Analysis of Sequenced Genomes of *Xanthomonas perforans* Identifies Candidate Targets for Resistance Breeding in Tomato

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ABSTRACT

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Bacterial disease management is a challenge for modern agriculture due to rapid changes in pathogen populations. Genome sequences for hosts and pathogens provide detailed information that facilitates effector-based breeding strategies. Tomato genotypes have gene-for-gene resistance to the bacterial spot pathogen *Xanthomonas perforans*. The bacterial spot populations in Florida shifted from tomato race 3 to 4, such that the corresponding tomato resistance gene no longer recognizes the effector protein AvrXv3. Genome sequencing showed variation in effector

profiles among race 4 strains collected in 2006 and 2012 and compared with a race 3 strain collected in 1991. We examined variation in putative targets of resistance among Florida strains of *X. perforans* collected from 1991 to 2006. Consistent with race change, *avrXv3* was present in race 3 strains but nonfunctional in race 4 strains due to multiple independent mutations. Effectors *xopJ4* and *avrBs2* were unchanged in all strains. The effector *avrBsT* was absent in race 3 strains collected in the 1990s but present in race 3 strains collected in 2006 and nearly all race 4 strains. These changes in effector profiles suggest that *xopJ4* and *avrBsT* are currently the best targets for resistance breeding against bacterial spot in tomato.

Additional keywords: type III effectors, avirulence, recombination.

Bacterial spot of tomato and pepper is caused by four distinct species within the genus *Xanthomonas*: *X. vesicatoria*, *X. euvesicatoria*, *X. perforans*, and *X. gardneri* (Jones et al. 2004). The disease is found in all areas in which tomato and pepper are grown and is most severe in areas with tropical and subtropical climates, including the southeastern United States. High temperatures, humidity, and rainfall favor disease development. The disease can cause yield losses of up to 50% in tomato (Scott et al. 1989). Current disease management strategies include resistant cultivars, use of disease-free seeds and transplants, and the preventative application of antibiotics, copper-based bactericides, and the plant defense elicitor acibenzolar-S-methyl (Huang et al. 2012). Disease management has been challenged by changes in the pathogen population, including increased resistance to bactericides and shifts to different *Xanthomonas* races and species.

Four tomato races have been described based on the presence of specific effector genes that interact with the corresponding resistance genes in tomato cultivars (Stall et al. 2009). Strains that elicit a hypersensitive response (HR) only in the tomato differential genotype Hawaii 7998, which contains the three recessive resistant genes

rx1, rx2, and rx3 are designated as tomato race 1 (T1). Strains that produce a susceptible reaction in all of the tomato differentials are designated race 2 (T2). All T1 strains to date have been *X. euvesicatoria* (Stall et al. 2009). Initially, all T2 strains were *X. vesicatoria* (Stall et al. 2009); however, *X. gardneri* strains were determined to also behave as race 2 (J. B. Jones, *unpublished data*). A third pathogenic race, identified in the early 1990s, was designated as race 3 (T3). These strains were placed in a new species, *X. perforans*, which is closely related to but distinct from *X. euvesicatoria*. *X. perforans* T3 strains cause disease on Hawaii 7998, like T2 strains, but carry the *avrXv3* effector gene that elicits an incompatible reaction on tomato genotypes with the corresponding *Xv3* resistance gene. Later, T3 strains were determined to contain another avirulence gene, *xopJ4* (*avrXv4*), which elicits a HR in *Solanum pennellii* LA 716 and other tomato genotypes with the *RxopJ4* (*Xv4*) resistant locus (Astua-Monge et al. 2000; Sharlach et al. 2013; Stall et al. 2009). *X. perforans* strains with *xopJ4* but lacking a functional *avrXv3* were later identified and designated as race 4 (T4). Surveys conducted in Florida in 2006 and 2012 showed a shift from T3 to T4, with 75% of the collected strains identified as T4 in 2006 (Horvath et al. 2012) and 100% T4 by 2012 (Timilsina et al. 2015).

Most *Xanthomonas* species use the type III secretion system to transfer pathogen effectors into their hosts. The acquisition or mutation of various effectors governs *Xanthomonas* host range and virulence (Mhedbi-Hajri et al. 2013). Approximately 45 type III effector groups are associated with *Xanthomonas* spp. (Potnis et al. 2015; White et al. 2009). Type III effectors are known or hypothesized to facilitate disease. Effectors that are perceived by host resistance proteins (*R* genes) induce a HR (Nimchuk et al. 2003) that confers race-specific resistance (Stall et al. 2009).

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Nucleotide sequence data is available in the NCBI database under accession numbers KU759430 to KU759471 for partial gene sequences of *gapA*, KU759370 to KU759372 for complete coding regions of *avrBsT*, KU759373 to KU759401 for partial sequence of *avrXv3*, KU759402 to KU759429 for partial sequence of *xopJ4*, and KU759472 to KU759498 for partial sequence of *xopQ*.

*The e-Xtra logo stands for "electronic extra" and indicates that four supplementary figures and one supplementary table are published online.

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The durability of plant resistance, regardless of how it was introduced, depends on the ability of the pathogen to evolve over time. For bacterial pathogens, durability depends on rates of mutation, the ability to horizontally exchange genetic material, the overall level of selection pressure, and the relative fitness of virulent strains (Stall et al. 2009). Effectors that are conserved in pathogen populations can be evaluated as potential targets for resistance breeding. Resistance breeding strategies that target evolutionarily conserved effectors coupled with the pyramiding of *R* genes could lead to durable resistance against pathogen populations.

The *Bs2* resistance gene recognizes the core effector AvrBs2, which is present in all four *Xanthomonas* species that cause bacterial spot of tomato (Horvath et al. 2012; Potnis et al. 2011). However, this pepper gene is not available in commercial tomato cultivars. A transgenic approach was used to develop resistant tomatoes expressing the *Bs2* transgene (Tai et al. 1999). The effectiveness of *Bs2* in commercial peppers can be greatly diminished by the appearance of mutations within *avrBs2* in *X. euvesicatoria* populations (Gassmann et al. 2000; Swords et al. 1996). In order to improve the durability of *Bs2* resistance in tomato, additional effectors should be targeted for pyramiding of resistance genes, line mixing, or alternating resistance lines (McDonald and Linde 2002). Resistance targeting effector AvrBsT, which is present in some strains of *X. euvesicatoria* and *X. vesicatoria*, has been reported in *Solanum lycopersicoides*, but the resistance gene has not been identified and introgressed into tomato (Wang 1992). Likewise,

resistance against the XopJ4 effector in *X. perforans* has been mapped to a 4.2-Mb cluster in *S. pennellii* (Sharlach et al. 2013). AvrBsT and XopJ4 are not considered core effectors because they are not present in all four bacterial spot species (Potnis et al. 2011). However, because resistance against these effectors is known, they are potential targets.

Whole genome sequence analyses of field strains of the four bacterial spot species revealed a high degree of variation in type III effectors within species (Schwartz et al. 2015). Even among the 11 core effectors, allelic variation was observed in XopAD, XopD, and XopQ in T4 *X. perforans* strains from Florida. Previously, multilocus analysis revealed recombination between strains of *X. perforans* and *X. euvesicatoria* that generated two different genetic groups of T4 *X. perforans* strains in Florida (Timilsina et al. 2015). Strains designated as group 1 were similar to the taxonomic type strain for *X. perforans*, strain 91-118, a T3 strain collected in Florida in 1991, while group 2 strains had sequence similarities with *X. euvesicatoria* (Timilsina et al. 2015). This pattern was also observed for the type III effector *xopQ*, such that *X. perforans* strains with the *X. euvesicatoria gapA* sequence also had the *X. euvesicatoria xopQ* sequence (Schwartz et al. 2015; Timilsina et al. 2015). The specific molecular function of XopQ in pathogenicity is unknown (Teper et al. 2014). Subsequent whole-genome comparisons of T4 strains identified two subgroups within group 1, groups 1A and 1B (Schwartz et al. 2015). In addition to allelic variation in core genes, the majority of T4 *X. perforans*

TABLE 1. *Xanthomonas perforans* strains used in this study and their genotypes

Strain	Source	Year	Host ^a	Tomato race	<i>avrXv3</i> ^b	<i>avrBsT</i>	<i>xopJ4</i>	<i>xopQ</i> allele	<i>gapA</i> allele ^c
<i>X. perforans</i>									
Xp894	Florida	1991	T	T3	+	–	+	1	1
Xp909	Florida		T	T3	+	–	+	1	1
Xp1183	Florida		T	T3	+	–	+	1	1
Xp1118	Florida	1992	T	T3	+	–	+	1	1
Xp1144	Florida		T	T3	+	–	+	1	1
Xp1126	Florida		T	T3	+	–	+	1	1
Xp1221	Florida	1993	T	T3	+	–	+	1	1
Xp1224	Florida		T	T3	+	–	+	1	1
Xp1241	Florida		T	T3	+	–	+	1	1
Xp1247	Florida		T	T3	+	–	+	1	1
Xp1262	Florida		T	T3	+	–	+	1	1
Xp1268	Florida		T	T3	+	–	+	1	1.2
Xp1275	Florida		T	T3	+	–	+	1	1.2
Xp1508	Florida		T	T3	+	–	+	1	1
Xp1345	Mexico		T	T3	+	–	+	1	1
Xp1485	Mexico		T	T3	+	–	+	1	1
Xp1550	Florida	1994	T	T3	+	–	+	1	1.2
Xp1564	Florida		T	T3	+	–	+	1	1.2
Xp1574	Florida		T	T3	+	–	+	1	1
Xp1757	Mexico		T	T3	+	–	+	1	1
Xp1775	NA	1995	T	T3	+	–	+	1	1
Xp1797	Florida		T	T3	+	–	+	1	1
Xp1805	Florida		T	T3	+	–	+	1	1
Xp1835	Florida	1996	T	T3	+	–	+	1	1
Xp1856	Florida		T	T3	+	–	+	1	1
Xp1861	Alabama		T	T3	+	–	+	1	1
Xp1911	Florida	1997	T	T3	+	–	+	1	1
Xp1912	Florida		T	T3	+	–	+	1	1
Xp1913	Florida		T	T3	+	–	+	1	3
Xp1920	Florida	1998	T	T3	+	–	+	1	1
Scott-1	Florida		T	T4	Ø	+	+	1	1
Xp1-5	Florida	2006	T	T3	+	+	+	1	1
Xp1-6	Florida		T	T3	+	+	+	1	1
Xp1-13	Florida		T	T3	+	–	+	1	1
Xp3-8	Florida		T	T3	+	+	+	1	1
Xp3-12	Florida		T	T3	+	+	+	1	1

(continued on next page)

^a T = tomato; P = pepper.

^b Ø, insertion sequence, could not be PCR amplified; Ø, potential pseudogene; ES, early stop codon detected in the sequence.

^c The *gapA* allele 1 differed from allele 1.2 by only one nucleotide and is within the same genetic group as allele 1.

^d Sequence information of all T4 strains collected in 2006, 2010, and 2012 were previously published by Timilsina et al. 2015 and Schwartz et al. 2015.

^e Housekeeping gene sequences of *X. vesicatoria* strains were published by Timilsina et al. 2015.

strains collected in 2006 and 2012 contained *avrBsT*. The *X. perforans* type strain 91-118 did not have *avrBsT*. This effector, when expressed in *X. perforans* and translocated into pepper mesophyll cells, elicits a HR, while when expressed in *X. vesicatoria* and translocated into tomato mesophyll cells, this effector protein suppresses defense responses and increases virulence (Kim et al. 2010). A group 2 T4 strain of *X. perforans* from bell pepper was isolated that lacked *AvrBsT* (Schwartz et al. 2015). When *avrBsT* was mutated in group 2 strains originally isolated from tomato, they also caused lesions in pepper. However, mutation of *avrBsT* in group 1A *X. perforans* strains did not confer pathogenicity in pepper, suggesting that host range of *X. perforans* strains is determined, in part, by genetic background (Schwartz et al. 2015). Both *XopQ* and *AvrBsT* appear to have a role in host range, because double deletion of these two effectors also allowed host expansion of *X. perforans* to *Nicotiana benthamiana* (Schwartz et al. 2015).

The long-term effectiveness of an *R* gene within a breeding program is generally dependent on the presence and fitness value of the corresponding effector gene within a defined pathogen population. However, in the case of tomato in Florida, unforeseen changes in the pathogen population due to the introduction of exotic species, mutations in effector genes, and even recombination among *Xanthomonas* species have necessitated the re-evaluation of resistance breeding strategies. The future of resistance breeding in tomato may fail if we do not understand the drivers of past changes within the pathogen population. The genomic variation found in Florida T4 *X. perforans* strains raised questions regarding variation in T3 *X. perforans* before and during the race shift. Is the *avrBsT*

gene exclusive to T4 strains, indicating that it was acquired simultaneous to the loss of *AvrXv3*? Did recombination with *X. euvesicatoria* occur at the same time as loss of *AvrXv3* or as gain of *AvrBsT*? In previous studies, only T4 strains had been genotyped (Schwartz et al. 2015; Timilsina et al. 2015). We hypothesized that the loss of *AvrXv3*, gain of *AvrBsT*, and recombination with *X. euvesicatoria* were related events, such that *AvrBsT* is exclusive to T4 recombinant strains. To test this hypothesis, we genotyped T3 strains from the 2006 and earlier surveys and compared them with T4 strains collected in 2006, 2010, and 2012. We sequenced the housekeeping gene *gapA* and the effector *xopQ*, as indicators of the recombination event with *X. euvesicatoria*, to determine the timing of recombination relative to the race shift. We examined sequences of *avrXv3* to determine the genetic basis of the race shift, and we sequenced *avrBsT* and *xopJ4* to determine their stability as potential targets for resistance breeding against *X. perforans*.

MATERIALS AND METHODS

Bacterial strains collection and pathogenicity testing.

Xanthomonas perforans strains used in this study were isolated over a period of 20 years from symptomatic plants (Table 1). In Florida, T3 and T4 strains of *X. perforans* were first identified in 1991 and 1998, respectively. Representative strains from each year between 1991-1998 and T3 strains from 2006 were selected for study. Additional T3 strains from Mexico and Alabama were also included for comparison.

Tomato and pepper differentials were inoculated to confirm pathogenicity and race (Stall et al. 2009). To determine race, bacterial

TABLE 1. (continued from preceding page)

Strain	Source	Year	Host ^a	Tomato race	<i>avrXv3</i> ^b	<i>avrBsT</i>	<i>xopJ4</i>	<i>xopQ</i> allele	<i>gapA</i> allele ^c
Xp3-16	Florida		T	T3	+	+	+	1	1
Xp5-8	Florida		T	T3	+	–	+	1	1
Xp5-9	Florida		T	T3	+	–	+	1	1
Xp5-14	Florida		T	T3	+	+	+	1	1
Xp5-15	Florida		T	T3	+	+	+	1	1
Xp6-19	Florida		T	T3	+	+	+	1	1
Xp10-20	Florida		T	T3	+	+	+	1	1
Xp20-5	Florida		T	T3	+	+	+	1	1
Xp20-7	Florida		T	T3	+	+	+	1	1
Xp3-15	Florida		T	T4 ^d	Ø	+	+	2	2
Xp4-20	Florida		T	T4	Ø	+	+	1	1
Xp5-6	Florida		T	T4	Ø	–	+	1	1
Xp7-12	Florida		T	T4	Ø	+	+	2	2
Xp8-16	Florida		T	T4	Ø	+	+	2	2
Xp9-5	Florida		T	T4	Ø	+	+	2	2
Xp10-13	Florida		T	T4	Ø	+	+	2	2
Xp11-2	Florida		T	T4	–	+	+	1	1
Xp15-11	Florida		T	T4	Ø	+	+	1	1
Xp17-12	Florida		T	T4	–	–	+	1	1
Xp18-15	Florida		T	T4	–	+	+	1	1
Xp2010	Florida	2010	P	T4	Ø	–	–	2	2
GEV839	Florida	2012	T	T4	Ø	+	+	2	2
GEV872	Florida		T	T4	ES	+	+	1	1
GEV893	Florida		T	T4	ES	+	+	1	1
GEV904	Florida		T	T4	ES	+	+	1	1
GEV909	Florida		T	T4	ES	+	+	1	1
GEV915	Florida		T	T4	ES	+	+	1	1
GEV917	Florida		T	T4	ES	+	+	1	1
GEV936	Florida		T	T4	ES	+	+	1	1
GEV940	Florida		T	T4	ES	+	+	1	1
GEV968	Florida		T	T4	ES	+	+	1	1
GEV993	Florida		T	T4	ES	+	+	1	1
GEV1001	Florida		T	T4	Ø	+	+	2	2
GEV1026	Florida		T	T4	ES	+	+	1	1
GEV1044	Florida		T	T4	Ø	+	+	2	2
GEV1054	Florida		T	T4	Ø	+	+	2	2
GEV1063	Florida		T	T4	Ø	+	+	2	2
<i>X. vesicatoria</i> ^c									
ETH17	Ethiopia	2011	T	T2	–	+	–	–	4
Xv144	Argentina	NA	T	T2	–	+	–	–	4

suspensions of approximately 5×10^8 CFU/ml (0.3 A at 600 nm) were infiltrated into tomato genotypes that included Hawaii 7998 with the *rxv* resistance gene, FL216 with the *Xv3* resistance gene, a tomato genotype carrying the *RXopJ4* resistance locus from *Solanum pinnellii* LA716, and the susceptible cultivar Bonnie Best. Similarly, bacterial suspensions were infiltrated into four pepper genotypes, 'Early Calwonder' (ECW), ECW-10R, ECW-20R, and ECW-30R (Bouzar et al. 1994). *X. euvesicatoria* 85-10 and *X. perforans* 91-118 were used as control strains.

Gene sequencing. Bacterial DNA was extracted, using a selective precipitation method with CTAB-NaCl extraction protocol (Ausubel et al. 1994). Previous studies using multilocus sequence analysis of six housekeeping genes showed different allelic forms of *gapA* and *gyrB* that differentiated *X. perforans* group 1 from group 2 (Timilsina et al. 2015). To assign *X. perforans* strains used in this study to a group, we sequenced a portion of *gapA*. To examine allelic variation in effectors, partial sequences of *avrXv3* and *xopJ4* (*avrXv4*), the effector genes responsible for determining tomato races in *X. perforans*, and *xopQ* were sequenced. We tested for the presence of *avrBsT* in T3 *X. perforans* strains, using internal and flanking primers. When found, the complete gene was amplified and sequenced. For sequence comparison, *avrBsT* from two *X. vesicatoria* strains, Xv144 and ETH17, isolated from Argentina and Ethiopia, respectively, was sequenced. Sanger sequencing was conducted at the Interdisciplinary Center for Biotechnology Research at the University of Florida. The target genes sequenced in this study are listed in Table 2 along with their respective primers.

Sequence analysis. Housekeeping and effector genes sequenced were compared with other reference *Xanthomonas* spp., including *X. euvesicatoria*, *X. gardneri*, *X. vesicatoria*, and *X. perforans* sequences available from the Plant Associated and Environmental Microbes Database (PAMDB) and the National Center for Biotechnology Information (NCBI). Sequence variation in the effector genes was screened comparing nucleotide polymorphisms using Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990). Allele designations were assigned manually based on sequence similarity. All sequences were aligned using the Muscle tool in MEGA software ver. 6 (Tamura et al. 2013). Sequences for the *gapA* housekeeping gene were used to construct a maximum likelihood phylogenetic tree using general time reversible model with gamma distributed invariant sites in MEGA software ver. 6 (Tamura et al. 2013). Model selection for phylogenetic analysis was determined using Akaike information criterion (Burnham and Anderson 2004).

Sequence submission. The genes sequenced for this study were submitted to the NCBI database. The accession numbers are KU759430 to KU759471 for partial gene sequences of *gapA*, KU759370 to KU759372 for complete coding regions of *avrBsT*, KU759373 to KU759401 for partial sequence of *avrXv3*, KU759402

to KU759429 for partial sequence of *xopJ4*, and KU759472 to KU759498 for partial sequence of *xopQ*. The reference gene sequences used during this study are available on the NCBI and PAMDB databases.

RESULTS

Pathogenicity and race determination. Strains previously assigned to tomato race 3 were confirmed as T3. These strains were pathogenic on tomato differentials 'Bonnie Best' and Hawaii 7998 but elicited a HR on the tomato cultivars carrying the *Xv3* resistance gene and the *RXopJ4* resistance gene (Table 1). One *X. perforans* strain isolated in 1998, Scott-1, was confirmed as T4. It did not elicit a HR on FL216, which carries the *Xv3* gene. PCR amplification of the *avrXv3* and *xopJ4* effector genes produced results consistent with the assigned tomato race.

Grouping of T3 strains by *gapA* sequence. The housekeeping gene *gapA* differentiates the two genetic groups of T4 *X. perforans* strains (Timilsina et al. 2015). Group 2 strains of T4 *X. perforans* have a *gapA* sequence identical to the *X. euvesicatoria* *gapA* gene, which differs by 4 or 5 nucleotides from the group 1 alleles. Sequencing of *gapA* in T3 *X. perforans* strains isolated between 1991 and 2006 revealed three different alleles (Fig. 1; Supplementary Fig. S1). Sequence comparisons showed a single nucleotide difference in *gapA* between strain 91-118 and strains Xp1268, Xp1275, Xp1550, and Xp1564, isolated in 1993 or 1994 (Table 1, allele 1.2). Strain Xp1913 differed from strain 91-118 by 19 nucleotides for *gapA*. This allele was most similar to a nonpathogenic strain, ETH12, isolated from tomato in Ethiopia (Kebede et al. 2014). All of the T3 strains collected in 2006 were identical to each other and to strain 91-118 for *gapA*. None of the T3 strains contained a *gapA* allele identical to the group 2 recombinant T4 *X. perforans* strains.

Effector gene sequences. Four effector genes, *avrXv3*, *xopJ4*, *xopQ* and *avrBsT*, were sequenced for the study. Effector gene sequences *avrXv3* and *xopJ4* were found to be identical in all examined T3 *X. perforans* strains. Whole genome sequences of T4 *X. perforans* strains were examined for the presence and allelic diversity of the *avrXv3* gene. Three different mutations were observed in *avrXv3* (Supplementary Fig. S2). In group 2 strains, there was a contig break in *avrXv3*, with remnants of an insertion sequence. Primers specific to *avrXv3* were not able to amplify the gene, and inoculations in tomato differentials indicated that *AvrXv3* was nonfunctional in these strains. Within group 1 strains, the two subgroups that were previously defined by whole-genome sequencing (Schwartz et al. 2015), showed different forms of mutations in *avrXv3*. In group 1A, an early stop codon was detected, due to a point mutation. In group 1B, *avrXv3* was either completely absent or truncated, rendering *AvrXv3* nonfunctional in both cases. In contrast, *xopJ4* had identical sequence in all T3 and T4 strains of *X. perforans*.

Like *gapA*, T4 *X. perforans* strains have two allelic forms of *XopQ* consistent with the two groups (Schwartz et al. 2015). The *xopQ* sequence of group 1 strains is identical to type strain 91-118, but group 2 strains have a *xopQ* sequence identical to *X. euvesicatoria*. The difference between these alleles of *XopQ* is 10 nucleotides, or 6 amino acids (Supplementary Fig. S3). For all T3 *X. perforans* strains examined, *xopQ* sequences were identical to strain 91-118 and group 1 T4 *X. perforans* strains, consistent with the *gapA* results.

Presence of *AvrBsT* in T3 *X. perforans*. Primers were designed to amplify the entire type III secreted effector gene *avrBsT* along with approximately 0.5-kb flanking regions on either side of the gene. These primers identified the presence of *avrBsT* gene in T3 strains of *X. perforans* isolated in 2006 but not prior to 2006 (Table 1). Although primers were designed for polymerase chain reaction (PCR) amplification of *avrBsT*, two different band sizes were observed during gel electrophoresis (Supplementary Fig. S4). The expected band size, that of the functional *avrBsT* gene in T4 *X. perforans* strains, was approximately 2 kb. Sequencing of the

TABLE 2. List of primers used in this study

Gene ^a	Direction	Sequence	Reference
<i>xopQ</i> *	Forward	AAGCTCGGCATGGTGGTCAT	This study
	Reverse	AGCAGGGTCAGCGGATCGTA	
<i>avrBsT</i> ^Δ	Forward	TTGGAACAACCTCTGGCG	This study
	Reverse	ATGCACCGCACTATGCACAT	
<i>avrBsT</i> *	Forward	TGTTGGTAATTGAGCCAGCACTTG	This study
	Reverse	AGTTGGATGACCGCCATGTCTC	
<i>avrXv3</i> *	Forward	AGCAACTGTCTGCCAGCCAGAACC	This study
	Reverse	TGAGCGAGAGCTACTATCGCCTCC	
<i>xopJ4</i> *	Forward	ACTCGCCAAATTGTCTATGC	This study
	Reverse	TACGACCTTTTCAGGGTTGG	
<i>gapA</i> *	Forward	GGCAATCAAGGTTGGYATCAACG	Almeida et al. 2010
	Reverse	ATCTCCAGGCACTTGTTSGARTAG	

^a An asterisk (*) indicates internal primers for genes *xopQ*, *avrBsT*, *avrXv3*, *xopJ4* and *gapA* and a carat (Δ) indicates a complete coding region for the *avrBsT* gene.

2-kb band amplified from T3 *X. perforans* strains showed an intact *avrBsT* gene that was identical to that in T4 *X. perforans* strains. A smaller, approximately 1.2-kb band was observed in strain Xp1861 isolated in 1996 from Alabama and in strain Xp1-13 isolated in

2006 from Florida. These PCR products were sequenced and were determined to have no homology to *avrBsT*, but a fragment of transposon sequence was found. The primers for *avrBsT* were designed to anneal the flanking sequence of the gene but were also

Fig. 1. Maximum likelihood phylogenetic analysis of *Xanthomonas perforans* strains using *gapA* gene sequence. Strains are color-coded based on the years they were isolated. T4 group 1 and T4 group 2 represent the *gapA* sequences for these respective group of strains collected in 2006 and 2012. Reference strains of *X. euvesicatoria*, *X. gardneri*, *X. perforans*, and *X. vesicatoria* were downloaded from the Plant Associated and Environmental Microbes Database and the National Center for Biotechnology Information database. Values on the branches indicate bootstrap values for each branch, expressed as percentages. The scale bar indicates the number of substitutions per site.

found to be identical to the putative transposon region. Primers internal to the coding region of the *avrBsT* gene were used to further confirm the presence or absence of *avrBsT* in all strains. The *X. perforans* strains collected from 1991 to 1998 lacked *avrBsT*, while all but two of the T3 strains collected in 2006 contained the gene. The *avrBsT* gene sequences from both T3 and T4 strains of *X. perforans* were identical to the *avrBsT* gene from *X. vesicatoria* Bv5-4a (GQ266402.1) but varied from the *avrBsT* gene from *X. euvesicatoria* 75-3 by 2 nucleotides (AF156163.1). We found the *X. perforans*-type *avrBsT* allele in other *X. vesicatoria* strains (Fig. 2). An identical *avrBsT* allele is also present in the cassava pathogen *X. axonopodis* pv. *manihotis* CIO151 (AKDA01000004).

DISCUSSION

Bacterial spot disease of tomato and pepper is caused by four different *Xanthomonas* species, but tomato in Florida is currently affected only by *X. perforans* and, in recent years, only tomato race 4 strains of *X. perforans* have been found (Schwartz et al. 2015). Prior to 1991, only tomato race 1 *X. euvesicatoria* was isolated from bacterial spot-affected tomato in Florida. The population shift from

T1 *X. euvesicatoria* to T3 *X. perforans* was attributed to bacteriocin activity of T3 *X. perforans* (Hert et al. 2005). The shift from T3 to T4 started in 1998, when the first T4 strain was found (strain Scott-1) and was complete by 2012 (Fig. 3). The reason for the population shift from T3 to T4 *X. perforans* remains unknown, as no known sources of resistance toward *X. perforans* are commercially available. Whole-genome sequence analysis of *X. perforans* identified a substantial variation in the effector profiles among T4 strains collected in 2006 and 2012 and a recombination event affecting *gapA* and *xopQ*, among other genes (Schwartz et al. 2015; Timilsina et al. 2015). The most notable change in effector profiles, in comparison with the T3 strain 91-118, was the presence of the *avrBsT* gene in most of the T4 *X. perforans* strains. The *avrBsT* effector is universally recognized by most varieties of pepper. Kim et al. (2010) demonstrated that *X. vesicatoria* Bv5-4a with *avrBsT* exhibited higher in planta titers postinoculation on a susceptible tomato line, compared with strains lacking *avrBsT*. Therefore, *X. perforans* strains with *avrBsT* may have gained increased virulence and fitness on tomato. Only three strains of group 2 T4 *X. perforans* have been found without the gene for *AvrBsT* to date. *X. perforans* is usually only pathogenic on tomato, but an *X. perforans* strain was isolated from symptomatic pepper that lacked *AvrBsT* (Schwartz et al. 2015) (Fig. 3; Supplementary Table S1). To determine if changes were occurring in the Florida population prior to the race shift, we characterized T3 strains of *X. perforans* that were collected from 1991 to 2006. We did not find recombinant *gapA* or *xopQ* genes in T3 *X. perforans*. In addition, all strains exhibited identical *avrXv3* and *xopJ4* gene sequences. Given the apparent homogeneity of the T3 *X. perforans* population, we were surprised to find *avrBsT* in T3 strains isolated in 2006. Thus, our results show that *AvrBsT* was acquired before the race shift or during the race shift by both T3 and T4 *X. perforans* strains.

All T3 *X. perforans* strains isolated in 2006 had the same *avrBsT* allele as T4 *X. perforans*. Interestingly, this allele has two adjacent nucleotides that are different from the *avrBsT* sequence in the *X. euvesicatoria* type strain 85-10. The two sites result in substitutions of two adjacent amino acids in the *AvrBsT* protein. The *avrBsT* gene from *X. perforans* was identical to the *avrBsT* gene of *X. vesicatoria* LMG 919, isolated from Zimbabwe (GCA_001469445.1). Identical *avrBsT* sequence was detected in other *X. vesicatoria* strains

Fig. 2. Maximum likelihood phylogenetic tree of *avrBsT*, showing sequence identity between alleles in *Xanthomonas vesicatoria* and *X. perforans* strains. Additionally, the *avrBsT* sequence from *X. axonopodis* pv. *manihotis* was identical to the *avrBsT* in bacterial spot-causing *Xanthomonas* strains.

Fig. 3. Infograph showing shifts in the *Xanthomonas* population on tomato in Florida over time. Prior to 1991, only *X. euvesicatoria* was identified. Later, the population changed from T3 *X. perforans* to T4 *X. perforans*. Three different mutations have been observed in the *AvrXv3* gene of T4 strains. The population acquired *AvrBsT*, which was first observed in 1998, in most strains in 2006, and in all strains examined from 2012.

isolated from Argentina and Ethiopia that were sequenced during this study. The effector is not universally present in *X. vesicatoria*, as the type strain of *X. vesicatoria*, ATCC 35937, appears to lack the *avrBsT* gene. *X. vesicatoria* is known to be resistant to bacteriocins produced by *X. perforans* and, given the sequence identity of *avrBsT* between these two species, *avrBsT* may have been acquired through horizontal gene transfer from strains of *X. vesicatoria* carrying *avrBsT*. This is a surprising find, because there are no reports of *X. vesicatoria* in Florida. However, the presence of multiple *Xanthomonas* species was associated with bacterial spot in Africa (Jibrin et al. 2015; Kebede et al. 2014; Timilsina et al. 2015). The presence of multiple *Xanthomonas* species within the same tomato-growing region increases the likelihood of genetic exchange between species. Further studies examining the global distribution of *avrBsT* in the *Xanthomonas* bacterial spot species could help to clarify the importance of this effector in *Xanthomonas* populations on tomato, determine whether there is selection for strains carrying *avrBsT*, and identify the *avrBsT* donor to *X. perforans*.

We previously found genetic exchange among bacterial species affecting housekeeping genes and the *xopQ* effector (Schwartz et al. 2015; Timilsina et al. 2015). Whole-genome sequencing confirmed that there are at least two genetic groups of T4 *X. perforans*, with group 2 identified as recombinants with *X. euvesicatoria*. Within group 1, two subgroups were observed. Group 1A was identified exclusively in 2012. Group 1B is a more genetically diverse group that includes the type strain 91-118 but does not include any strains collected in 2012. The groups are defined based on core genome phylogeny, yet each group has distinct unique mutations in the *avrXv3* gene resulting in a shift from T3 to T4. Group 1A had a point mutation resulting in an early stop codon, group 1B either lacked the entire *avrXv3* coding region or a section of the gene making it a likely pseudogene, and group 2 contained insertion sequences in *avrXv3* (Fig. 3). The unique mutations within the genetically homogenous Groups 1A and 2 suggest that the distinct mutations in *avrXv3* occurred in a strain ancestral to each of these groups and any further variation in effector profiles occurred after the loss of *AvrXv3*.

We looked for evidence of recombination with *X. euvesicatoria* in T3 strains by sequencing the *gapA* and *xopQ* genes. We found no variation in *xopQ* within T3 strains. T3 strains had three *gapA* alleles, but none of them were the recombinant allele from *X. euvesicatoria*. The most common allele was identical to that of *X. perforans* type strain 91-118, while the second allele varied by only one nucleotide and was isolated in 1993 and 1994 only. The third allele found in strain Xp1913, isolated in 1996 in Alabama, exhibited a *gapA* gene sequence that differed by 19 nucleotides from the other sequenced *X. perforans* strains. This *gapA* sequence is closely related to an unknown *Xanthomonas* species isolated from Ethiopia and originally identified as *X. euvesicatoria* but found to be nonpathogenic on tomato (Kebede et al. 2014). Here, Xp1913 was identified as a T3 *X. perforans* by inoculation on differential tomato lines and based on sequence similarity of the *avrXv3* and *XopJ4* genes to other sequenced *X. perforans* strains.

The development of tomato cultivars with individual dominant *R* genes to bacterial spot has been undermined by changes in bacterial spot species and their effectors. A long-term breeding program to introgress *Xv3* into tomato genotypes began in 1991 after the displacement of T1 *X. euvesicatoria* by T3 *X. perforans* in Florida. In 1998, *X. perforans* overcame this resistance as a result of a mutation in *avrXv3*, prior to commercial deployment of *Xv3*. More concerning was that by 2006, 75% of the Florida population contained mutations in *avrXv3* without any apparent selection pressure. Similarly, in pepper, *Bs2* was introgressed into commercial pepper genotypes in an effort to produce cultivars with durable resistance to the bacterial spot pathogen. Although pepper cultivars containing *Bs2* provided high levels of resistance for a few years, bacterial spot began to develop on the cultivars resulting in significant disease. Gassmann et al. (2000) characterized *Xanthomonas*

strains associated with these outbreaks and determined that point mutations occurred in *avrBs2* that resulted in the protein not being recognized by *Bs2* but still allowed *AvrBs2* to function as a virulence factor in pepper. Although *Bs2* has been transferred to tomato (Horvath et al. 2012), it is unlikely that this gene alone will provide durable resistance. Therefore identifying multiple *R* genes that target *Xanthomonas* effectors will be required.

Breeding for resistance against pathogens depends on selecting appropriate effector gene targets. A single nucleotide variation in an effector may undermine the usefulness of the resistance gene. Thus, for breeding purposes, effectors that have conserved sequences within a diverse pathogen population would be preferred. One or more factors driving the race shift from T3 to T4 in the *X. perforans* population in Florida remains unknown. However, our data suggest that mutations in *avrXv3* were associated with other simultaneous changes in the population that resulted in additional variation in effector profiles. Nevertheless, we confirmed that the *xopJ4* gene is conserved in all *X. perforans* strains regardless of tomato race or genetic group and in strains collected over a period of 20 years. Effector *XopJ4* is currently a stable effector in *X. perforans*. Likewise, the acquisition of the *avrBsT* gene in *X. perforans* makes this gene another potential target for resistance breeding. Preliminary field data indicate that *AvrBsT* is a virulence factor in *X. perforans* (P. Abrahamian J. B. Jones, and G. E. Vallad, unpublished data), and this effector is largely present in recent *X. perforans* collections. Additionally, corresponding host resistance has been studied against both *XopJ4* and *AvrBsT* effectors. A resistance locus, *RXopJ4*, that recognizes *xopJ4* was mapped (Sharlach et al. 2013) and identified in the wild tomato accession *Solanum pennellii* LA716. The locus is a 4.2-Mb segment conferring resistance to *Xanthomonas* strains carrying *xopJ4* and the resistance gene was fine-mapped to a 190-kb region. Likewise, heat-shock protein 70a was recently reported to interact with the *avrBsT* effector to induce programmed cell death in pepper (Kim and Hwang 2015). An incompatible reaction in the presence of *avrBsT* gene was observed in *S. lycopersicoides* and its hybrid, *S. lycopersicum* (Wang 1992), as well as in most pepper cultivars (Potnis et al. 2011), although the resistance locus has not been identified. Additional studies are needed to identify the specific *R* genes against *xopJ4* and *avrBsT*. In conclusion, these genes could potentially be pyramided with the *Bs2* resistance gene to develop cultivars with more durable resistance against *X. perforans* in Florida.

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In the article “Analysis of Sequenced Genomes of *Xanthomonas perforans* Identifies Candidate Targets for Resistance Breeding in Tomato” by S. Timilsina, P. Abrahamian, N. Potnis, G. V. Minsavage, F. F. White, B. J. Staskawicz, J. B. Jones, G. E. Vallad, and E. M. Goss, *XopJ4* for strain Xp2010 was changed from + to – in Table 1 and *X. euvesicatoria* 85-10 was changed to *X. euvesicatoria* 75-3 in the last paragraph of the Results section and in Figure 2. The correct strain is shown below in revised Figure 2.

Revised Figure 2

Fig. 2. Maximum likelihood phylogenetic tree of *avrBsT*, showing sequence identity between alleles in *Xanthomonas vesicatoria* and *x. perforans* strains. Additionally, the *avrBsT* sequence from *X. axonopodis* pv. *manihotis* was identical to the *avrBsT* in bacterial spot-causing *Xanthomonas* strains.

Previously Published Version

Fig. 2. Maximum likelihood phylogenetic tree of *avrBsT*, showing sequence identity between alleles in *Xanthomonas vesicatoria* and *x. perforans* strains. Additionally, the *avrBsT* sequence from *X. axonopodis* pv. *manihotis* was identical to the *avrBsT* in bacterial spot-causing *Xanthomonas* strains.