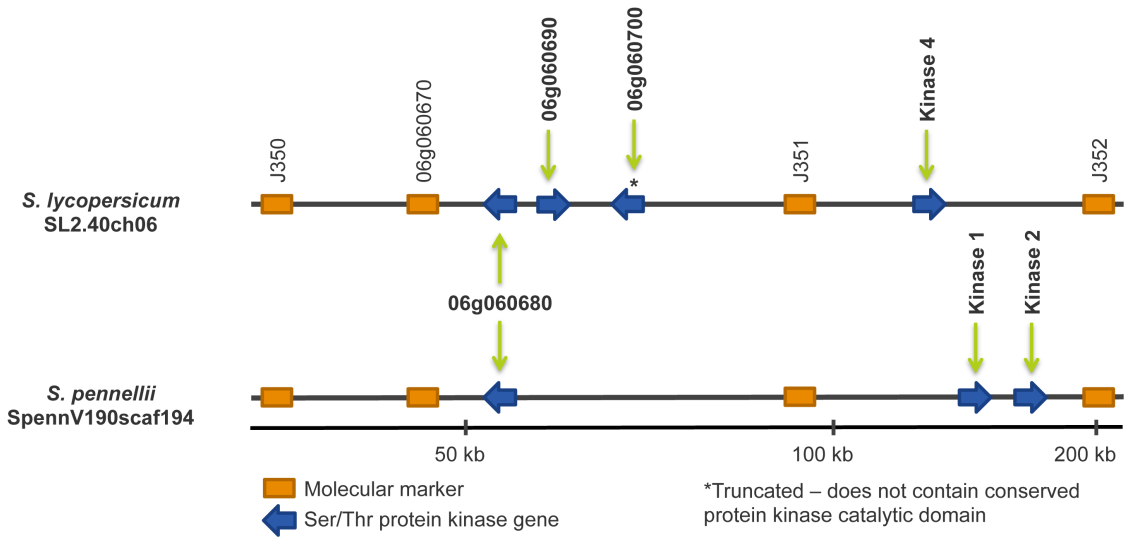
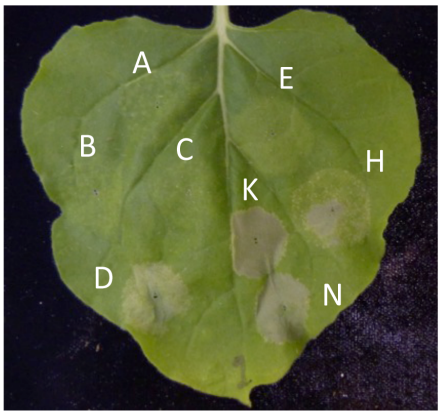


Figure 2-1. Candidate disease resistance genes within the 190-kb *RXopJ4* locus in *S. lycopersicum* and *S. pennellii*. Distances are not to scale but are roughly indicated in kilobases (kb). The *Sl* genome assembly was obtained from the Sol Genomics Network (Bombarely et al., 2011). The *Sp* genome assembly was provided by Anthony Bolger, Alisdair Fernie, and Björn Usadel at the Max Planck Institute for Molecular Plant Physiology, Golm, Germany. Genomic regions were annotated using Eukaryotic GeneMark.hmm version bp 3.9e (Lomsadze et al., 2005) and BLASTP (Altschul et al., 1990). Of 37 predicted genes in *Sl* and 29 in *Sp*, candidate serine-threonine protein kinase genes are shown as blue arrows, with the arrow direction denoting the coding strand. Molecular markers used for mapping the *RXopJ4* locus (Chapter 1; Sharlach et al., 2012) are shown as orange rectangles.

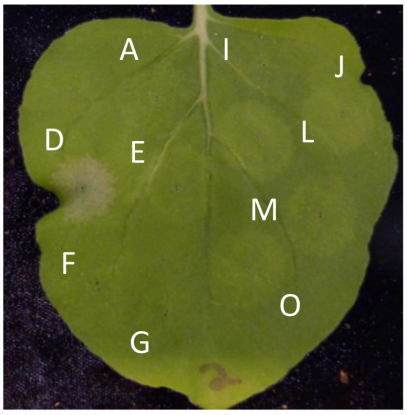
a

A. pE1776 empty vector (EV)
B. EV + AvrBs2-HA
C. EV + Bs2-HA
D. AvrBs2-HA + Bs2-HA
E. EV + XopJ4-HA
F. EV + Sopen06g062440-6xHA
G. EV + Sopen06g060680-6xHA
H. EV + Sopen kinase 1-6xHA
I. EV + Sopen kinase 2-6xHA
J. XopJ4-HA + Sopen06g060680-6xHA
K. XopJ4-HA + Sopen kinase 1-6xHA
L. XopJ4-HA + Sopen kinase 2-6xHA
M. J + Sopen06g062440-6xHA
N. K + Sopen06g062440-6xHA
O. L + Sopen06g062440-6xHA

b



AvrBs2 + Bs2 | XopJ4 + Sopen kinase 1



Sopen06g060680/kinase 2 +/- XopJ4, +/- Sopen06g062440 (CC-NBS-LRR)

c

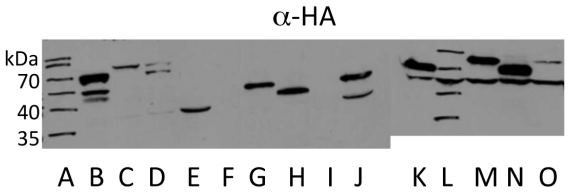


Figure 2-2. Candidate kinase 1 produces a hypersensitive response when transiently coexpressed with XopJ4 in *Nicotiana benthamiana*. **a** List of constructs used in the transient assay. AvrBs2-HA and XopJ4-HA were expressed using the binary vector pMD1, while all other constructs were in pE1776. Sopen06g062440 encodes a CC-NB-LRR protein that was previously hypothesized to be required for RXopJ4-mediated resistance. **b** Hypersensitive response in *N. benthamiana* induced by transient coexpression of XopJ4 and Sopen kinase 1 (K,N). Coexpression of AvrBs2 and Bs2 (D) was used as a positive control. Photographs were taken at 50 hpi. **c** Western blot showing protein expression levels. Expected protein sizes (in kilodaltons, kDa) are as follows: AvrBs2-HA, 80; Bs2-HA, 106; XopJ4-HA, 41; Sopen06g062440-6xHA, 82; Sopen06g060680-6xHA, 51; Sopen kinase 1-6xHA, 49; Sopen kinase 2-6xHA, 41. Sopen06g062440-6xHA (lane F) and Sopen kinase 2-6xHA (lane I) were not expressed.

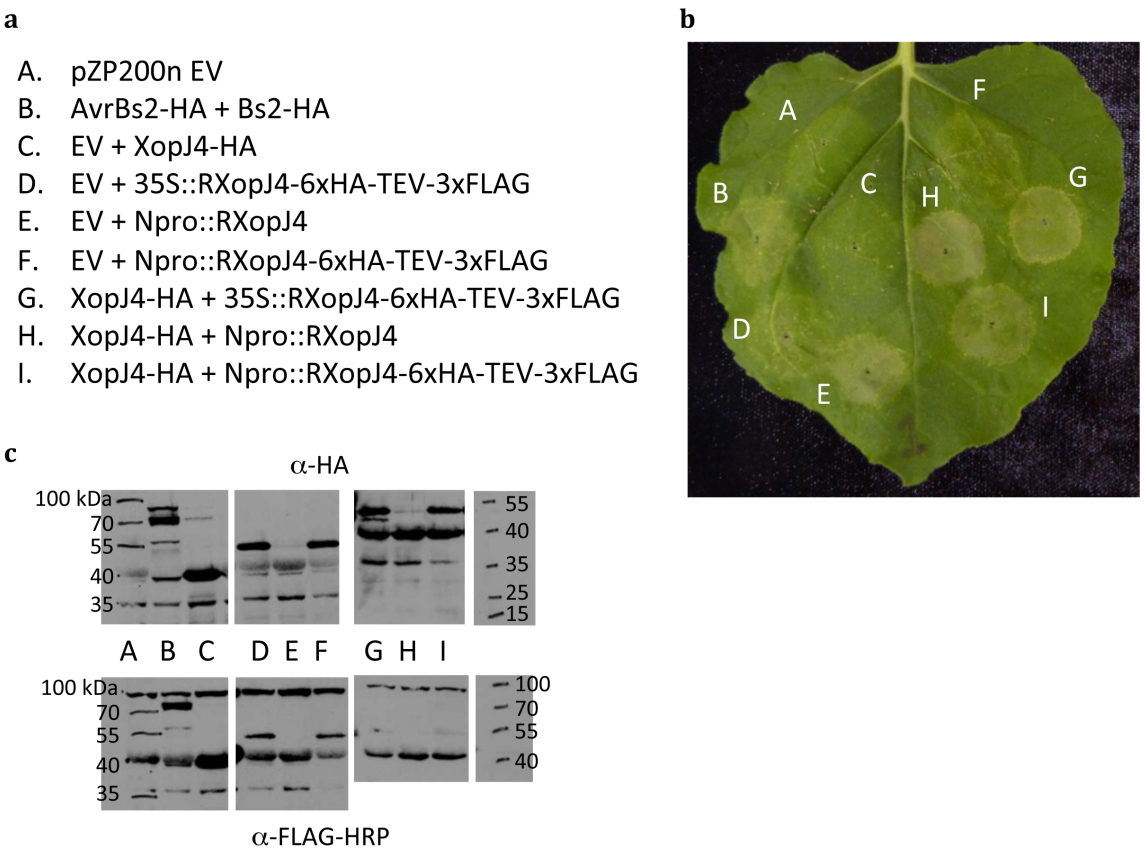


Figure 2-3. The *RXopJ4* native promoter functions in a transient assay in *Nicotiana benthamiana*. **a** List of constructs used in the transient assay. AvrBs2-HA and XopJ4-HA were expressed using the binary vector pMD1, Bs2-HA was in pE1776, and all other constructs were in pZP200n. **b** Hypersensitive responses in *N. benthamiana* induced by transient coexpression of XopJ4 and RXopJ4 under the control of 35S (G) or native promoter (Npro; H,I). Coexpression of AvrBs2 and Bs2 (B) was used as a positive control. Photograph was taken at 43 hpi. **c** Western blot showing protein expression levels. Expected protein sizes (in kilodaltons, kDa) are as follows: AvrBs2-HA, 80; Bs2-HA, 106; XopJ4-HA, 41; RXopJ4-6xHA-TEV-3xFLAG, 55. Note that the RXopJ4 protein expressed in lanes E and H is untagged and thus is not detectable by α-HA or α-FLAG antibodies.

a

A. pZP200n EV
B. EV + XopJ4-HA
C. EV + XopJ4 C219A-HA
D. EV + XopJ4 K289R-6xHA
E. EV + Npro::RXopJ4-6xHA-TEV-3xFLAG
F. EV + Npro::RXopJ4
G. EV + Npro::RXopJ4::Nterm
H. XopJ4-HA + Npro::RXopJ4-6xHA-TEV-3xFLAG
I. XopJ4-HA + Npro::RXopJ4
J. XopJ4-HA + Npro::RXopJ4::Nterm
K. XopJ4 C219A-HA + Npro::RXopJ4-6xHA-TEV-3xFLAG
L. XopJ4 K289R-HA + Npro::RXopJ4-6xHA-TEV-3xFLAG

b

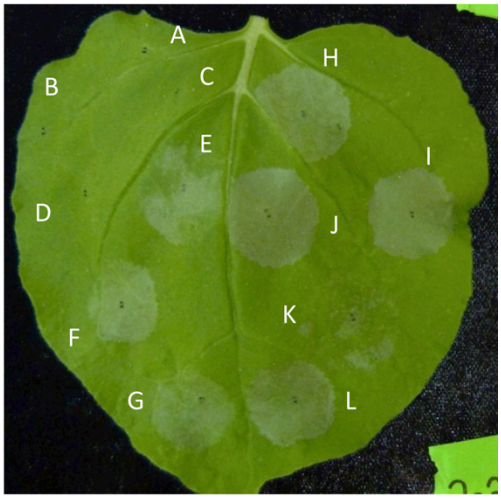


Figure 2-4. The *RXopJ4* native terminator (Nterm) functions in a transient assay in *Nicotiana benthamiana*; XopJ4 lysine 289 is not required for recognition by RXopJ4. **a** List of constructs used in the transient assay. XopJ4-HA and C219A-HA were expressed using the binary vector pMD1, XopJ4 K289R-6xHA was in pE1776, and all other constructs were in pZP200n. **b** Hypersensitive responses in *N. benthamiana* induced by transient coexpression of XopJ4 and RXopJ4. Photograph was taken at 45 hpi.

Figure 2-5. Protein sequence of the candidate RXopJ4 kinase. The putative catalytic domain and activation loop are shown; these were predicted using the Conserved Domain Database of the National Center for Biotechnology Information (NCBI).

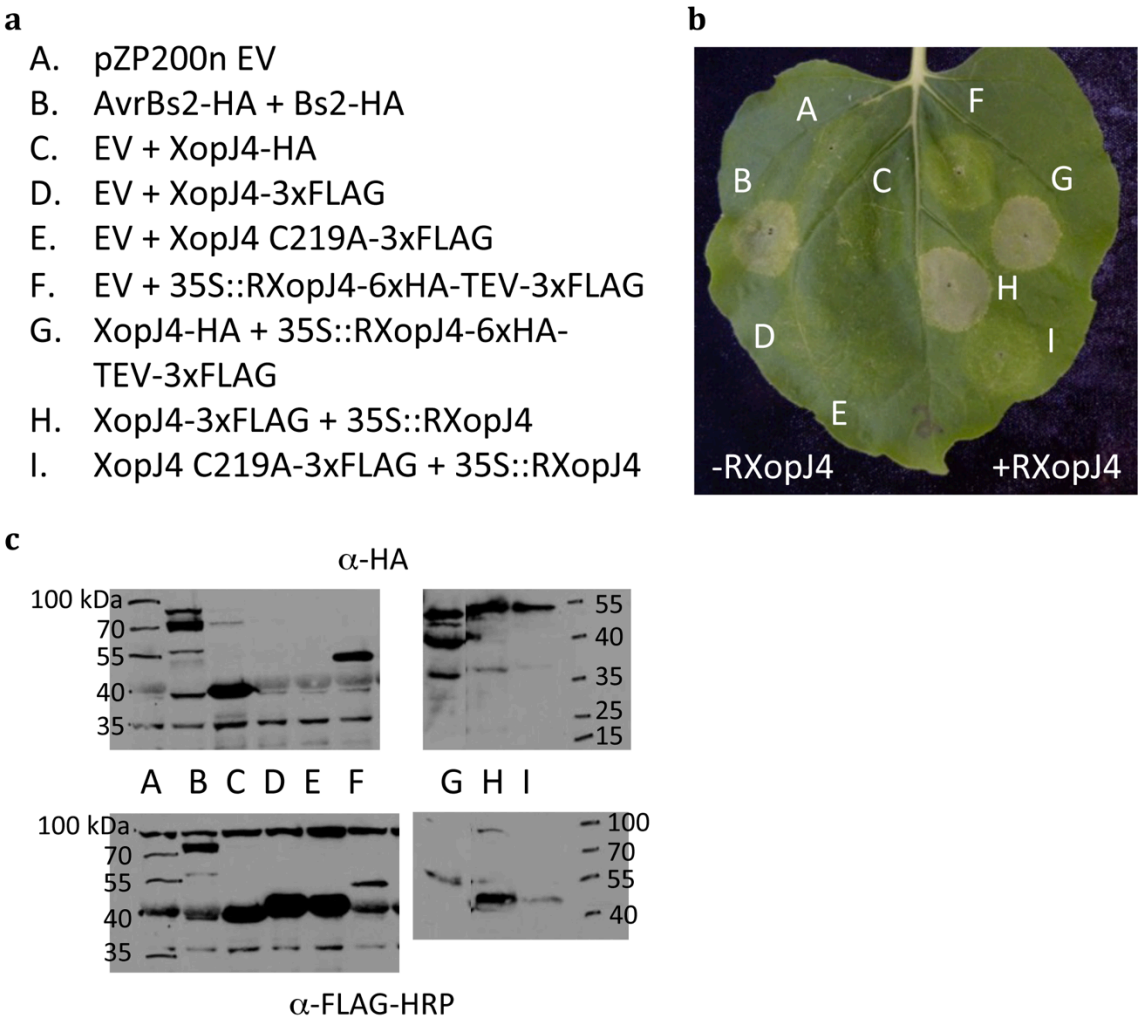


Figure 2-6. The putative catalytic cysteine of XopJ4 is required for recognition by RXopJ4 in a transient assay in *Nicotiana benthamiana*. **a** List of constructs used in the transient assay. AvrBs2-HA and XopJ4-HA were expressed using the binary vector pMD1, XopJ4-3xFLAG was in pE1776, and all other constructs were in pZP200n. **b** Hypersensitive responses in *N. benthamiana* induced by transient coexpression of RXopJ4 with XopJ4 WT (G,H) but not C219A mutant (I). Coexpression of AvrBs2 and Bs2 (B) was used as a positive control. Photograph was taken at 43 hpi. **c** Western blot showing protein expression levels.

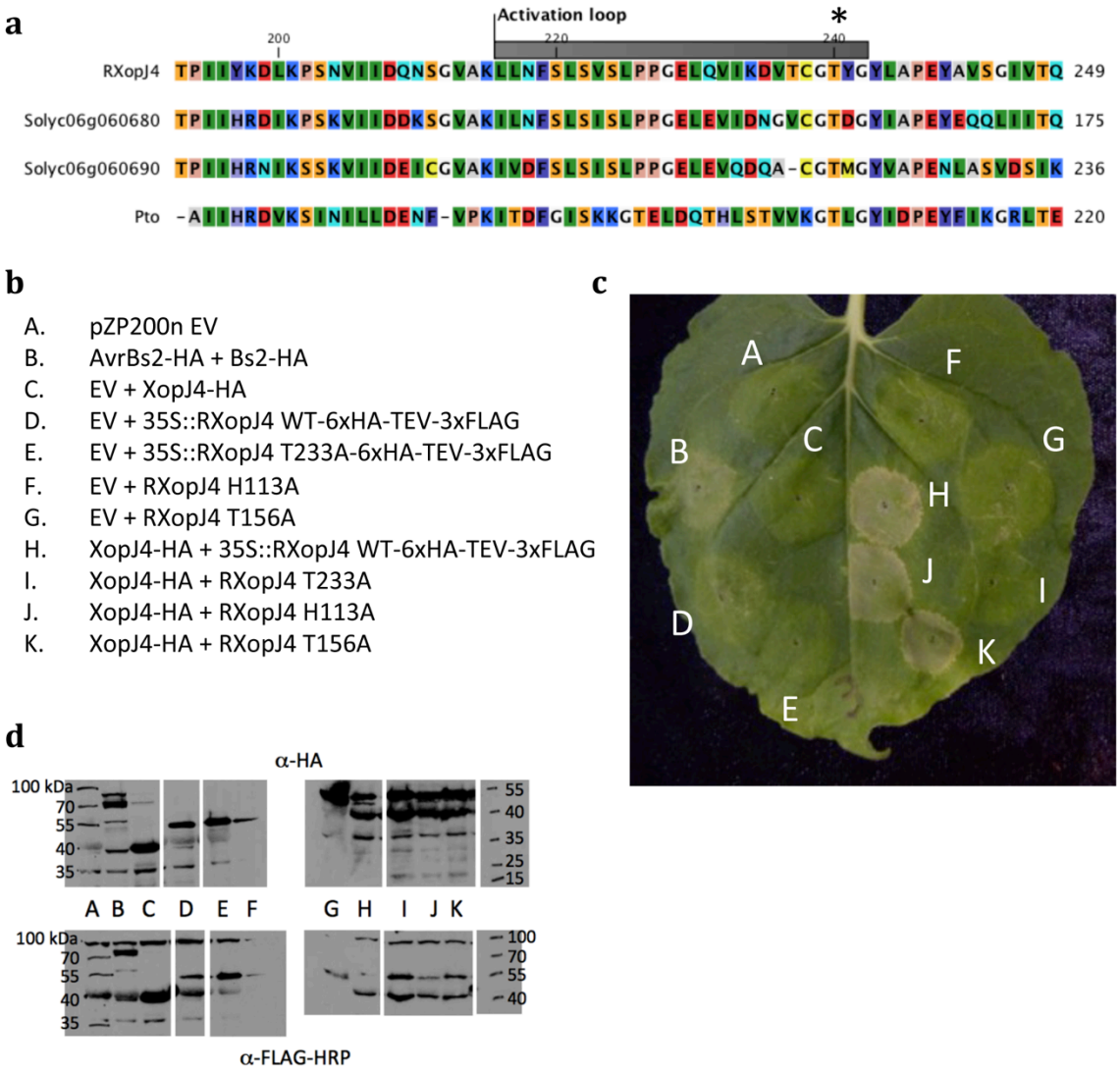


Figure 2-7. A conserved threonine residue of RXopJ4 is required for recognition of XopJ4 in a transient assay in *Nicotiana benthamiana*. **a** Alignment of the activation loop regions of RXopJ4, Solyc06g060680, Solyc06g060690, and Pto. The asterisk indicates the position of a corserved threonine residue. The alignment was constructed using CLC Main Workbench. **b** List of constructs used in the transient assay. AvrBs2-HA and XopJ4-HA were expressed using the binary vector pMD1, and all other constructs were in pZP200n. **c** Hypersensitive responses in *N. benthamiana* induced by transient coexpression of XopJ4 with RXopJ4 WT, H113A, and T156A (H,J,K), but not T233A mutant (I). Coexpression of AvrBs2 and Bs2 (B) was used as a positive control. Photograph was taken at 43 hpi. **d** Western blot showing protein expression levels.

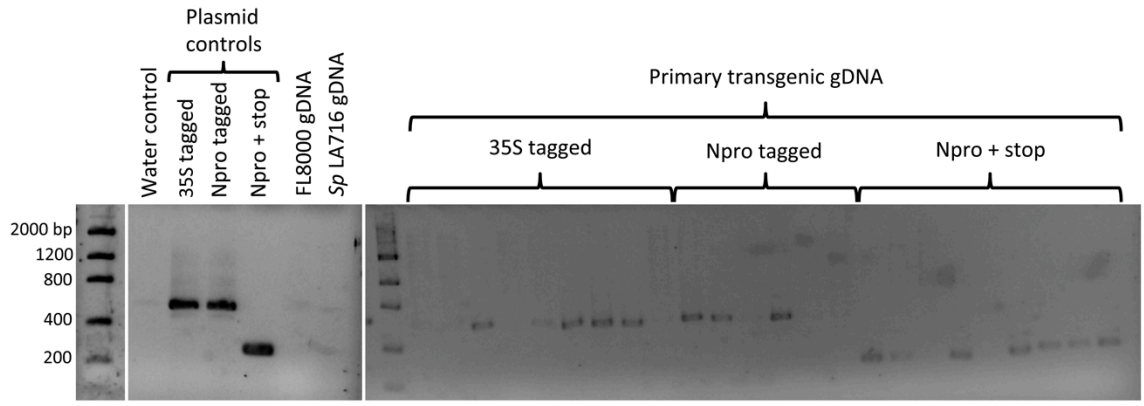


Figure 2-8. The *RXopJ4* transgene is detectable in FL8000 transgenic tomato lines by polymerase chain reaction (PCR). PCR was performed using One*Taq*® DNA Polymerase (New England Biolabs, Ipswich, MA, USA) and products were separated on a 2.5% agarose gel and stained with ethidium bromide. Band sizes in ladder are indicated in base pairs (bp). Reactions were performed using the primers *RXopJ4* (*Sopen kinase 1*) 967 F and *Nos Term* R and the indicated templates. Tagged constructs produced an amplicon of 557 bp, while untagged constructs produced an amplicon of 236 bp.
35S tagged = pZP200n(*35S::RXopJ4::6xHA-TEV-3xFLAG*)
Npro tagged = pZP200n(*Npro::RXopJ4::6xHA-TEV-3xFLAG*)
Npro + stop = pZP200n(*Npro::RXopJ4*)
gDNA = genomic DNA

Table 2-1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	References
Bacteria		
Agrobacterium tumefaciens		
GV3101	Rif^R, Gm^R	(Holsters et al., 1980)
C58C1	Rif^R, Gm^R	T. Clemente
Escherichia coli		
NEB 5-alpha	DH5α derivative, subcloning efficiency	New England Biolabs
NEB Turbo	K12 strain, high efficiency	New England Biolabs
Plasmids		
pENTR™/D-TOPO®	Km^R, Gateway®-compatible entry vector	Life Technologies
pE1776	Km^R, MAS promoter + triple chimeric OCS UAS + MAS UAS	(Leister et al., 2005)
pE1776(*Bs2::HA*)		(Leister et al., 2005)
pE1776(*Sopen06g060680::6xHA*)		This study
pE1776(*Sopen kinase 1::6xHA*)	This gene is hereafter referred to as *RXopJ4*	This study
pE1776(*Sopen kinase 2::6xHA*)		This study
pE1776(*xopJ4::6xHA*)		This study
pE1776(*xopJ4::6xHA* K289R)	pE1776(*xopJ4::6xHA*) with arginine substitution at Lys 289	This study
pMD1	Km^R, pBI121 (CLONTECH) derivative; CaMV 35S promoter, nopaline synthase 3' sequence	(Tai et al., 1999)
pMD1(*avrBs2::HA*)		(Tai et al., 1999)
pMD1(*xopJ4::HA*)		This study
pMD1(*xopJ4::HA* C219A)	pMD(*xopJ4::HA*) with alanine substitution at Cys 219	(Roden, Eardley, et al., 2004), This study
pZP200n	$Spec^R$, Km^R plant marker gene	(Hajdukiewicz et al., 1994)
pZP200n(*35S::RXopJ4::6xHA-TEV-3xFLAG*)		This study
pZP200n(*35S::RXopJ4::6xHA-TEV-3xFLAG* T233A)	Alanine substitution at Thr 233	This study
pZP200n(*Npro::RXopJ4::6xHA-TEV-3xFLAG*)	Contains 2,061 bp of native promoter	This study
pZP200n(*Npro::RXopJ4*)	Contains 2,061 bp of native promoter	This study
pZP200n(*Npro::RXopJ4::Nterm*)	Contains 927 bp of native terminator	This study

Rif rifampicin, *Km* kanamycin, *Gm* gentamicin, *Spec* spectinomycin

Table 2-2 PCR primers used in this study

Primer name	Sequence (5'-3')
Sopen06g060680 F	*cacc*ATGGTGATGCCTTTCTTCAG
Sopen06g060680 SpeI R	**ACTAGT**TTTTGACCAAGAGCGAAAAG
Sopen kinase 2 F	*cacc*ATGCAGACACTTTTTTCCTTTG
Sopen kinase 2 SpeI R	**ACTAGT**TATATGGTCTGGTTTCTCCTTTG
Sopen kinase 1 F	*cacc*ATGCAGTTCTTCAGGGAACTC
Sopen kinase 1 R	TTACTTATGTAACTGATTCTGGCCAAGAGTG
Sopen kinase 1 SpeI R	**ACTAGT**CTTATGTAACTGATTCTGGCCAAGAGTG
Sopen kinase 1 T233A F	TAATAAAAGATGTTACTTGCGGAGCATATGGATATTTAGCTCCAGAA
Sopen kinase 1 T233A R	TTCTGGAGCTAAATATCCATATGCTCCGCAAGTAACATCTTTTATTA
Sopen kinase 1 -2061 F	*cacc*ATTGTTCAAATACGCTTAGG
Sopen kinase 1 914 R	GCTTTATCTGCTATGTCCATTAC
Sopen kinase 1 967 F	CAACAATTGGAAGATTGCTGGG
Nos Term R	TCGCAAGACCGGCAACAGGA
Sopen kinase 1 -648 F	*cacc*GATGAATGAGGTAAATACATC
Sopen kinase 1 +927 R	CCACAAAGGAACAGTTTGCTC
xopJ4 F	*cacc*ATGAAAAACATATTTAGGT
xopJ4 SpeI R	ATT**ACTAGT**GCTACGACTC
xopJ4 K289R F	GGTGTGCTTCCACCTATATTTTACAGGCACTGTCAGTCT
xopJ4 K289R R	AGACTGACAGTGCCTGTAAAATATAGGTGGAAGCACACC

The 5' CACC in the forward primers is shown in lower-case, italicized letters and is required for introducing PCR products into the TOPO® entry cloning system. SpeI restriction sites are shown in bold and allow cloned fragments to be ligated into an entry vector containing sequences that encode the epitope tags 6xHA-TEV-3xFLAG (TEV, tobacco etch virus protease cleavage site).

Chapter 3:
Core effectors of *Xanthomonas perforans*: Gene knockout construction, preliminary virulence phenotypes, and recognition by *Nicotiana* and *Solanum americanum* accessions

Introduction

In recent years, the ability to rapidly and inexpensively sequence bacterial genomes has broadened our understanding of the type III effector proteins present in various pathogen species, as well as variation in effector repertoires among pathogen populations in the field (Arnold and Jackson, 2011). The genomes of diverse *Pseudomonas syringae* pathovars and strains have been sequenced, enabling the compilation of a pangenome for the species. Analysis of effector distributions among strains has shown that a set of core effectors function to target antimicrobial vesicle trafficking, while more variable effectors tend to interfere with kinase-based immune signaling pathways (Lindeberg et al., 2012). Many species of *Xanthomonas* with diverse host ranges and tissue specificities have also been sequenced, revealing a preliminary set of core effectors common to all species, which may play roles in suppressing conserved defense pathways among different plant hosts, and effectors that are unique to single species or groups of species, which may reveal important insights into processes of host adaptation (White et al., 2009).

Studies of various phytopathogenic bacteria have shown that deletion of a single effector seldom has a notable effect on pathogen virulence, with some exceptions. For example, XopN, an effector common to all sequenced xanthomonads, is required for full virulence on several hosts (White et al., 2009); deletion of the *xopN* gene from *X. euvesicatoria* 85-10 resulted in 10-fold lower bacterial growth and reduced symptom development in tomato. Kim et al. (2009) also showed that XopN inhibits the expression of defense-related genes in tomato and suppresses callose deposition in both tomato and *Arabidopsis*. In some cases, the specific mechanisms of plant defense suppression are known, while in others only downstream effects have been characterized. For example, the *P. syringae* effector AvrPto interacts directly with the pattern recognition receptors FLS2 and EFR to block PAMP-triggered immunity at an early stage (Xiang et al., 2008). Combinatorial knockouts in *P. syringae* have also helped to reveal redundant effector groups that target common defense processes (Kvitko et al., 2009; Cunnac et al., 2011).

Although understanding the functions of pathogen type III effectors is an ongoing and challenging area of research, a different set of questions are more pertinent for agricultural applications. Which effectors are retained in pathogen populations across space and time and are under positive selection? Do these core effectors contribute to pathogen virulence? This strategy can help to inform which effectors to target for the development of durable genetic resistance. The approach of core effector identification has been adopted by Bart et al. (2012) and others (Lindeberg et al., 2012). Understanding the distributions and virulence

contributions among the effectors of the oomycete pathogen *Phytophthora infestans* is aiding the development of durably resistant potato cultivars (Vleeshouwers et al., 2011).

X. perforans became the dominant species causing bacterial spot disease of tomato in Florida in the 1990s (Jones, Bouzar, et al., 1998). In an effort to understand which effectors may be most important for the virulence of *X. perforans*, and thus the most effective targets for the development of durable genetic resistance, we assembled a collection of fourteen field isolates collected from each of the five fresh market tomato production zones of Florida during the fall of 2006 (Figure 3-1, Table 3-1). We performed Illumina sequencing, *de novo* genome assembly, and effector prediction on each strain and compared their effector repertoires. Also included in these analyses were *Xp* 91-118, the type strain for this species, and *Xp* 4B, a strain collected during a field study that serves as our experimental strain. We identified a preliminary set of seventeen core effectors common to all isolates, as well as eleven effectors whose presence varied among strains. We then constructed gene knockouts of several of the core effectors we identified. The gene knockouts were markerless to allow for construction of multiple gene knockouts in a single strain to assess redundancies and synergisms in effector repertoires.

We inoculated susceptible tomato plants with these effector deletion strains and found that a double knockout strain lacking both *avrBs2* and *xopJ4* showed reduced disease symptoms compared to either individual knockout, while the *xopF2* and *xopAP* effector genes may make minimal individual contributions to virulence. To identify potential solanaceous *R* genes recognizing core effectors, we cloned eight effector genes into binary vectors for *Agrobacterium*-mediated transient expression in various *Nicotiana* and *Solanum* species. Preliminary inoculations with these constructs suggest differential recognition of seven core effectors by some accessions, which may facilitate future efforts to identify the corresponding resistance genes.

Results and Discussion

Genome sequencing of *Xanthomonas perforans* field isolates reveals a preliminary set of core type III effectors

We assembled a set of fourteen *X. perforans* field isolates collected during a survey of bacterial spot disease-infected fields throughout Florida and Georgia in the fall of 2006 (Figure 3-1, Table 3-1). We used Illumina technology to generate 100-bp paired-end genome sequence data for these strains as well as the strain *Xp* 4B, a strain isolated during a field study in 2007. We chose to use *Xp* 4B for effector gene knockouts because it produces reliable symptoms of bacterial spot and is an efficient recipient in bacterial conjugations. *De novo* assembly of the sequence data produced high-quality draft genomes (See Materials and Methods).

To predict type III effector genes using these draft genomes, we used the pipeline described by Bart et al. (2012), employing a database of 1,019 known and putative effector sequences from plant and animal pathogens. BLAST searches were performed against this database, and homologs were considered present if they were found at 45% identity over 50% of the coding sequence. Amino acid sequence alignments were performed to enable pseudogene identification. This analysis revealed a set of seventeen core effectors that are present in all fourteen field isolates, as well as *Xp* 4B and *Xp* 91-118, the previously sequenced *Xp* type strain (Potnis et al., 2011) (Table 3-2). Total effector gene content per strain ranged from 21 (strain 17-12) to 26 (strains 7-12, 8-16, 10-13, and 15-11). Since the set of core effectors are the most likely to be conserved in *X. perforans* strains across space and time, as well as to play important virulence roles, we chose a subset of these core effectors for further analyses, including gene knockouts to assess virulence roles and transient expression assays to search for recognition by resistance proteins in solanaceous species.

We chose this subset of eleven effectors for several reasons (Table 3-3). AvrBs2 and XopJ4 were selected based on prior knowledge of their roles in virulence (Zhao et al., 2011; Roden, Eardley, et al., 2004) as well as the known existence of resistance genes recognizing these effectors (Tai et al., 1999; Sharlach et al., 2012). XopA was of interest because it appears not to be a type III effector per se, but is secreted via the type III secretion system and may be involved in effector delivery to plant host cells (Noël et al., 2002). XopAP and XopR were shown to be type III-secreted effectors in *Ralstonia solanacearum* and *Xanthomonas oryzae* pv. *oryzae*, respectively, and are intriguing due to their conservation across phytopathogen species. Other effectors have been shown to be type III-dependent in *Xanthomonas* and some have demonstrated virulence roles in other species. XopL and XopN are both core effectors in *X. axonopodis* pv. *manihotis* (Bart et al., 2012) and are present in a wide range of sequenced *Xanthomonas* species (White et al., 2009). XopL was recently shown to function as an E3 ubiquitin ligase. This activity was required for the induction of cell death in *Nicotiana benthamiana*, while the N-terminal LRR domain of XopL was found to play a role in suppressing PAMP-triggered immunity (Singer et al., 2013). XopN has an irregular, α-helical repeat structure; it interacts with a tomato receptor-like kinase and with 14-3-3 proteins that are likely to play roles in immune signaling (Kim et al., 2009).

Construction of unmarked type III effector gene deletions in *X. perforans*

To construct unmarked deletions of type III effector genes, we employed the suicide vector pLVC18 (Lindgren et al., 1986; Sharlach et al., 2012). The pLVC18 origin of replication is not functional in xanthomonads, and thus can only be maintained if it is integrated into the chromosome by homologous recombination with an endogenous genomic sequence. This vector encodes resistance to the antibiotic tetracycline, and, importantly, sensitivity to sucrose encoded by the *sacBR* genes;

this characteristic can be used to select for a second homologous recombination event (Logue et al., 2009).

We used PCR to amplify upstream and downstream sequences flanking the open reading frame of the effector to be deleted. We cloned these fragments into separate entry vectors, used restriction digestion to ligate them into the same entry vector, and then employed the Gateway recombination system to clone the fragments into the pLVC18 suicide vector. We conjugated the resulting vector into *Xp* 4B, selected exconjugants (single recombination event) on tetracycline, and selected for a second recombination event on sucrose. Depending on where the second recombination event occurs, the resulting strain can either revert to the wild-type copy of the effector gene or contain an unmarked deletion of the gene (Figure 3-2). We used PCR and Sanger sequencing to identify deletion mutants among candidate colonies selected on sucrose (Figure 3-3). For the Δ*xopJ4* strain we verified the deletion by Southern blotting (Figure 3-4). This served to validate our deletion method and was also an important confirmation, since this strain was used for the mapping of the *RXopJ4* resistance gene and will be used to test transgenic tomato plants containing the *RXopJ4* candidate kinase gene (Chapters 1 and 2).

Preliminary virulence phenotypes of effector gene knockouts

To assess the ability of effector gene knockout strains to cause disease on susceptible tomato plants, we used a dip inoculation method. This method may be more sensitive to subtle differences in virulence than hand inoculation methods, since bacteria must survive on the leaf surface and gain entry into leaves in order to proliferate and cause disease. Furthermore, larger disease lesions develop when dip inoculation is combined with humid conditions; this may allow *X. perforans* to grow and cause disease to its maximum potential.

The *Bs2* resistance gene, which recognizes the effector AvrBs2, has been identified and incorporated into transgenic cultivated tomatoes, and has shown effective disease resistance in field trials (Horvath et al., 2012), and the *RXopJ4* resistance gene has been tentatively identified (Chapter 2). Therefore we are interested in the potential effects of deploying plants that contain both *Bs2* and *RXopJ4* on bacterial populations in the field. We constructed a double mutant in the effector genes *xopJ4* and *avrBs2* and compared its ability to produce disease symptoms on FL8000 plants to the *Xp* 4B wild-type strain and each of the single mutants. Despite considerable variation in symptom development, an overall trend was clear: While each single mutant had reduced virulence compared to wild-type, the double mutant produced even fewer lesions (Figure 3-5). Future work will determine the reproducibility of these results, quantify bacterial growth, and test complemented mutants to verify that the apparent differences in virulence are due to the mutated effector genes. While still preliminary, this result suggests that plants containing the *Bs2* and *RXopJ4* genes may possess durable resistance to *X. perforans* in the field, since strains with mutations in both of the recognized effectors may experience dramatically reduced fitness.

Dip inoculations with *xopAP* and *xopF2* deletion mutants also suggest that these effectors may play roles in virulence (Figure 3-6). In this experiment, a Δ*hrcV* strain was used as a control; this strain produces no symptoms because, without HrcV, an inner membrane component of the type III secretion needle complex, it is unable to secrete effectors and suppress plant defenses (Rossier et al., 1999). The *xopN* deletion strain also serves as a control, since this effector has a well-established virulence role on tomato in *X. euvesicatoria* (Kim et al., 2009).

Transient expression of core effectors in *Nicotiana* and *Solanum americanum* accessions reveals potential sources of cognate resistance genes

In an effort to identify *R* genes that can recognize core effectors of *X. perforans*, we cloned six of the core effectors into the binary vector pMD1 for *Agrobacterium*-mediated transient expression in various solanaceous plants (Table 3-3). The pMD1 vector contains a 35S promoter to drive overexpression in plant cells, as well as a C-terminal HA tag to enable detection of proteins (Tai et al., 1999). We inoculated seven species of *Nicotiana* and three accessions of *Solanum americanum* with *Agrobacterium* strains carrying each of the six effector genes.

Like the *Bs2* gene from *Capsicum chacoense* (Tai et al., 1999), we expect resistance genes from other solanaceous plants to function in tomato. In fact, both the pattern recognition receptor EFR and the *Verticillium* R protein Ve-1 have been shown to function across families when transferred from *Arabidopsis* to tomato and tomato to *Arabidopsis*, respectively (Lacombe et al., 2010; Fradin et al., 2011). Since *Solanum americanum* is in the same genus as tomato and *Nicotiana* is also in the Solanaceae family, *R* genes found in these plants are likely to be effective in tomato. Angel and Schoelz (2013) have used a collection of *Nicotiana* accessions to identify resistance genes against *Tomato bushy stunt virus* and assay for recognition of specific viral proteins. *Solanum americanum* is a widely distributed wild species with many accessions collected from different regions. These accessions exhibit considerable genetic diversity and are also being evaluated for differential recognition of effector proteins from *Phytophthora infestans* (J. D. G. Jones, personal communication).

The preliminary results of our survey for recognition of *X. perforans* core effectors are shown in Figure 3-7 and summarized in Table 3-4. Plants were also infiltrated with strains carrying the effector genes *avrBs2* and *xopJ4*, since these constructs were created in previous studies (Tai et al., 1999; Chapter 2). Although we have already identified *R* genes that recognize these effectors, it may be informative to learn how widely they are recognized among the Solanaceae and, eventually, whether the responsible *R* genes and pathways are conserved. Coexpression of AvrBs2 and Bs2 was used as a control to determine whether each plant was successfully transformed by *Agrobacterium* and produced protein. *S. americanum* 2272 was also tested but showed only nonspecific chlorosis in response to all bacterial strains, and thus was not studied further. *N. glauca* was also problematic due to its small leaves—HR could not be confidently identified in this

species. The remaining ten accessions tested all showed a hypersensitive response (HR) to *Xp* 4B but not to the Δ*hrcV* strain, indicating that the HR depends on the perception of one or more type III effector proteins.

We compared the recognition spectra of *N. benthamiana* from two different sources: the Nb-1 accession commonly used in the Staskawicz Laboratory (BJS), for which a draft genome sequence is available (Bombarely et al., 2012), and an unknown accession from M. B. Mudgett at Stanford University (MBM). We previously determined that *Nb* (MBM) produces a HR in response to XopJ4, while Nb-1 (BJS) does not (Roden, Eardley, et al., 2004; Chapter 2). Therefore we hypothesized that other effectors might also be differentially recognized by these two accessions. We found that Nb-1 but not *Nb* (MBM) may recognize XopQ. Recognition of XopQ by *N. benthamiana* was not surprising, since it is a homolog of the effector HopQ1-1, the known avirulence determinant of *Pseudomonas syringae* pv. *tomato* DC3000 on *N. benthamiana* (Kvitko et al., 2009). XopQ produced a strong HR on *N. tabacum* var. Turk and *N. rustica*; these species may be promising candidates for *R* genes, especially since XopQ is conserved in a wide variety of sequenced xanthomonads (Roden, Belt, et al., 2004; White et al., 2009). There appear to be two alleles of *xopQ* present among the sequenced field isolates of Xp, with six nonsynonymous polymorphisms between them; future work will determine whether both alleles are recognized. Previous analyses suggested that XopQ alone may not be required for full virulence in *X. euvesicatoria* 85-10 (Roden, Belt, et al., 2004), but a forthcoming knockout of *xopQ* in *Xp* 4B will reveal whether this is also true for *Xp* and under what conditions.

XopX is another potential target for durable resistance, although the HR it incited on six of the ten accessions tested was not particularly robust and will need to be verified. It is possible that, rather than eliciting a defense response, XopX actually suppresses defenses and causes a cell death response to *Agrobacterium* inoculation, as described by Metz et al. (2005). Notably, XopJ4 elicited a strong HR on several *Nicotiana* accessions. It would be interesting to ascertain whether these contain homologs of the *RXopJ4* kinase. An initial attempt to clone a homolog from *N. tabacum* was unsuccessful. XopF2, while more restricted in its distribution among xanthomonads (Roden, Belt, et al., 2004; White et al., 2009), is conserved at the amino acid level among the sequenced *Xp* field isolates, with the exception of two polymorphisms in the strain 17-12. XopF2 may contribute to the virulence of *Xp* on tomato (Figure 3-6), and therefore may be another useful target, particularly due to its differential recognition among *S. americanum* accessions.

The sexual compatibility of the *S. americanum* accessions accessions should make identification of the responsible *R* genes straightforward. Mapping may be more challenging in *Nicotiana*, since chromosome numbers differ among species and only some are compatible with one another (J. Schoelz, personal communication). *N. longiflora* may recognize XopF2; if *N. plumbaginifolia* (not yet tested) does not recognize this effector, these two species can be successfully crossed to map the resistance locus. *N. alata* TW7 is likely to be sexually compatible with several other species in our collection; it could be crossed to a species that does

not recognize XopQ. XopI recognition in *S. americanum* 268152 may be mapped using a cross to another *S. americanum* accession, although the reaction to XopI was ambiguous and must be confirmed.

Followup studies will confirm these reactions, test more accessions from both groups, and assess protein expression levels to ensure that we are not missing recognition due to lack of expression or stability. We have obtained *N. tabacum eds1* RNAi lines from B. Baker; we can use to these to test whether recognition of XopJ4, XopQ, and XopX in this species is likely to depend on TIR-NB-LRR proteins (Wiermer et al., 2005).

Materials and Methods

Bacterial strains and growth conditions

The fourteen sequenced field isolates are listed in Table 3-1. All were collected by Jeffrey B. Jones and Robert E. Stall of the University of Florida. Strains *Xp* 91-118 (Tudor-Nelson et al., 2003) and 4B were also provided by Jones and Stall. *Xp* 4B was isolated during a field trial on *Bs2*-containing tomatoes in 2007 but contains an intact *avrBs2* effector gene. Rifampicin-resistant mutants were selected for all strains to facilitate selective growth for DNA extractions, conjugations, and in planta growth assays.

Escherichia coli and *Agrobacterium tumefaciens* strains were grown on Luria agar supplemented with the appropriate antibiotics at 37°C and 28°C, respectively. *Xanthomonas perforans* strains were grown on nutrient yeast glycerol agar (NYGA) at 28°C. Bacterial DNA transformation was conducted using chemically competent *E. coli* (New England Biolabs, Ipswich, MA). Plasmids were introduced into *A. tumefaciens* GV3101 or *X. perforans* 4B by triparental mating with a helper *E. coli* strain containing the pRK600 plasmid.

Illumina sequencing, genome assembly and effector prediction

Genomic DNA was prepared from field isolates using the QIAamp DNA Mini Kit (QIAGEN Sciences, Germantown, MD, USA). Quality control, shearing, library preparation, and sequencing were carried out as described by Bart et al. (2012). Assemblies were performed on CLC Genomics Workbench v 4.9 using the *de novo* assembly algorithm with a length fraction of 0.9 and a similarity of 1.0. Resulting assemblies ranged from 50 to 132 contigs per genome, with each contig representing at least 100-fold sequence coverage. For effector prediction, we used the database and pipeline described by Bart et al. (2012). Computational analyses were performed by Andrew Kassen and Rebecca Bart.

Construction of effector gene knockouts and transient expression vectors

All primers used to construct and detect effector gene knockouts are listed in Table 3-5. The *xopJ4* gene knockout and transient expression constructs were created as previously described (Chapters 1 and 2). Other knockouts were constructed using similar methods, as outlined in Figure 3-2. Briefly, both upstream and downstream fragments of the effector gene to be deleted were amplified from *Xp* 4B genomic DNA. PCR products were cloned into separate pENTR™/D-TOPO® entry vectors (Life Technologies, Grand Island, NY). Both vectors were digested with NotI and XbaI (*avrBs2* and *xopN* knockouts) or BamHI (*xopAP* and *xopF2* knockouts), allowing ligation of the upstream fragment to the downstream fragment using the Rapid DNA Ligation Kit (Roche Applied Science, Indianapolis, IN). Ligation products were transformed into NEB 5-alpha cells. Sanger sequencing was used to verify that the resulting entry vector contained both upstream and downstream inserts. The entry vector was then introduced into a Gateway®-compatible pLVC18 destination vector using LR Clonase™ (Life Technologies). The pLVC18 vector contains a tetracycline resistance gene, as well as the *sacBR* genes, which confer sensitivity to sucrose in Gram-negative bacteria. The *sacBR* genes allow for the construction of unmarked deletions, since cells that have undergone double homologous recombination show restoration of sucrose resistance (Logue et al., 2009).

The resulting pLVC18 suicide vector containing both upstream and downstream fragments of the effector gene was conjugated into *Xp* 4B. Exconjugants (merodiploid strains) were selected on NYGA supplemented with 100 μg/ml rifampicin and 10 μg/ml tetracycline and were also checked for sucrose sensitivity. One merodiploid strain was grown overnight on rifampicin alone, then plated at low density on NYGA with rifampicin and 5% sucrose. Several resulting colonies were checked for deletion of the effector gene (since reversion to wild-type is also possible) using PCR with primers annealing within the upstream and downstream sequences (Figure 3-3). Sanger sequencing was used to verify PCR products from candidate knockout strains. Douglas Dahlbeck constructed the Δ*hrcV* strain. The *hrcV* gene in this strain was not deleted, but rather disrupted, by the integration of a pLVC18 suicide vector containing the sequence from 4-960 bp of the 1923-bp *hrcV* ORF, as well as kanamycin resistance.

To construct vectors for *Agrobacterium*-mediated transient expression of effectors, the full open reading frame of each effector, with its stop codon replaced by a SpeI restriction site (to facilitate possible future cloning projects), was cloned into a pENTR™/D-TOPO® entry vector. Using LR Clonase™, the entry vector was then introduced into the Gateway®-compatible pMD1 destination vector containing the 35S promoter and a C-terminal HA tag (See Chapter 2). The resulting construct was conjugated into *A. tumefaciens* GV3101.

Plant inoculations

Agrobacterium-mediated transient expression assays were carried out as described in Chapter 2. To assess the ability of effector knockout strains to produce disease symptoms in susceptible tomato, whole leaves of five-week-old FL8000 plants were dipped for one minute in a gently stirring inoculum containing 10^6 cfu/ml of *Xp* 4B in 1 mM $MgCl_2$ and 0.025% Silwet L-77 surfactant. In order to simulate field conditions, inoculated plants were kept in constant humidity for approximately 36 h, and then kept under humidity only at night (12 h) for 7 days. Disease symptoms were evaluated 7-10 days post inoculation.

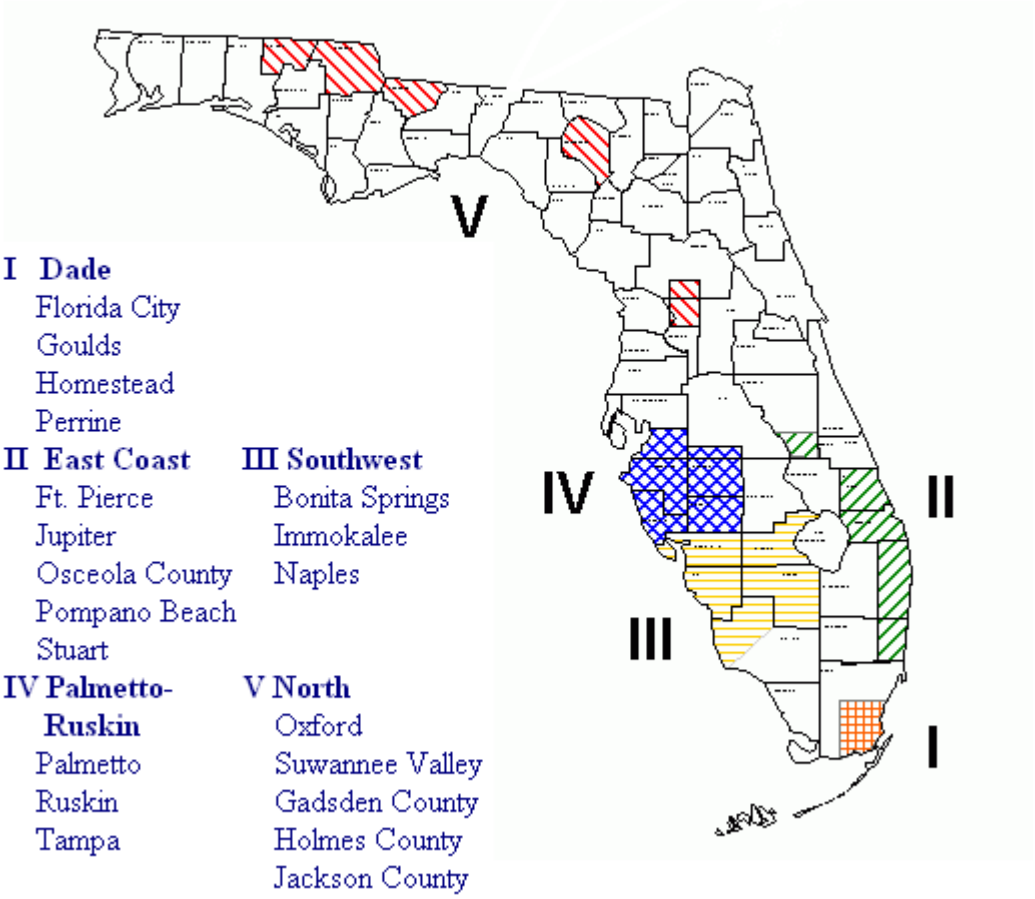


Figure 3-1. Map of the state of Florida showing the five production areas for fresh market tomatoes. Counties within each production area are listed on the left. Figure courtesy of J. B. Jones.

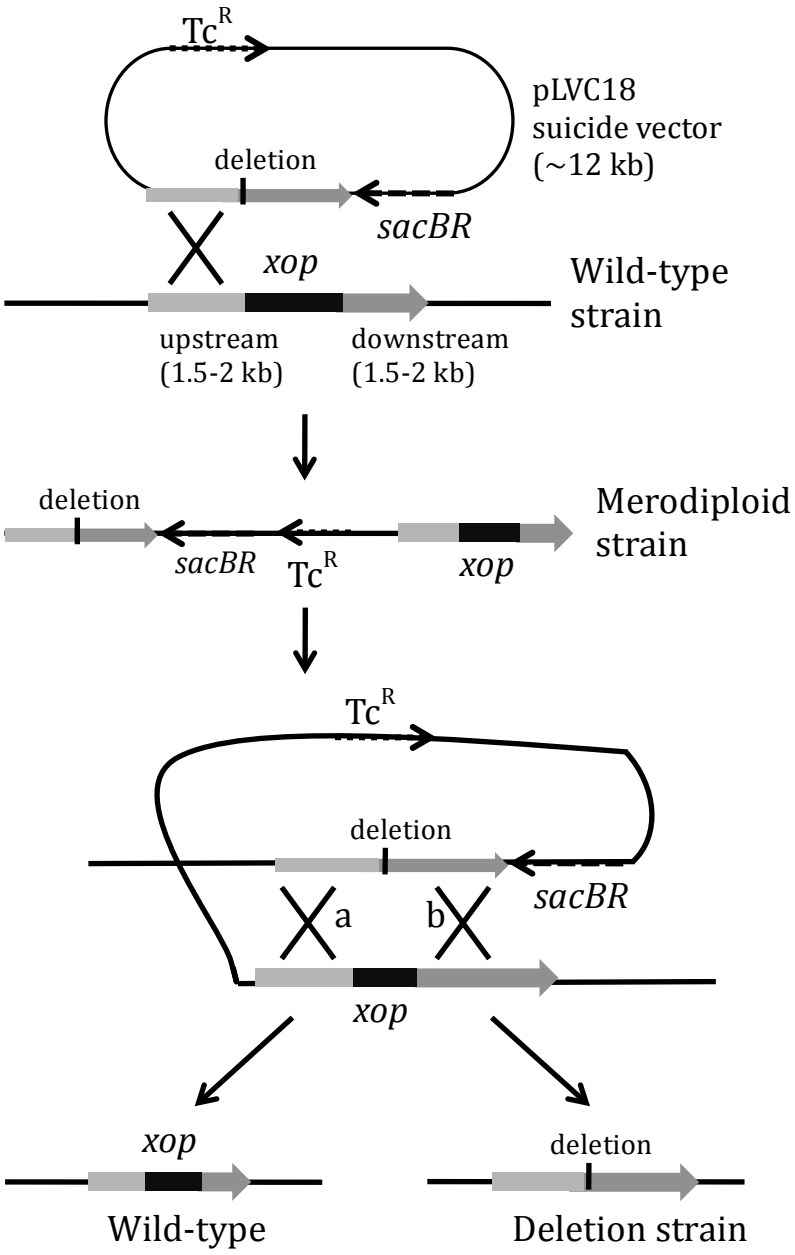


Figure 3-2. Procedure for generating unmarked deletions of type III effector genes in *X. perforans* 4B. Integration of the suicide vector into the chromosome by homologous recombination results in a merodiploid strain (initial exconjugant). Exconjugants are selected on tetracycline (Tc) and checked for sucrose sensitivity. Excision of the vector from the genome by another recombination event results in a sucrose-resistant and Tc-sensitive strain that is either wild-type or contains a deletion at the site of the effector gene. These two outcomes can be distinguished by PCR. Figure adapted from Logue et al. (2009).

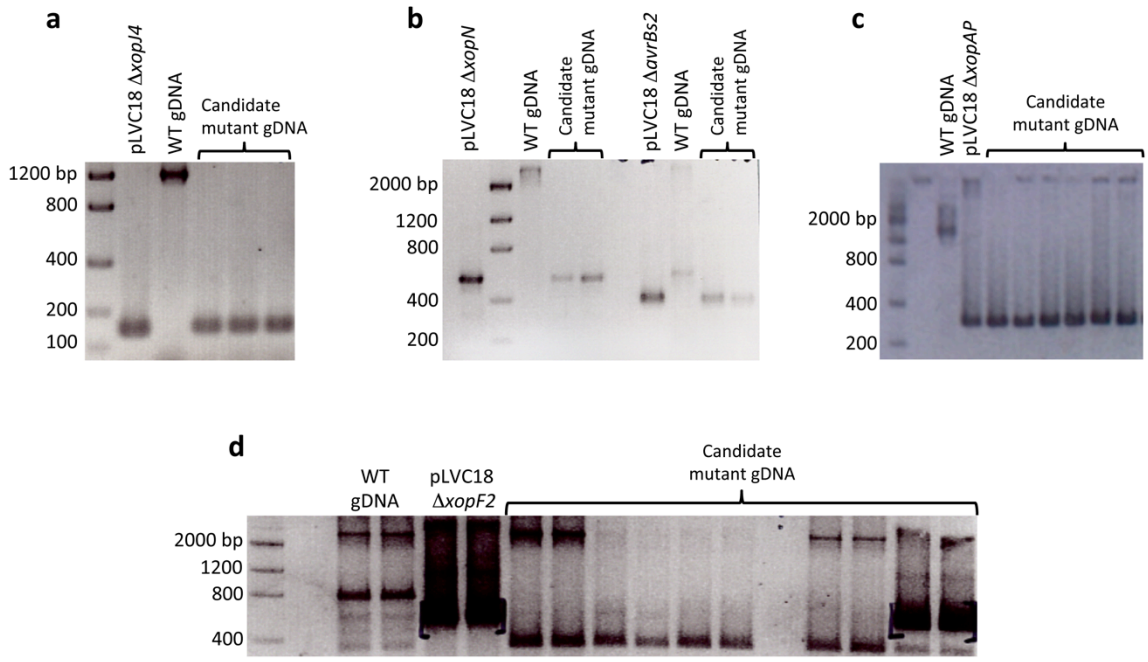


Figure 3-3. PCR reactions used to detect effector gene knockouts in *Xp* 4B. Reaction products were separated on 2.5% agarose gels and stained with ethidium bromide. Band sizes in ladder are indicated in base pairs (bp). gDNA, genomic DNA.
a Detection of *xopJ4* deletion using primers *xopJ4* -50 F and *xopJ4* +50 R. **b** Detection of *xopN* deletion using primers *xopN* -201 F and *xopN* +359 R. Detection of *avrBs2* deletion using primers *avrBs2* -219 F and *avrBs2* +282 R. **c** Detection of *xopAP* deletion using primers *xopAP* -246 F and *xopAP* +230 R. **d** Detection of *xopF2* deletion using primers *xopF2* -393 F and *xopF2* +29 R.

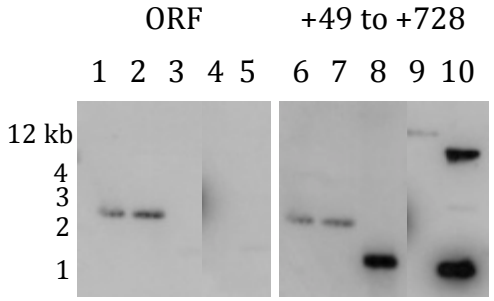


Figure 3-4. Southern blot of *X. perforans* 4B genomic DNA showing deletion of the *xopJ4* effector gene. Genomic DNA was digested with BstBI and separated by gel electrophoresis. DNA was then hybridized to a nylon membrane and probed with the following ^{32}P-labeled PCR products: the full 1,080-bp open reading frame (ORF) of *xopJ4* (left panel) or a 680-bp downstream fragment (+49 to +728) of *xopJ4* (right panel). The wild-type copy of *xopJ4* is present in a 2298-bp restriction fragment (both panels), while the deletion of *xopJ4* results in a 1252-bp fragment that hybridizes to the downstream sequence (right panel). Lanes 1 and 6, *Xp* 4B WT; lanes 2 and 7, merodiploid exconjugant containing pLVC18 Δ*xopJ4*; lane 3 and 8, *Xp* 4B Δ*xopJ4*; lanes 4 and 9, pLVC18 destination vector; lanes 5 and 10, pLVC18 Δ*xopJ4*.

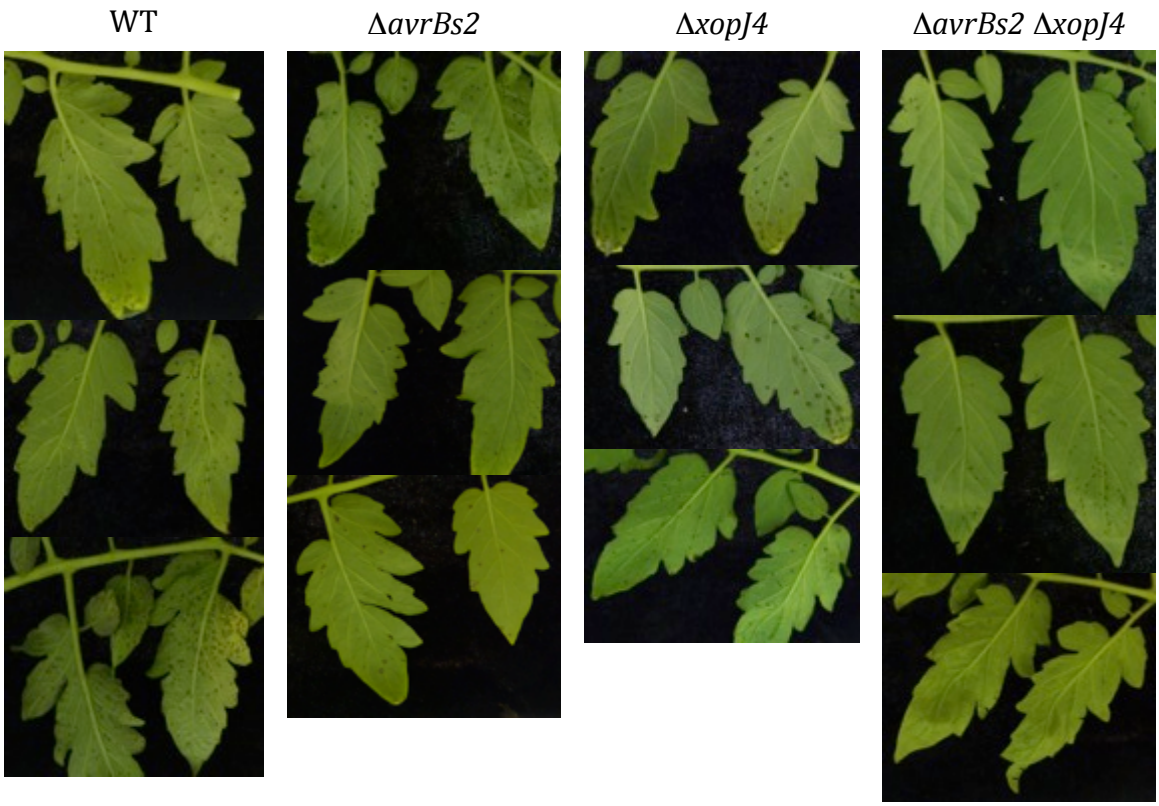


Figure 3-5. A strain lacking both the *avrBs2* and *xopJ4* effector genes may show a synergistic loss of virulence on susceptible tomato plants. Whole leaves of five-week-old FL8000 tomato plants were dip-inoculated with *X. perforans* 4B at a concentration of 1 x 10^6 cfu/ml. Photographs were taken at 16 dpi.

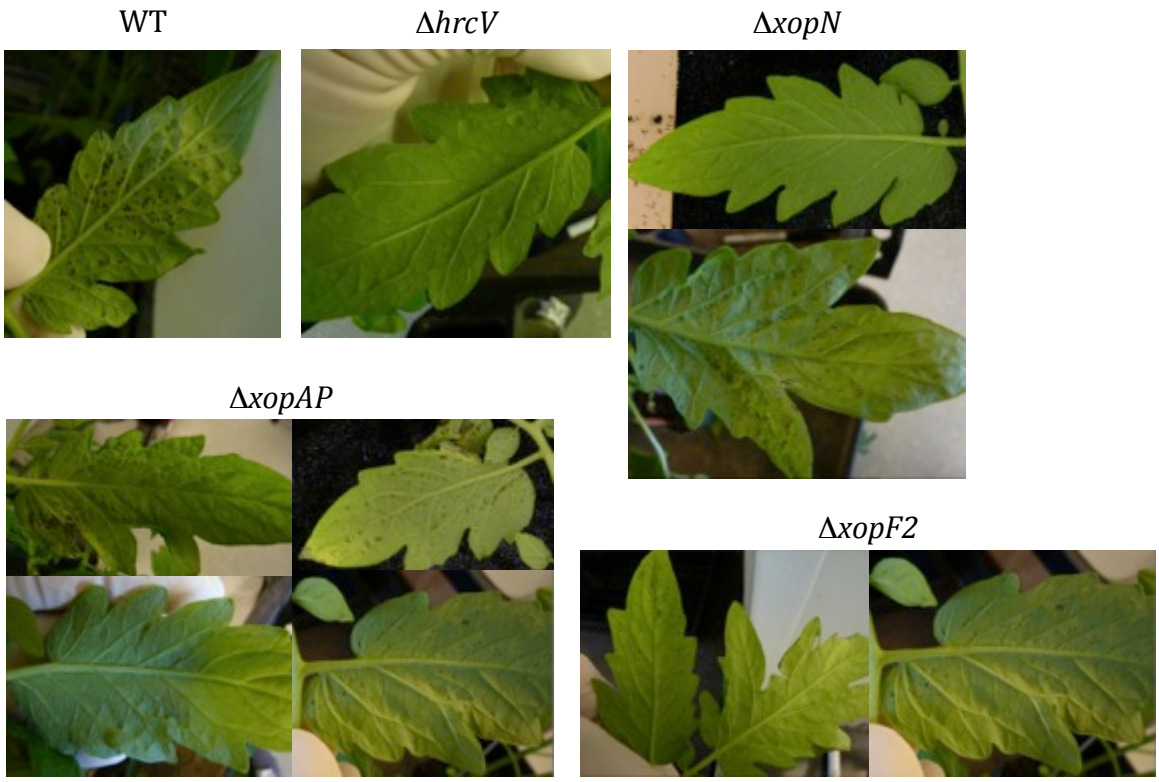


Figure 3-6. Possible reduced virulence of strains lacking the effector genes *xopAP* and *xopF2*. Whole leaves of five-week-old FL8000 tomato plants were dip-inoculated with *X. perforans* 4B at a concentration of 1 x 10^6 cfu/ml. Photographs were taken at 9 dpi. The Δ*hrcV* and Δ*xopN* mutant strains are shown as controls for lack of effector secretion and a mutant with a known virulence role, respectively.

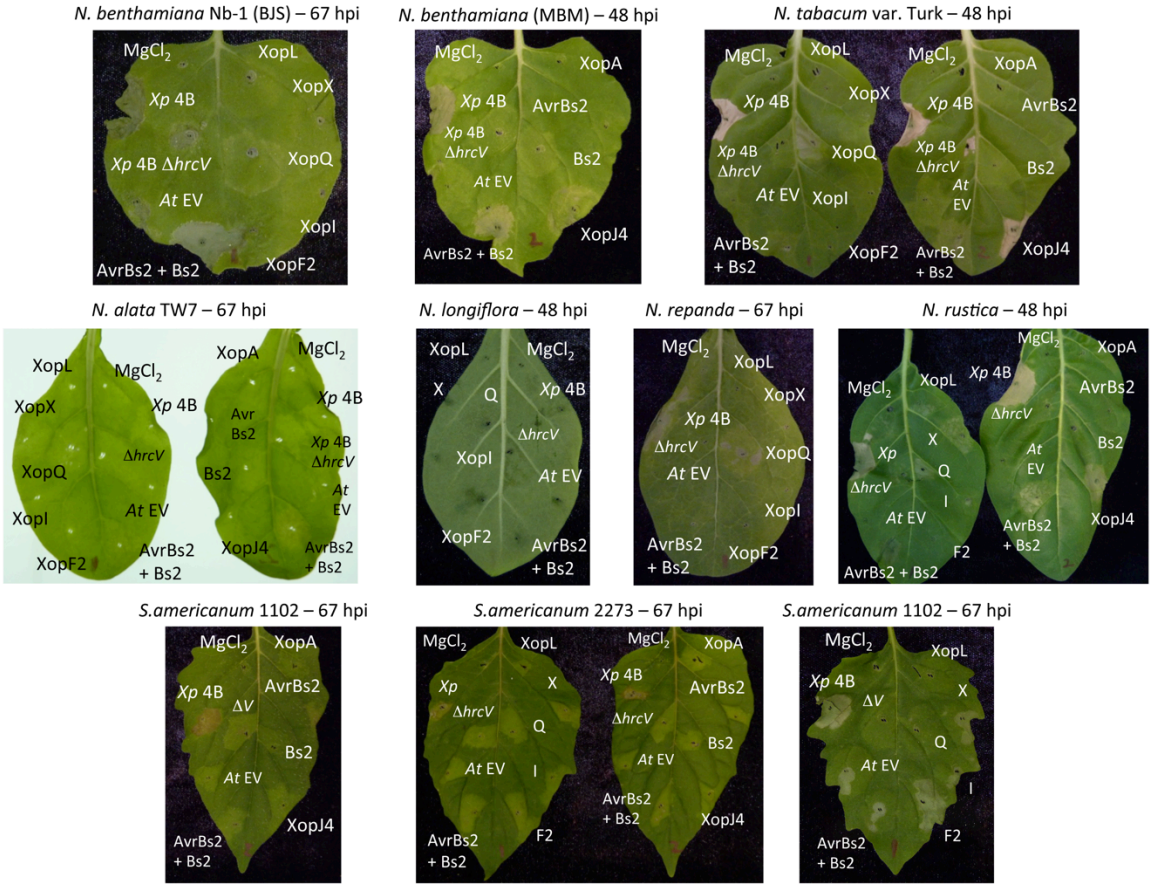


Figure 3-7. Hypersensitive responses (HR) of *Nicotiana* and *Solanum americanum* accessions to *Agrobacterium*-mediated transient expression of *X. perforans* core effectors. Leaves were hand-infiltrated with *X. perforans* at a concentration of 3 x 10^8 cfu/ml and *A. tumefaciens* at a concentration of 5 x 10^8 cfu/ml. $MgCl_2$ is shown as a control for responses to mock inoculation. Lack of HR in response to *Xp* 4B Δ*hrcV* (Δ*V*) indicates that HR is due to recognition of one or more type III effectors secreted by *Xp* 4B. *A. tumefaciens* GV3101 strains contain the empty vector pE1776, pE1776 Bs2-HA, or pMD1 carrying C-terminally HA-tagged AvrBs2, XopL, XopX (X), XopQ (Q), XopI (I), XopF2 (F2), XopA, or XopJ4 under the control of the 35S promoter. Photographs were taken at 48 or 67 hpi as indicated, and show the adaxial leaf surfaces, except for *N. alata* TW7 and *N. longiflora*, which show the abaxial surfaces, since HR in these accessions was weak and difficult to visualize

Table 3-1 *Xanthomonas perforans* field isolates used in this study

Strain	Collection date	County	Production zone	Fruit type
1-7	10/4/06	Gadsden	V	Large fruit
2-12	10/4/06	Gadsden	V	Large fruit
3-15	10/4/06	Decatur (Georgia)	V	Large fruit
4-20	10/4/06	Decatur (Georgia)	V	Large fruit
5-6	10/4/06	Decatur (Georgia)	V	Large fruit
7-12	10/16/06	Manatee	IV	Large fruit
8-16	10/16/06	Manatee	IV	Large fruit
9-5	10/16/06	Manatee	IV	Large fruit
10-13	10/16/06	Manatee	IV	Large fruit
11-2	10/19/06	Palm Beach	II	Heirloom varieties
15-11	11/29/06	Miami-Dade	I	Plum
17-12	12/5/06	Collier	III	Roma
18-15	12/5/06	Collier	III	Grape
19-10	12/6/06	Collier	III	Grape

Table 3-2 Type III effector repertoires of *Xanthomonas perforans* field isolates

Production zone	N/A	N/A	V					IV				II	I	III		
Strain	4B	91-118	1-7	2-12	3-15	4-20	5-6	7-12	8-16	9-5	10-13	11-2	15-11	17-12	18-15	19-10
AvrBs2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
XopA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
XopAP	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
XopAR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
XopC2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
XopE1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
XopF1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
XopF2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
XopI	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
XopJ4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
XopK	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
XopL	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
XopN	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
XopQ	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
XopR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
XopV	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
XopX	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
XopAK	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
XopD	ψ	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
SKWP3	/	?	+	?	+	+	+	+	+	+	+	+	+	/	/	+
SKWP4	/	/	+	+	+	+	+	+	+	+	+	+	+	/	/	+
XopAD	+	+	+	+	+	+	+	+	+	+	+	+	+	/	/	+
XopE2	+	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+
XopJ2	+	+	+	/	+	+	/	+	+	+	+	+	+	/	+	+
XopP	ψ	+	+	+	/	/	+	+	+	/	+	/	+	+	+	+
XopZ1	ψ	+	/	/	/	+	/	+	+	/	+	+	+	/	/	/
XopAE	+	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/
XopAF	+	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/

Effector repertoires were deduced on the basis of homology to known animal and plant pathogen type III effector proteins. Seventeen core effectors are present in all sequenced field isolates (purple box). +, presence of homolog; /, absence of homolog; ψ, inversion or pseudogene; ?, cannot be determined using current sequence data.

Table 3-3 Core type III effectors of *Xanthomonas perforans* selected for gene knockouts and transient expression assays

Effector	Gene knockout	Transient expression	Function	Reference
AvrBs2	✔	✔	Glycerophosphoryl diester phosphodiesterase	(Kearney and Staskawicz, 1990)
XopA	In progress	✔	Putative harpin	(Noël et al., 2002)
XopAP	✔		Lipase class III	(Mukaihara et al., 2010)
XopF2	✔	✔	Unknown	(Roden, Belt, et al., 2004)
XopI		✔	F-box domain	(Potnis et al., 2011)
XopJ4	✔	✔	Putative acetyltransferase/SUMO protease	This study, (Roden, Eardley, et al., 2004)
XopL		✔	E3 ubiquitin ligase	(Singer et al., 2013)
XopN	✔		ARM/HEAT repeat	(Kim et al., 2009)
XopQ	In progress	✔	Inosine uridine nucleoside N-ribohydrolase	(Roden, Belt, et al., 2004)
XopX	In progress	✔	Unknown	(Metz et al., 2005)

Table 3-4 Hypersensitive responses (HR) of *Nicotiana* and *Solanum americanum* accessions to *Agrobacterium*-mediated transient expression of *X. perforans* core effectors

	AvrBs2 + Bs2	AvrBs2	XopI	XopJ4	XopF2	XopL	XopQ	XopX
***N. benthamiana* Nb-1 (BJS)**	++	-	-	-	-	-	$+^{L}$	$+^{L}$
***N. benthamiana* (MBM)**	++	-	-	+++	-	-	-	$+^{L}$
***N. tabacum* var. Turk**	++	-	-	+++	-	-	+++	$+^{P}$
***N. alata* TW7**	$+^{L}$	-	-	+	-	-	$+^{N}$	-
N. longiflora	+	-	-	-	+	-	-	-
N. repanda	+	-	-	-	-	-	$++^{L,P}$	-
N. rustica	++	-	-	+++	-	$++^{P}$	+++	$++^{P}$
***S. americanum* 1102**	+	+	-	-	-	-	-	-
***S. americanum* 2273**	+	+	-	-	$+^{L}$	-	-	$+^{L}$
***S. americanum* 268152**	++	-	$+^{L,N}$	-	++	-	-	$++^{N}$

-, no reaction; +, weak HR; ++, moderate HR; +++, strong HR. L, late HR (3 dpi); N, possibly nonspecific; P, patchy. Reactions were observed at ~48 hpi unless otherwise noted. Boxes shaded in gray represent reactions observed in two independent experiments.

Table 3-5 PCR primers used in this study

Primer name	Sequence (5'-3')
avrBs2 -1808 F	*cacc*CCGTCACATAGCGTGGTT
avrBs2 -21 XbaI R	**TCTAGA**GGGCGGCCAGTAAAGCGCGTG
avrBs2 +84 XbaI F	*cacc***TCTAGA**AGGGATTGGGAGAGATCA
avrBs2 +2079 R	CGCACATGAAGCCGCCAAA
avrBs2 -219 F	GCCATGCTGCCGTTCAAGGAAACGC
avrBs2 +282 R	GCTGCGGGTCATTCCGATCAGGCCAC
hrcV 4 F	*cacc*GCGAGCGACAAGGTATTTCG
hrcV 960 R	GCGCCAGATCGTATAGCCAC
xopA F	*cacc*ATGATCAATTCATTGAATACG
xopA SpeI R	**ACTAGT**CTGCATCGATGCAGTGTCGC
xopAP -1902 XhoI F	*cacc***CTCGAG**CTCCACACCACGCGATAG
xopAP -62 BamHI R	CGTC**GGATCC**GGCGCAGCACTGAGACCTGG
xopAP +106 BamHI F	*cacc***GGATCC**CCGGCTCCAGGCACTGCTTG
xopAP +2086 EcoRI R	CGTC**GAATTC**GTGATCAGCGCTCCCTCCAG
xopAP -246 F	GATATAAACGAATCTCTGCG
xopAP +230 R	CATCAGCGCAAATACACAGG
xopF2 -1615 F	*cacc*GTCGACCGTCGTTTTCGTTCAGCC
xopF2 -73 BamHI R	CGTC**GGATCC**GAATGGTTCGCTTGCGTG
xopF2 +27 BamHI F	*cacc***GGATCC**GGCCATAACCAAACGCACGC
xopF2 +970 R	CGTCTCTAGACAACGACCATGACGACACCAG
xopF2 F	*cacc*ATGAAGCTCCAACGCCAGAAC
xopF2 SpeI R	**ACTAGT**AGGCCTACCCTGTTGCCACTG
xopF2 -393 F	GGTGGCTTCGTTCTTTTC
xopF2 +29 R	GCGTGCGTTTGGTTATGG
xopI F	*cacc*ATGCCGATCACCCGAACCGG
xopI SpeI R	**ACTAGT**CATGTCCATATACCTGCGCG
xopJ4 -50 F	CCAATTCTGGGTCAGGCAT
xopJ4 +50 R	AGGGTGATTATGCACTTATTC
xopL F	*cacc*ATGCCACCAACCGAGGGC
xopL SpeI R	**ACTAGT**CTGATGGCCTGAAGGTTCCGG
xopN -1746 F	*cacc*CAACGCGCCAACGAAAAA
xopN -19 XbaI R	**TCTAGA**GTATACGGCAGATAGATTGC
xopN +2 XbaI F	*cacc***TCTAGA**GTGATGATCCGGCATGTT
xopN +1971 R	GCTGGCGTTCTTGATGTA
xopN -201 F	CATACGCGTCTGGTAGTTC
xopN +359 R	CTTGCACACATGTCGCTCAC
xopQ F	*cacc*ATGCAGCCCACCGCAATCCG
xopQ SpeI R	**ACTAGT**GCGCCCGCGTTGCCCCTCGTC
xopX F	*cacc*ATGGAGATCAAGAAACAGC
xopX SpeI R	**ACTAGT**GGACGAAGGTGCAGTGCTGG

The 5' CACC in the forward primers is shown in lower-case, italicized letters and is required for introducing PCR products into the TOPO® entry cloning system. Restriction sites are shown in bold.

Chapter 4:
Screening wild *Solanum* germplasm for resistance to *Xanthomonas perforans*

Introduction

Ten thousand years ago, humans began the grand evolutionary experiment now known as the domestication of food crops from wild plant species. Selection for desirable agricultural traits was accompanied by dramatic genetic bottlenecks that resulted in cultivated germplasm with greatly reduced diversity compared to wild populations (Tanksley and McCouch, 1997). This low diversity has limited the variation available to breeders and has sometimes had dire consequences, most famously in the case of the Irish potato famine in the nineteenth century (Strange and Scott, 2005). The creation of seedbanks for wild relatives of crop species during the late twentieth century was spurred in part by the corn leaf blight outbreak in the Southern United States in 1970. Today there are more than 700 seed banks worldwide, representing over 2.5 million entries. These include at least 30,000 tomato accessions, about seventy percent of which are wild species (Tanksley and McCouch, 1997).

Collections of wild tomato relatives have been successfully utilized to discover new sources of genetic resistance to several agronomically important diseases, includding *Tomato yellow leaf curl virus* (Tomás et al., 2011). Although wild pepper accessions have been screened for resistance to bacterial spot disease, sources of resistance among wild tomatoes remain relatively unexplored (Stall et al., 2009).

Two known gene-for-gene resistances to bacterial spot disease of tomato, *Bs2* and *RXopJ4*, have already been identified (Tai et al., 1999; Sharlach et al., 2012). Transgenic *Bs2* tomato plants have been field-tested (Horvath et al., 2012), while the *RXopJ4* gene has not been definitively identified, but will be confirmed and tested in transgenic tomato plants in the near future (Chapter 2). Together, these two genes are likely to provide more durable resistance to disease caused by *X. perforans* in the southeastern United States than either individual gene, based on preliminary observations of reduced symptom development with a Δ*avrBs2* Δ*xopJ4* double mutant strain (Chapter 3). However, more resistance genes will be necessary to combat the ability of the pathogen to evade detection by mutating recognized effector genes.

In order to identify new sources of resistance to bacterial spot disease, we performed disease assays on 224 wild accessions of *Solanum* from the collection of the Tomato Genetics Resource Center. We identified nine candidate resistant accessions and verified four of them by quantifying levels of bacterial growth. Two of the accessions, *S. habrochaites* LA2860 and *S. pimpinellifolium* LA0722, also exhibited HrcV-dependent HR in response to *Xp* 4B, suggesting the recognition of a type III effector by these accessions. Future efforts will focus on verifying disease

resistance, determining effector recognition, assessing prospects for durability, and crossing resistant accessions to FL8000 to begin the process of gene mapping.

Results and Discussion

Screening wild *Solanum* accessions for bacterial spot disease resistance using *X. perforans* 4B

We have screened a total of 224 wild *Solanum* accessions of the 1,196 present in the collection of the C. M. Rick Tomato Genetics Resource Center (TGRC) (Table 4-1). These constitute approximately one-fifth of the accessions and include fourteen of the seventeen species present in the entire collection. Initially, 139 accessions were screened using a hand infiltration method. This method proved to be problematic for several reasons. First, hand-infiltration is time-consuming and difficult. Some species are more amenable to infiltration than others; species with narrow, deeply lobed leaflets, such as *S. chilense*, were especially challenging. Second, disease lesions resulting from this method were very small and hard to visualize, particularly in photographs. Third, lesions usually did not appear until at least ten and sometimes fourteen days post infiltration. Because of these difficulties, a considerable number of accessions were initially scored as resistant, but many of these were found to be susceptible upon re-inoculation and/or quantification of bacterial growth.

Therefore, we later turned to a dip inoculation method. This method more closely mimics natural conditions than hand infiltration, since the bacteria must survive on the leaf surface and gain entry into leaves through stomata. We facilitated bacterial survival and lesion development by keeping the plants in constant humidity for 36 hours after inoculation, and in humidity at night for five days thereafter. This resulted in dramatically more robust and reliable disease symptoms than hand infiltration. We used dip inoculation to re-screen eight accessions originally deemed resistant (LA0722, LA2113, LA2860) or tentatively susceptible (LA1317, LA1319, LA1325, LA1326, LA1731). Of these, six accessions – LA2113, LA1317, LA1319, LA1325, LA1326 and LA1731 – were revealed as susceptible by dip inoculation, while two – LA0722 and LA2860 – were confirmed to be resistant. We also screened an additional 85 accessions by dip inoculation and discovered seven more candidate resistant accessions (Table 4-1, Figure 4-1).

The geographic origins and collection years of the nine candidate resistant accessions are shown in Figure 4-2. The three strictly wild accessions, *S. habrochaites* LA2860, *S. neorickii* LA1329, and *S. pimpinellifolium* LA0722, as well as three of the *S. lycopersicum* accessions (LA2308, LA2670, and LA2845) were collected in Ecuador or Peru. This region of the Andes mountains is the center of origin of tomato; the crop experienced a severe domestication bottleneck that resulted in reduced genetic diversity when it was brought to Central America, where its earliest cultivation probably occurred (Bai and Lindhout, 2007). Two of the other

resistant *S. lycopersicum* accessions were collected in Brazil, while one was collected in Sri Lanka. The Sri Lankan accession, LA2703, is listed as a Latin American cultivar in the TGRC record. The five other *S. lycopersicum* accessions are described as *S. lycopersicum* var. *cerasiforme*, or cherry tomato, which likely resulted from hybridization of cultivated tomato and the compatible red-fruited wild species *S. pimpinellifolium*, and is known to possess greater genetic diversity than *S. lycopersicum* (Ranc et al., 2008). Quantitative resistance to bacterial spot disease has already been described in *S. lycopersicum* var. *cerasiforme* PI 114490 (Hutton et al., 2010).

Anecdotally, *S. lycopersicum* var. *cerasiforme* LA2402, one of the Brazilian accessions, may exhibit disease resistance; according to the TGRC record, it was "said to be harvested in [Oswaldo Rockenback's] home garden and produced during a long time up to 8 kg, no diseases apparent." Genetic resistance to powdery mildew (*Oidium neolycopersici*) has been characterized in the *S. lycopersicum* var. *cerasiforme* accession LC-95 from Ecuador (Bai et al., 2008). The other wild species, *S. habrochaites*, *S. neorickii*, and *S. pimpinellifolium*, have also proven to be valuable sources of disease resistance to pests and pathogens. *S. neorickii* (formerly *Lycopersicon parviflorum*) possesses powdery mildew resistance (Bai et al., 2003), while resistance to *Tomato yellow leaf curl virus* (Tomás et al., 2011) and *Phytophthora infestans* (Johnson et al., 2012) have been identified in *S. habrochaites*, and spider mite resistance was found in *S. pimpinellifolium* TO-937 (Salinas et al., 2012).

In fact, the *S. pimpinellifolium* accession PI128216 is known to possess the *Xv3* resistance gene, which recognizes the *Xp* effector AvrXv3 [also known as XopAF (Potnis et al., 2011)]; unfortunately, this resistance is unlikely to be useful in the field. It seems that grape tomatoes containing *Xv3* have already applied selective pressure to reduce the prevalence of *avrXv3* among pathogen populations (Stall et al., 2009). The draft genome sequence of *Xp* 4B suggests that it contains *avrXv3* (Chapter 3), but it probably does not produce an active protein, since *Xp* 4B is virulent on FL8000 plants, which contain *Xv3* (Chapter 1). Since *Xp* 4B is also predicted to contain several other non-core effectors and pseudogenes (Chapter 3), it may not be the most useful strain for disease resistance screening. Field isolate 17-12 may be a wiser choice for future studies, since it contains only four non-core effectors. The candidate resistant accessions identified here can be screened with this strain to help determine whether they may recognize core *Xp* effectors. Since all three of the wild species evaluated here are sexually compatible with *S. lycopersicum*, at least when used as the male parent (Lefrancois et al., 1993), crosses have been initiated to enable future mapping studies.

Quantification of bacterial growth in candidate resistant accessions

We used bacterial growth assays to confirm and quantify the disease resistance of four candidate resistant accessions – *S. pimpinellifolium* LA0722, *S. lycopersicum* LA1543, *S. neorickii* LA1329, and *S. lycopersicum* LA2845 (Figure 4-3). All four

accessions supported lower levels of bacterial growth than the commercial tomato line *S. lycopersicum* FL8000, which served as a susceptible control, but showed higher levels than transgenic VF36 *Bs2* plants, which are known to recognize the *Xp* 4B effector AvrBs2 and thus served as a resistant control. *S. pimpinellifolium* LA0722 was tested several times in different individual plants by dip inoculation and never exhibited disease lesions; its resistance was also confirmed in two independent growth assays (Figure 4-3a,b). *S. neorickii* LA1329 shows only five-to-ten-fold lower bacterial growth than FL8000, on average; this does not exclude the presence of a gene-for-gene resistance in this accession but should be confirmed by re-testing. *S. lycopersicum* LA2845 appears to support ten-fold lower levels of bacteria than FL8000 (Figure 4-3c), but this result was based on a single leaflet; this should also be confirmed by re-testing with more replicates.

Disease resistance in *S. pimpinellifolium* LA0722 and *S. habrochaites* LA2860 is accompanied by a hypersensitive response

Gene-for-gene resistance is usually, but not always, associated with a hypersensitive response (HR), a rapid programmed cell death that occurs at the site of infection and can be visualized macroscopically when a high inoculum of bacteria is used under laboratory conditions (Mur et al., 2008). We reasoned that an HR in response to *Xp* 4B inoculation in candidate resistant accessions would serve as a confirmation of a gene-for-gene resistance and possibly reveal, through the use of effector gene knockout strains (Chapter 3), which effector was recognized by each accession.

We tested *S. pimpinellifolium* LA0722, *S. neorickii* LA1329, *S. lycopersicum* LA1543, and *S. habrochaites* LA2860 for HR induced by *Xp* 4B inoculation. Only LA0722 and LA2860 exhibited HR (Figure 4-4, data not shown). The lack of HR in the other two accessions does not exclude the possibility of gene-for-gene resistance; indeed, LA1543 exhibited ~50-fold lower levels of bacterial growth than FL8000 (Figure 4-3a). Furthermore, HR and disease resistance are known to be genetically separable, although HR may be involved in signaling leading to systemic acquired resistance (Coll et al., 2011).

HR was observed in LA0722 in response to WT *Xp* 4B as well as five single effector knockout strains; only the Δ*xopJ4* strain was tested in LA2860, but it also incited an HR. Importantly, a Δ*hrcV* strain did not elicit HR in either accession, indicating that type III secretion, and thus delivery of a type III effector protein, is required for the HR. In the future, the construction of more effector knockout strains should reveal which effector is recognized by each of these two accessions. Alternatively, these accessions may be inoculated with strains of *Xp* possessing different repertoires of effectors, or *Agrobacterium*-mediated transient expression may be used to determine which effector produces HR, though this assay can be challenging in tomato plants (Wroblewski et al., 2005).

Materials and Methods

Bacterial strains and growth conditions

Xp 4B and its mutant derivatives Δ*hrcV*, Δ*avrBs2*, Δ*xopJ4*, Δ*xopN*, Δ*xopAP* and Δ*xopF2* are described in previous chapters. All strains were grown on nutrient yeast glycerol agar (NYGA) supplemented with 100 μg/ml rifampicin plus 25 μg/ml kanamycin (Δ*hrcV* strain and Δ*xopJ4* complemented strains) at 28°C for 48-72 h prior to inoculation.

Plant inoculations and bacterial growth assays

For disease assays using hand inoculation, leaves were hand-infiltrated with a bacterial suspension containing 1 x 10^4 cfu/ml and 1 mM $MgCl_2$. Plants were placed in a growth chamber at 24°C with 12 h day/night cycle. Disease symptoms were assayed 10-15 days post inoculation. For dip inoculations, whole leaves were dipped for one minute in a gently stirring inoculum containing 10^6 cfu/ml of *Xp* 4B in 1 mM $MgCl_2$ and 0.025% Silwet L-77 surfactant. In order to simulate field conditions, inoculated plants were kept in constant humidity for approximately 36 h, and then kept under humidity only at night (12 h) for five days. Disease symptoms were evaluated 7-10 days post inoculation. For HR assays, leaves were hand-infiltrated with a bacterial suspension containing 3 x 10^8 cfu/ml and 1 mM $MgCl_2$. Responses were observed 24-72 h post inoculation.

To quantify bacterial growth in dip inoculation experiments, whole leaflets were weighed and then homogenized in 2 ml of 1 mM $MgCl_2$ using a mortar and pestle. Appropriate dilutions were plated on NYGA supplemented with 50 μg/ml rifampicin and 50 μg/ml cycloheximide (to prevent fungal growth) and incubated for 3 days at 28°C. Bacterial counts were calculated as colony forming units (cfu) per gram of leaf tissue. Quantification of bacterial growth for hand-infiltration experiments followed a similar protocol, except that 0.8-cm2 punches were taken from leaflets and homogenized in 1 mM $MgCl_2$ in a BeadBeater.

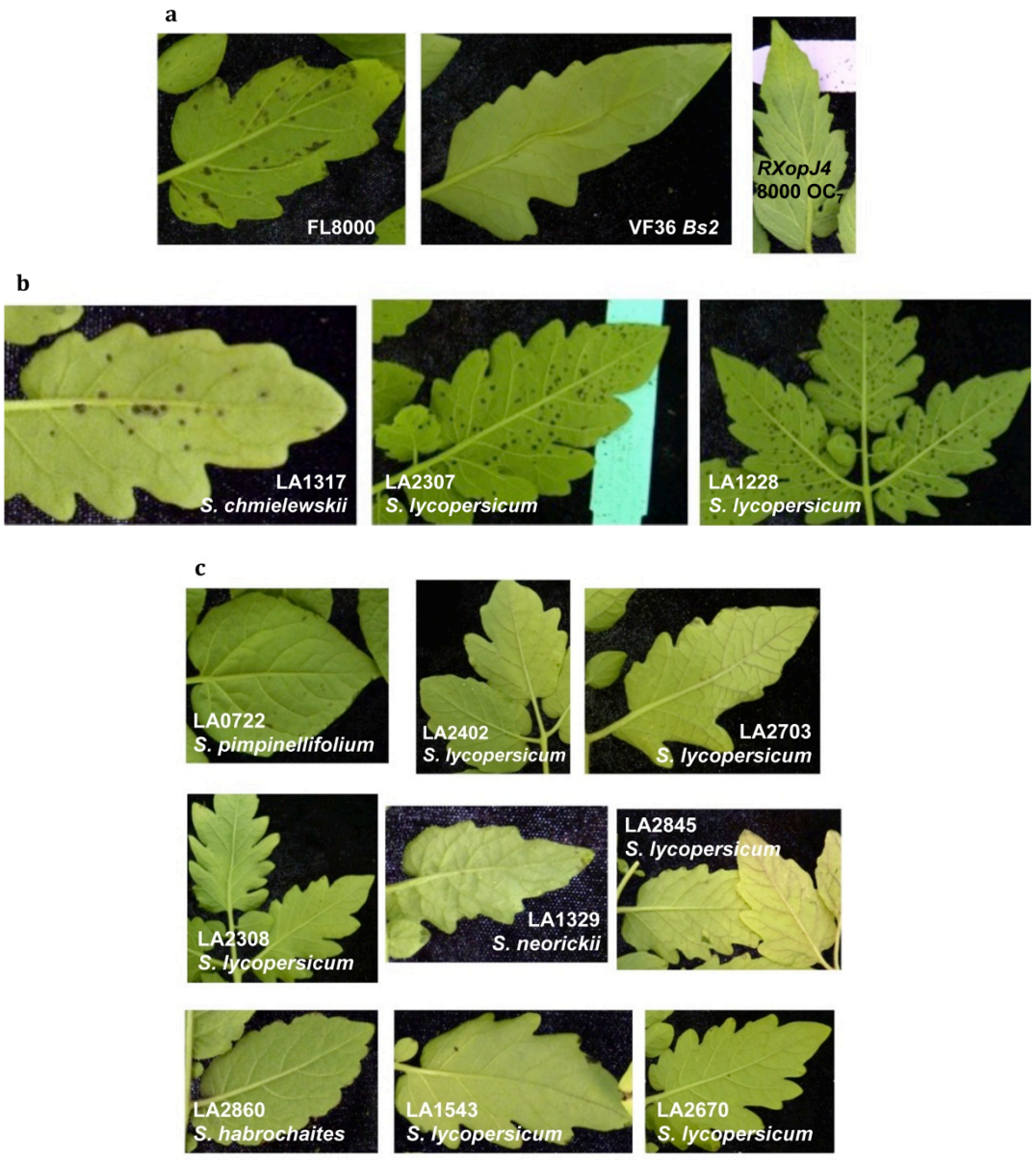


Figure 4-1. Disease phenotypes of wild *Solanum* accessions. Plants were dip-inoculated with *Xp* 4B at a concentration of 10^6 cfu/ml. Photographs were taken 7-10 days post inoculation. **a** Susceptible FL8000 and resistant VF36 *Bs2* and *RXopJ4* 8000 OC_7 control plants. **b** Susceptible wild accessions show varying degrees of symptom development. **c** Candidate resistant accessions show no symptoms of bacterial spot disease.

Accession	Species	Collection Site	Year
LA2860	*S. habrochaites*	Cariamanga, Ecuador	1985
LA1543	*S. lycopersicum*	Upper Parana, Brazil	1973
LA2308	*S. lycopersicum*	San Francisco, Peru	1980
LA2402	*S. lycopersicum*	Florianopolis, Brazil	1981
LA2670	*S. lycopersicum*	Puno, Peru	1984
LA2703	*S. lycopersicum*	Kandy, Sri Lanka	1985
LA2845	*S. lycopersicum*	Mercado Moyobamba, Peru	1986
LA1329	*S. neorickii*	Yaca, Peru	1970
LA0722	*S. pimpinellifolium*	Trujillo, Peru	1959

Figure 4-2. Collection sites of candidate resistant wild accessions. Accession information is from the website of the Tomato Genetics Resource Center at the University of California, Davis: http://tgrc.ucdavis.edu. Maps were constructed by J. Chiu using Google Maps (https://maps.google.com).

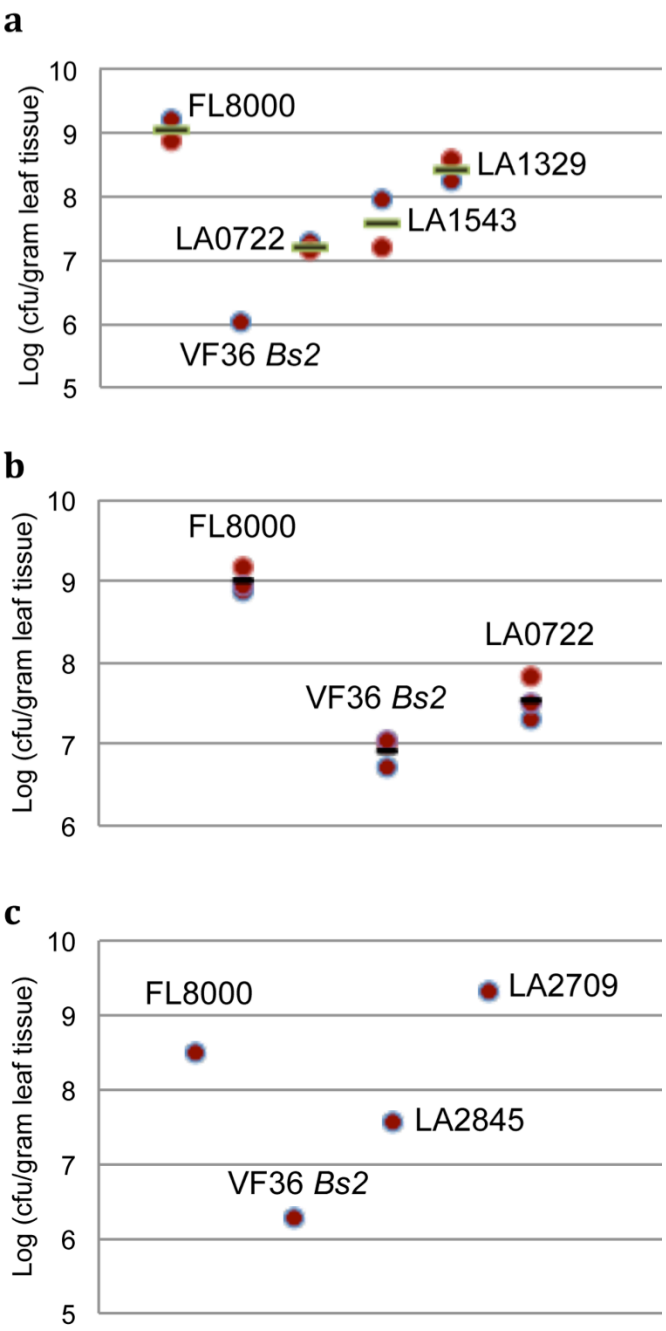


Figure 4-3. Quantification of bacterial growth in candidate resistant wild accessions. Plants were dip-inoculated with *Xp* 4B as described in Materials and Methods. FL8000 and VF36 *Bs2* plants served as susceptible and resistant controls. **a** Bacterial growth was quantified at 7 dpi using one leaflet from each of two plants per accession (red dots); the mean for each accession is represented by a black horizontal line. **b** Bacterial growth was quantified at 10 dpi using three leaflets from the same plant (red dots); the mean for each accession is represented by a black horizontal line. **c** Bacterial growth was quantified at 9 dpi using one leaflet from each plant. *S. lycopersicum* LA2709 developed disease symptoms and served as a susceptible control accession.

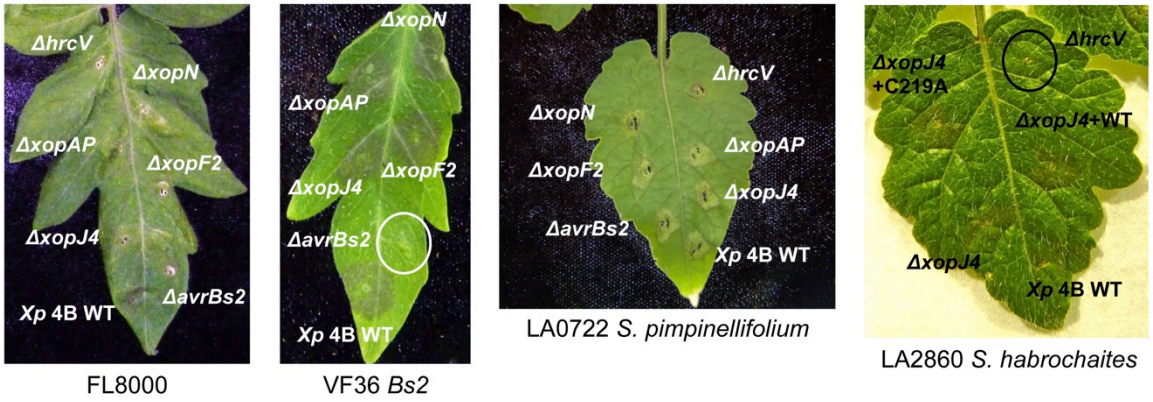


Figure 4-4. Hypersensitive responses of candidate resistant accessions *S. pimpinellifolium* LA0722 and *S. habrochaites* LA2860. Leaves were hand-infiltrated with the indicated *Xp* strains at 3 x 10^8 cfu/ml. Plants were kept in a greenhouse with supplemental lighting. Photographs were taken at 24-72 hpi.

Table 4-1 Disease and hypersensitive response (HR) scores of wild *Solanum* accessions

Accession	Species	Disease Score (Hand infiltration)	Disease Score (Dip inoculation)	HR Score
LA0441	*S. arcanum*	Susceptible	N	N
LA1346	*S. arcanum*	Susceptible	N	N
LA1360	*S. arcanum*	Susceptible	N	N
LA1626	*S. arcanum*	Susceptible	N	N
LA1708	*S. arcanum*	Susceptible	N	N
LA1984	*S. arcanum*	Susceptible	N	N
LA2150	*S. arcanum*	Susceptible	N	-
LA2152	*S. arcanum*	Susceptible	N	N
LA2163	*S. arcanum*	Susceptible[5]	N	N
LA2172	*S. arcanum*	Susceptible	N	N
LA2185	*S. arcanum*	Susceptible	N	N
LA0166	*S. cheesmaniae*	Susceptible[5]	N	-
LA0428	*S. cheesmaniae*	Susceptible	N	N
LA0429	*S. cheesmaniae*	Susceptible	N	N
LA0531	*S. cheesmaniae*	Susceptible[3,5]	N	N
LA1039	*S. cheesmaniae*	Susceptible	N	N
LA1041	*S. cheesmaniae*	Susceptible	N	N
LA1406	*S. cheesmaniae*	Susceptible	N	N
LA1407	*S. cheesmaniae*	Susceptible	N	N
LA1409	*S. cheesmaniae*	Susceptible	N	N
LA1412	*S. cheesmaniae*	Susceptible	N	N

LA1450	*S. cheesmaniae*	Susceptible	N	N
LA1930	*S. chilense*	Resistant[1]	N	N
LA1932	*S. chilense*	Susceptible	N	N
LA1958	*S. chilense*	Susceptible	N	N
LA1960	*S. chilense*	Susceptible	N	N
LA1963	*S. chilense*	Susceptible	N	N
LA1967	*S. chilense*	Susceptible[5]	N	N
LA1968	*S. chilense*	Susceptible[5]	N	-
LA1969	*S. chilense*	Susceptible	N	N
LA1971	*S. chilense*	Susceptible	N	N
LA2748	*S. chilense*	Susceptible	N	N
LA2750	*S. chilense*	Susceptible	N	N
LA2753	*S. chilense*	Susceptible	N	N
LA2765	*S. chilense*	Susceptible	N	N
LA2771	*S. chilense*	Susceptible	N	N
LA2778	*S. chilense*	Susceptible	N	N
LA2880	*S. chilense*	Susceptible	N	N
LA2884	*S. chilense*	Susceptible	N	N
LA2930	*S. chilense*	Susceptible	N	N
LA3114	*S. chilense*	Susceptible	N	N
LA1028	*S. chmielewskii*	Susceptible	N	N
LA1306	*S. chmielewskii*	Susceptible	N	N
LA1317	*S. chmielewskii*	Susceptible	Susceptible	N
LA1325	*S. chmielewskii*	Susceptible	Susceptible	N
LA1330	*S. chmielewskii*	Susceptible	N	N
LA2663	*S. chmielewskii*	Susceptible	N	N
LA2677	*S. chmielewskii*	Susceptible	N	N
LA2680	*S. chmielewskii*	Susceptible	N	N
LA2695	*S. chmielewskii*	Susceptible	N	N
LA3661	*S. chmielewskii*	Susceptible[5]	N	+
LA0103	*S. corneliomulleri*	Susceptible	N	N
LA0107	*S. corneliomulleri*	Susceptible	N	N
LA0444	*S. corneliomulleri*	Susceptible	N	N
LA1274	*S. corneliomulleri*	Susceptible	N	N
LA1292	*S. corneliomulleri*	Susceptible	N	-
LA1305	*S. corneliomulleri*	Susceptible[2]	N	N
LA1331	*S. corneliomulleri*	Susceptible	N	N
LA1339	*S. corneliomulleri*	Susceptible[3,5]	N	N
LA1647	*S. corneliomulleri*	Susceptible	N	N
LA1677	*S. corneliomulleri*	Susceptible	N	N
LA1910	*S. corneliomulleri*	Susceptible	N	N
LA1937	*S. corneliomulleri*	Susceptible	N	N
LA1945	*S. corneliomulleri*	Susceptible[4]	N	N

LA1973	*S. corneliomulleri*	Susceptible	N	N
LA0317	*S. galapagense*	Susceptible[5]	N	-
LA0438	*S. galapagense*	Resistant[1]	N	N
LA0483	*S. galapagense*	Susceptible	N	N
LA1136	*S. galapagense*	Susceptible	N	N
LA1137	*S. galapagense*	Susceptible	N	N
LA1141	*S. galapagense*	Susceptible	N	N
LA1401	*S. galapagense*	Susceptible	N	N
LA1410	*S. galapagense*	Susceptible	N	N
LA0407	*S. habrochaites*	Susceptible	N	N
LA1223	*S. habrochaites*	Susceptible	N	N
LA1266	*S. habrochaites*	Susceptible	N	N
LA1347	*S. habrochaites*	Susceptible	N	N
LA1353	*S. habrochaites*	Susceptible[5]	N	+
LA1361	*S. habrochaites*	Susceptible[5]	N	N
LA1559	*S. habrochaites*	Susceptible[5]	N	N
LA1624	*S. habrochaites*	Susceptible[5]	N	N
LA1718	*S. habrochaites*	Susceptible[5]	N	N
LA1721	*S. habrochaites*	Susceptible[5]	N	N
LA1731	*S. habrochaites*	Susceptible[5]	Susceptible	N
LA1753	*S. habrochaites*	Susceptible[5]	N	N
LA1777	*S. habrochaites*	Susceptible	N	N
LA1918	*S. habrochaites*	Susceptible[5]	N	N
LA1928	*S. habrochaites*	Susceptible	N	N
LA2098	*S. habrochaites*	Susceptible	N	N
LA2103	*S. habrochaites*	Susceptible	N	N
LA2109	*S. habrochaites*	Susceptible	N	N
LA2119	*S. habrochaites*	Susceptible	N	N
LA2128	*S. habrochaites*	Susceptible[5]	N	N
LA2155	*S. habrochaites*	Susceptible	N	N
LA2158	*S. habrochaites*	Susceptible	N	N
LA2167	*S. habrochaites*	Susceptible	N	N
LA2204	*S. habrochaites*	Susceptible	N	N
LA2329	*S. habrochaites*	Susceptible	N	N
LA2409	*S. habrochaites*	Susceptible	N	N
LA2650	*S. habrochaites*	Susceptible	N	N
LA2860	*S. habrochaites*	Resistant	Resistant	+
LA2864	*S. habrochaites*	Susceptible	N	N
LA1365	*S. huaylasense*	Susceptible	N	N
LA1982	*S. huaylasense*	Susceptible	N	N
LA2408	*S. lycopersicoides*	Susceptible[5]	N	+
LA2951	*S. lycopersicoides*	Resistant	N[6]	N
LA0126	*S. lycopersicum*	Susceptible	N	N

LA0292	*S. lycopersicum*	N	Susceptible	N
LA0404	*S. lycopersicum*	Susceptible	N	N
LA0409	*S. lycopersicum*	Susceptible	N	N
LA0446	*S. lycopersicum*	Susceptible	N	N
LA0466	*S. lycopersicum*	Susceptible	N	N
LA0468	*S. lycopersicum*	Susceptible	N	N
LA0473	*S. lycopersicum*	Susceptible	N	N
LA0477	*S. lycopersicum*	Susceptible	N	N
LA1021	*S. lycopersicum*	Susceptible	N	N
LA1162	*S. lycopersicum*	Susceptible	N	N
LA1204	*S. lycopersicum*	N	Susceptible	N
LA1206	*S. lycopersicum*	N	Susceptible	N
LA1226	*S. lycopersicum*	Susceptible[5]	N	-
LA1228	*S. lycopersicum*	N	Susceptible	N
LA1231	*S. lycopersicum*	N	Susceptible[5]	N
LA1251	*S. lycopersicum*	Susceptible	N	N
LA1268	*S. lycopersicum*	N	Susceptible	N
LA1286	*S. lycopersicum*	N	Susceptible	N
LA1307	*S. lycopersicum*	N	Susceptible	N
LA1312	*S. lycopersicum*	N	Susceptible	N
LA1314	*S. lycopersicum*	N	Susceptible	N
LA1320	*S. lycopersicum*	N	Susceptible	N
LA1323	*S. lycopersicum*	N	Susceptible	N
LA1338	*S. lycopersicum*	N	Susceptible	N
LA1385	*S. lycopersicum*	N	Susceptible	N
LA1388	*S. lycopersicum*	N	Susceptible	N
LA1420	*S. lycopersicum*	N	Susceptible	N
LA1425	*S. lycopersicum*	N	Susceptible	N
LA1429	*S. lycopersicum*	N	Susceptible	N
LA1453	*S. lycopersicum*	N	Susceptible	N
LA1456	*S. lycopersicum*	N	Susceptible	N
LA1461	*S. lycopersicum*	N	Susceptible	N
LA1464	*S. lycopersicum*	N	Susceptible	N
LA1482	*S. lycopersicum*	N	Susceptible	N
LA1509	*S. lycopersicum*	N	Susceptible	N
LA1511	*S. lycopersicum*	N	Susceptible	N
LA1543	*S. lycopersicum*	N	Resistant[3]	-
LA1620	*S. lycopersicum*	N	Susceptible	N
LA1622	*S. lycopersicum*	N	Susceptible	N
LA2078	*S. lycopersicum*	N	Susceptible	N
LA2095	*S. lycopersicum*	N	Susceptible	N
LA2131	*S. lycopersicum*	N	Susceptible	N
LA2138A	*S. lycopersicum*	N	Susceptible	N

LA2283	*S. lycopersicum*	N	Susceptible	N
LA2285	*S. lycopersicum*	N	Susceptible	N
LA2304	*S. lycopersicum*	N	Susceptible	N
LA2307	*S. lycopersicum*	N	Susceptible	N
LA2308	*S. lycopersicum*	N	Resistant	N
LA2392	*S. lycopersicum*	N	Susceptible	N
LA2402	*S. lycopersicum*	N	Resistant	N
LA2670	*S. lycopersicum*	N	Resistant	N
LA2688	*S. lycopersicum*	N	Susceptible	N
LA2703	*S. lycopersicum*	N	Resistant	N
LA2709	*S. lycopersicum*	N	Susceptible[3]	N
LA2710	*S. lycopersicum*	N	Susceptible	N
LA2845	*S. lycopersicum*	N	Resistant[3]	N
LA4028	*S. lycopersicum*	Susceptible	N	N
LA0247	*S. neorickii*	Susceptible	N	N
LA0735	*S. neorickii*	N	Susceptible	N
LA1319	*S. neorickii*	Susceptible	Susceptible[5]	N
LA1321	*S. neorickii*	N	Susceptible	N
LA1322	*S. neorickii*	Susceptible[4]	N	N
LA1326	*S. neorickii*	Susceptible	Susceptible	-
LA1329	*S. neorickii*	N	Resistant[3]	-[7]
LA1626A	*S. neorickii*	Susceptible	N	N
LA1716	*S. neorickii*	Susceptible[4]	N[6]	N
LA2072	*S. neorickii*	N	Susceptible	N
LA2073	*S. neorickii*	N	Susceptible	N
LA2074	*S. neorickii*	N	Susceptible	N
LA2075	*S. neorickii*	N	Susceptible	N
LA2113	*S. neorickii*	Resistant	Susceptible[8]	N
LA2133	*S. neorickii*	Susceptible	N	N
LA2190	*S. neorickii*	Susceptible	N	N
LA2191	*S. neorickii*	N	Susceptible	N
LA2192	*S. neorickii*	N	Susceptible	N
LA2193	*S. neorickii*	N	Susceptible	N
LA2194	*S. neorickii*	N	Susceptible	N
LA2195	*S. neorickii*	N	Susceptible	N
LA2197	*S. neorickii*	N	Susceptible	N
LA2198	*S. neorickii*	Susceptible	N	N
LA2200	*S. neorickii*	N	Susceptible	N
LA2201	*S. neorickii*	N	Susceptible	N
LA2202	*S. neorickii*	N	Susceptible	N
LA2315	*S. neorickii*	N	Susceptible	N
LA2317	*S. neorickii*	N	Susceptible	N
LA2318	*S. neorickii*	N	Susceptible	N

LA2319	*S. neorickii*	Susceptible	N	N
LA2403	*S. neorickii*	N	Susceptible[7]	N
LA2613	*S. neorickii*	N	Susceptible	N
LA2614	*S. neorickii*	N	Susceptible	N
LA2639A	*S. neorickii*	N	Susceptible[7]	N
LA2641	*S. neorickii*	N	Susceptible	N
LA2727	*S. neorickii*	N	Susceptible	N
LA2847	*S. neorickii*	N	Susceptible	N
LA2848	*S. neorickii*	N	Susceptible	N
LA2862	*S. neorickii*	N	Susceptible	N
LA2865	*S. neorickii*	N	Susceptible	N
LA2913	*S. neorickii*	N	Susceptible	N
LA4020	*S. neorickii*	N	Susceptible	N
LA4021	*S. neorickii*	N	Susceptible	N
LA4022	*S. neorickii*	N	Susceptible	N
LA4023	*S. neorickii*	N	Susceptible	N
LA0751	*S. pennellii*	Resistant	N[6]	N
LA1733	*S. pennellii*	N	Susceptible	N
LA1926	*S. pennellii*	Susceptible[5]	N	-
LA1946	*S. pennellii*	N	Susceptible	N
LA0111	*S. peruvianum*	Susceptible[5]	N	-
LA0153	*S. peruvianum*	Resistant	N[6]	N
LA1336	*S. peruvianum*	Susceptible	N	N
LA1954	*S. peruvianum*	N	Susceptible	N
LA2732	*S. peruvianum*	N	Susceptible	N
LA2744	*S. peruvianum*	N	Susceptible	N
LA0722	*S. pimpinellifolium*	Resistant	Resistant[3,5]	+
LA1245	*S. pimpinellifolium*	N	Susceptible	N
LA1589	*S. pimpinellifolium*	Susceptible	N	N
LA2401	*S. pimpinellifolium*	Susceptible	N	N
LA2533	*S. pimpinellifolium*	Susceptible	N	N

[1]Accession should be re-tested by dip inoculation.
[2]Did not develop disease lesions. However, quantification of bacterial growth indicated susceptibility.
[3]Result was confirmed by quantification of bacterial growth.
[4]Symptoms were difficult to evaluate. Dip inoculation and quantification of bacterial growth may yield clearer results.
[5]Result was verified by at least two independent experiments.
[6]Not re-tested by dip inoculation because seeds failed to germinate.
[7]Ambiguous result – should be repeated.
[8]Appears susceptible only by dip inoculation. May reflect difficulty of hand infiltration and disease scoring in this accession.
N, Not tested.
Shaded boxes indicate the nine candidate resistant accessions shown in Figure 4-1.

Conclusions and Outlooks

In this work I set out to map and identify a bacterial spot disease resistance gene, *RXopJ4,* from the wild species *Solanum pennellii.* The cognate effector of *RXopJ4, xopJ4,* was identified more than a decade ago, and the *RXopJ4* resistance locus was mapped to chromosome 3. This misidentification was likely due to a nonspecific cell death response in plants containing *S. pennellii* introgressions on chromosome 3, rather than to the specific recognition of the XopJ4 effector and resulting disease resistance. By including a Δ*xopJ4* mutant strain and a complemented strain in my analyses of HR and disease resistance, I was able to correct the record and show that the locus lies within a 190-kb interval on chromosome 6. Initial mapping efforts were hampered by a severe linkage drag in the *RXopJ4*-containing *S. pennellii* introgression lines (ILs) 6-2 and 6-2-2. Although a resistant seventh outcross line was considerably healthier and was suitable for the mapping study, this difficulty highlighted the limitations of traditional breeding approaches for incorporating traits from wild species into crop plants. The mapping of *RXopJ4* also underscored the utility of plant genome sequence data for accelerating the discovery of genes conferring desirable agronomic traits. Without access to the emerging genome sequence of tomato, the process of developing molecular markers for this study would have been far more laborious, and it may not have been possible to define the locus to a mere 190 kb.

Likewise, progress in sequencing the *S. pennellii* genome expedited the process of *RXopJ4* candidate gene identification. Rather than probing a genomic library of *S. pennellii* with cosegregating markers, I was able to quickly ascertain the sequences of the twenty-nine genes present at the *RXopJ4* locus, identify three candidate serine-threonine kinase genes, and clone them into binary vectors for transient co-expression with the XopJ4 effector in *Nicotiana benthamiana.* The demonstration that an apparently intracellular, 365-amino-acid kinase is responsible for XopJ4 recognition suggests that this type of resistance protein is not simply an exception to the rule of NB-LRR and receptor-like kinase proteins. Although *Pto* of tomato was the first gene-for-gene resistance gene to be cloned, until recently it was the only example of an *R* gene encoding an intracellular kinase without an LRR or transmembrane domain. Now, with the knowledge that resistance triggered by the effectors HopZ1a and XopJ4 requires *Arabidopsis* and *S. pennellii* kinase genes, we may speculate that this is a more common recognition mechanism than previously thought.

Since Pto depends on the CC-NB-LRR protein Prf for AvrPto and AvrPtoB recognition, and HopZ1a recognition requires both a CC-NB-LRR protein (ZAR1) and a kinase, it is possible that the RXopJ4 kinase also interacts with a NB-LRR protein to recognize XopJ4 and/or initiate resistance. If so, this NB-LRR is likely to be conserved among *S. pennellii, S. lycopersicum,* and *N. benthamiana.* Future biochemical and genetic studies may reveal the identity of this NB-LRR protein, or may show that RXopJ4 functions through an altogether different mechanism with its own distinct signaling partner(s). Future experiments will also help to elucidate the

nature of the RXopJ4-XopJ4 recognition: Do the two proteins directly interact? Does XopJ4 acetylate or otherwise modify XopJ4 to trigger resistance? Is RXopJ4 a decoy for the virulence target(s) of XopJ4? Is it a functional kinase? Of course, there is a more pertinent and immediate question: Is this candidate kinase indeed the *RXopJ4* gene responsible for XopJ4-mediated resistance to bacterial spot disease of tomato? I am eagerly monitoring the growth of the T_1 generation of transgenic *RXopJ4* plants, which will allow me to answer this question in the near future.

Even before completing the fine mapping of *RXopJ4,* I began work on a second, broader line of inquiry—namely, an exploration of the core type III effectors of *Xanthomonas perforans,* including their virulence contributions and their potential recognition by solanaceous plants. For help with this work I am indebted to Staskawicz Lab members Andrew Kassen and Rebecca Bart for their computational expertise, as well as undergraduate students Lily Liu and Joshua Chiu for their capable hands and conscientious dispositions. It is perhaps premature to conclude that the sequencing of only fourteen field isolates, all collected in the same year, albeit from different regions of Florida and Georgia, can reveal a set of core *X. perforans* effectors. In the near future our confidence in the identification of core effectors will expand with the sequencing of more diverse *Xp* isolates. In the meantime, we may postulate that effectors conserved across different species of *Xanthomonas,* that are known virulence factors in other species and/or have demonstrated virulence targets in host plants, may contribute to the virulence of *Xp* in the field. Initial disease assays with effector gene knockout strains have hinted that this is a reasonable supposition, but needs to be thoroughly followed up. Similarly, transient expression of core effectors in *Nicotiana* and *Solanum americanum* accessions has yielded promising sources of cognate resistance genes, but this work must be carefully validated.

We also undertook a parallel and more extensive analysis of disease resistance among wild *Solanum* accessions that are closely related to cultivated tomato. This allowed us to discover two accessions, *S. habrochaites* LA2860 and *S. pimpinellifolium* LA0722, that showed reproducible disease resistance by two different inoculation methods, and, in addition, are likely to recognize type III secreted effectors of *Xp* 4B, based on the results of HR assays. It will be important to ascertain whether core *Xp* effectors are recognized by these accessions, since this may dictate the utility of their disease resistance for durability in the field. Although not all of the nine candidate resistant accessions have been tested for HR, some of them appear to possess disease resistance in the absence of HR. These could be cases of effector-triggered immunity that are not associated with HR, or more genetically complex, quantitative sources of resistance, which may be useful in the field. Through the specific, directed identification of a single disease resistance gene, and the more open-ended endeavor of searching for new sources of resistance, my work has furthered our understanding of plant-pathogen interactions, as well as yielding propitious additions to the plant breeding tool kit for bacterial spot disease resistance.

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