Genome-Wide Association Study of Resistance to *Xanthomonas gardneri* in the USDA Pepper (*Capsicum*) Collection

Neha Potnis,^{1,2} Sandra E. Branham,² Jeffery B. Jones,³ and W. Patrick Wechter^{2,†}

¹ Department of Entomology and Plant Pathology, Auburn University, Auburn, AL

² U.S. Vegetable Laboratory, U.S. Department of Agriculture Agricultural Research Service, Charleston, SC

³ Department of Plant Pathology, University of Florida, Gainesville, FL

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ABSTRACT

Bacterial spot *Xanthomonas* species cause significant disease outbreaks on tomato and pepper in tropical and subtropical regions throughout the world. Host resistance has been one of the key components of integrated disease management approaches to mitigate plant pathogens. Although a number of resistance genes have been identified in pepper against bacterial spot xanthomonads, emergence of bacterial spot pathogen variants capable of overcoming these sources and changing pathogen distributions reinforce the importance of identifying novel candidates to incorporate into breeding programs. We conducted a genome-wide association study (GWAS) on a diverse U.S. Department of Agriculture collection of pepper germplasm

including different species of *Capsicum* to identify novel sources of disease resistance against a highly virulent *X. gardneri* strain isolated from a recent outbreak. GWAS identified highly significant single nucleotide polymorphisms associated with defoliation in response to infection with *X. gardneri*. Functionally relevant candidate genes encoded products involved in disease resistance/susceptibility, hormone signaling, and basal resistance against multiple pathogens in various host-pathogen systems. The *X. gardneri*-resistant genotypes and quantitative trait loci identified in this study provide alleles that could be used for a resistance gene pyramiding effort against different species of bacterial spot xanthomonads in pepper.

Bell and chili peppers (*Capsicum* spp.) are significant economic crops grown throughout the world. In 2016, the U.S. Department of Agriculture (USDA) estimated bell pepper production at 15.6 million pounds grown on 46,300 acres and valued at more than \$735 million (NASS 2017). Bacterial spot of peppers is one of the most devastating and difficult to manage diseases on peppers over the past several decades (Jones et al. 2004; Obradovic et al. 2008). Bacterial spot symptoms are seen on aboveground plant parts of pepper and include necrotic lesions that expand upon disease progression and result in large necrotic areas. The disease can cause severe defoliation, resulting in reduced plant vigor, exposure of fruit to sunburn, reduced yield, as well as spots and rots on fruit. Yield losses in severely affected crops under conducive environmental conditions can be 100% (Ritchie 2007; Zitter 1985). The causal agent of bacterial spot has been reclassified several times and was separated into four distinct species: *Xanthomonas euvesicatoria*, *X. vesicatoria*, *X. perforans*, and *X. gardneri* (Jones et al. 2004).

Geographic distribution of the four species has changed dramatically over the years (Potnis et al. 2015). *X. euvesicatoria* and *X. vesicatoria* have been dominant species on pepper. There have been increasing numbers of outbreaks caused by the emerging species *X. gardneri* and *X. perforans* on tomato and/or pepper, such as the 2010 outbreak in the Midwest, and identification of novel

lineages of *X. perforans* with added ability to cause disease on pepper (Ma et al. 2011; Schwartz et al. 2015). A survey conducted between 2010 and 2013 on tomato and pepper production areas in Ohio and Michigan indicated that *X. euvesicatoria* was the dominant species on pepper, with *X. gardneri* being present in 23% of the samples. Thus, the ability of *X. gardneri* to infect both tomato and pepper and its predominance in the recent outbreaks in the Midwest are of concern in both tomato and pepper production (Ma 2015). *X. gardneri* is noted for its aggressiveness, causing large star-shaped lesions on the fruit. Such fruits with large lesions are unacceptable in fresh as well as processed markets (Miller 2012).

The increasing number of bacterial spot outbreaks caused by emerging variants of xanthomonads highlights the importance of finding practical, immediate solutions for growers. Current strategies rely heavily on bactericides including copper. However, these compounds are ineffective under strong disease pressure and have a negative impact on the environment, so we must find sustainable alternatives. Thus, host resistance will be a critical component of disease management strategies. Resistance breeding programs have been successful in developing commercial pepper lines with hypersensitive and quantitative resistance (Stall et al. 2009). However, durability of resistance has been elusive as a result of the changing geographic distribution of the pathogen and emergence of new pathogenic variants.

Six dominant resistance (R) genes have been identified in pepper: namely *Bs1*, *Bs2*, *Bs3*, *Bs4C*, *Bs7*, and *BsT* (Stall et al. 2009). Avirulence genes corresponding to these resistance genes have been identified. As plant resistance was identified in pepper genotypes, pathogenic races were identified based on differential reactions on resistant genotypes. The dominant resistance genes, *Bs1* (from *C. annuum* plant introduction [PI] 163192), *Bs2* (from *C. chacoense* PI 260435), and *Bs3* (from *C. annuum* PI 271322), which confer resistance to certain races of *X. euvesicatoria*, have been introgressed into commercial bell pepper cultivars. Race shifts in bacterial spot xanthomonads in response to resistance gene deployments have been common. The avirulence gene *avrBs1*, which interacts with resistance gene *Bs1* in pepper, was linked with copper resistance on a large self-transmissible plasmid. *avrBs1* was

†Corresponding author: W. P. Wechter; Pat.Wechter@ars.usda.gov

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N. Potnis and S. E. Branham contributed equally to this work.

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found to be inactivated by insertion sequence *IS476*, leading to the emergence of spontaneous mutants resulting in race shift. The bacterial gene *avrBs2* that interacts with *Bs2* in pepper has been considered an important virulence factor that when mutated would render the bacterium less aggressive (Kearney and Staskawicz 1990). However, strains were isolated with non-synonymous point mutations in *avrBs2* that resulted in AvrBs2 not being recognized by *Bs2*, but maintaining virulence (Gassman et al. 2000). The avirulence gene *avrBs3*, which elicits a hypersensitive response in pepper with the *Bs3* resistance gene, is found on a plasmid. Loss of *avrBs3* plasmid from race 1 strains, resulting in race shift to race 3, was reported by Kousik and Ritchie (1996) in field plots of pepper possessing the *Bs3* gene. Bacterial strains exhibiting virulence on cultivars carrying one or more dominant resistance genes have been isolated (Bouzar et al. 1994; Kousik and Ritchie 1998). Currently, 11 pathogenic races of *Xanthomonas* on pepper have been identified. Although resistance gene pyramiding has offered advantages in certain combinations, none of these combinations have been successful in controlling disease without the need for additional control measures.

In addition to hypersensitive type resistance, quantitative resistance has also been identified. The level of quantitative resistance identified in pepper accessions with two to three recessive R genes varies in response to different species of bacterial spot pathogen. Breeding efforts have enabled transfer of quantitative resistance to commercial varieties in an additive fashion governed by *bs5* and *bs6*. Although PIs containing these additive recessive genes exhibit a higher level of resistance toward *X. euvesicatoria*, *X. gardneri* strains caused an aggressive reaction on plants containing individual as well as combined recessive resistance genes (Stall et al. 2009).

Bacterial spot can be caused by four distinct species, complicating a resistance-based management strategy. Identifying resistance genes effective against a constantly changing population of the four species, as well as a changing geographic distribution, remains challenging. Introgression of resistance genes with different modes of gene action can provide a durable solution to this continuous host-pathogen arms race. Failure of commercial pepper varieties with recessive genes to be effective against *X. gardneri*, as well as increasing outbreaks caused by *X. gardneri* around the world, warrants the identification of novel sources of resistance in pepper against emerging species, including *X. gardneri*, and the incorporation of these novel resistances in breeding programs. Studies have utilized the power of correlating high-density single nucleotide polymorphism (SNP) markers with phenotypic traits through genome-wide association studies (GWAS). GWAS of various plant-pathogen systems have facilitated the mapping of novel alleles associated with disease resistance (Dilla-Ermita et al. 2017; Jia et al. 2012; Tung et al. 2010; Wang et al. 2012, 2014; Zhang et al. 2016; Zhu et al. 2008).

The goal of the current project was to find novel sources of resistance to *X. gardneri* in pepper and to identify potential candidate genes involved in bacterial spot disease resistance. We screened a diverse USDA panel of pepper germplasm for resistance to *X. gardneri* and found highly resistant germplasm. In addition, using high-throughput sequencing technologies to facilitate GWAS, we identified novel resistance quantitative trait loci (QTLs) associated with bacterial spot of pepper.

MATERIALS AND METHODS

Plant material and growth conditions. The USDA pepper PIs (287 accessions) were obtained from the U.S. plant germplasm repository at the Plant Genetic Resources Conservation Unit in Griffin, Georgia. These 287 PIs included 158 PIs belonging to *C. annuum*, nine PIs belonging to *C. annuum* var. *glabriusculum*, five PIs belonging to *C. baccatum*, 35 PIs belonging to *C. baccatum* var. *pendulum*, one PI belonging to *C. baccatum* var. *praetermissum*,

one PI belonging to *C. baccatum* var. *umbilicatum*, one PI belonging to *C. chacoense*, 44 PIs belonging to *C. chinense*, 24 PIs belonging to *C. frutescens*, one PI belonging to *C. galapagoense*, four PIs of *C. pubescens*, and four belonging to *Capsicum* spp. The designation of the species of *Capsicum* mentioned above and in the figures is based on the metadata obtained from the U.S. plant germplasm repository. Twelve individuals from each PI accession were grown in Metro-Mix 360 (Sun Gro Horticulture, Agawam, MA) in 50-cell trays (PRO050R5; Myers Industries Lawn and Garden Group, Middlefield, OH) until the cotyledon stage, at which time the plants were transferred into plastic 10 × 10 × 10-cm pots and fertilized with Osmocote (Everris NA Inc., Dublin, OH). Plants were grown in two replicates of six plants each in a greenhouse (average temperature of 25°C, ranging between 18 and 38°C) for 4 to 5 weeks prior to inoculation.

***X. gardneri* inoculum preparation.** A highly virulent strain of *X. gardneri* (USVLXG1), isolated from infected tomatoes from an outbreak of the disease in Ohio, was streaked to single colonies on nutrient agar (NA) plates. USVLXG1 was maintained as a 15% glycerol stock at –80°C in the Wechter Laboratory at the U.S. Vegetable Laboratory (USDA Agricultural Research Service, Charleston, SC). After 2 days, single colony isolates were then streaked onto fresh NA plates and grown at 27°C in an incubator for 48 h. Cells were scraped from the plates and resuspended in sterile tap water to an optical density of 0.3 at 600 nm, which equates to ~1 × 10⁸ CFU ml^{–1}. This stock then was diluted again to a final concentration of 1 × 10⁶ CFU ml^{–1} with sterile tap water, then amended with 0.04% of the wetting agent Silwet L-77 (Helena Chemical Co., Collierville, TN). Inoculated plants were maintained in the greenhouse using standard practices for the remainder of the experiment.

***X. gardneri* inoculation and host resistance evaluation.** The entire foliar portion of each plant was inverted and submerged in the inoculum for 30 s. Plants were then randomized and returned to the greenhouse bench. The cultivars Early California Wonder (susceptible), 56R (tolerant), and 30R (resistant) were included in each test as controls. Two-hundred eighty-seven accessions were tested along with pepper genotypes with known reactions to *X. gardneri*, which are commonly used as recipient parents for generating genetic populations and include the cultivars Early Calwonder (ECW; susceptible to *Xg*), ECW-20R (containing resistance alleles for the *Bs2* gene), ECW-30R (containing resistance alleles for the *Bs2* gene), and ECW-56R (containing recessive resistance alleles for *bs5* and *bs6*). Disease severity was evaluated 14 days postinoculation by ratings based on the percentage of area covered by bacterial spot lesions on three middle leaves of each plant. The disease severity scale was as follows: 0, no lesions observed; 1, trace but less than 1% leaf area diseased; 2, 1 to 10% of leaf area diseased; 3, 11 to 20% of leaf area diseased; 4, 21 to 35% of leaf area diseased; 5, 36 to 50% of leaf area diseased; 6, 51 to 65% of leaf area diseased; 7, 66 to 80% of leaf area diseased; 8, 81 to 99% of leaf area diseased; and 9, 100% of leaf area diseased/if defoliated (Kousik and Ritchie 1998; Kousik et al. 1996). We also used the percentage of defoliation for each plant as an indicative trait for the level of disease resistance because this is a classic symptom observed with *X. gardneri* on susceptible pepper. Based on the disease ratings, the PIs were classified into four phenotypic categories: highly resistant (disease ratings of 0 to 2), moderately resistant (disease ratings of >2 to 4), moderately susceptible (disease ratings of >4 to 6), and highly susceptible (disease ratings of >6 to 9).

Statistical analyses. Adjusted means for defoliation and disease severity were used as the phenotypic traits for GWAS analysis. Adjusted genotypic means for each *C. annuum* PI, using the checks to correct for environmental variation between incomplete blocks, were calculated with the ‘easynova’ package (Arnhold 2013) in R software version 3.2.5 (R Core Team 2016) with the design parameter set to augmented incomplete block. The same checks (30R, 56R, and ECW) were used in every incomplete

block to reduce error by controlling for blocking effect and experimental error variance with an augmented incomplete block design (Federer and Raghavarao 1975). Variance components, for estimation of broad-sense heritability, were determined with a restricted maximum likelihood mixed model using ASReml-R version 3.0 (Gilmour et al. 2009) in R version 3.2.5. The model for both defoliation and disease severity rating included the following: accession (random), check (fixed), the interaction between check and block (fixed), block (random), replicate nested in block (random), and plant nested in accession (random).

Genomic DNA extraction and polymerase chain reaction screening. Genomic DNA was extracted from young leaf tissue of a single representative plant per accession. DNA was extracted from 100 mg of leaf tissue using the DNeasy Plant Mini kit (Qiagen, Germantown, MD). The tissues were homogenized in 1.5-ml screw-cap microcentrifuge tubes in a solution containing 400 µl of buffer AP1 and 4 µl of RNase A using a FastPrep FP120 homogenizer on setting 4 m/s for 20 s (Thermo Fisher Scientific, Waltham, MA) with a quarter-inch ceramic bead (MP Biomedicals, Santa Ana, CA) in each microcentrifuge tube. The DNeasy protocol was then followed to completion. PIs rated as highly resistant, moderately resistant, or moderately susceptible (ratings 0 to 6) were subjected to polymerase chain reaction (PCR) screening for the presence of known resistance alleles of *Bs2* and *Bs3* (using primers and settings optimized by Romer et al. 2010 and Troung et al. 2011).

Sequencing and SNP calling. We followed a selective genotyping approach (Darvasi and Soller 1992) to choose a subset of 187 PIs from the 287 phenotyped accessions for genotyping by sequencing (GBS). Accessions from all four phenotypic categories were chosen, including all of the highly resistant accessions and more than half of the accessions from each of the remaining categories (moderately resistant, moderately susceptible, and highly susceptible). In addition, we also included control lines including ECW and near-isogenic lines derived from ECW containing known resistance alleles for *Bs2* (20R), *Bs3* (30R), and *Bs5/6* (56R) as well as CM334 (for which the complete genome is available) for GBS. Purified DNA was then quantified using the Quant-iT dsDNA assay kit, broad range (Thermo Fisher Scientific), on a Qubit Fluorometer (Thermo Fisher Scientific). DNA samples were sent to two facilities for library preparation and sequencing: one plate (95 samples) was sent to the Genomic Diversity Facility at Cornell University (Ithaca, NY) and the second plate was processed by the DNA Sequencing Facility at the University of Wisconsin–Madison (Madison, WI). Libraries (96-plex) were prepared by digestion of genomic DNA with *ApeKI* (Elshire et al. 2011) and were sequenced as 100-bp, single-end reads on an Illumina HiSeq 2000/2500 system. Processing of raw reads and SNP calling was completed with the GBSv2 pipeline of TASSEL 5.2.30 (Bradbury et al. 2007; Glaubitz et al. 2014) using default parameters. Unique sequence tags were aligned to the *C. annuum* hot pepper reference genome (Kim et al. 2014) with the Burrows-Wheeler aligner version 0.5.9 (Li and Durbin 2009, 2010). Likely genotyping errors were filtered with VCFtools version 0.1.15 (Danecek et al. 2011), for each grouping of PIs described below, by removing SNPs with a minor allele frequency (MAF) of less than 0.01 and/or >90% missing data.

Genotypes for GWAS. Missing allele calls for SNPs polymorphic in the *C. annuum* PIs (postinitial filtering) were imputed in BEAGLE version 4.1 (Browning and Browning 2007, 2016) without a reference panel and using default settings with 10 burn-in iterations and 15 phasing iterations. High-quality SNPs with a MAF > 0.05 were used for GWAS.

Population structure. Genetic distances were visualized for all peppers in the GWAS panel ($n = 187$) using principal coordinates analysis (PCoA). An additional PCoA of accessions excluding *C. baccatum* PIs was generated to provide better separation of the clusters corresponding to the more closely related species in the genus (*C. annuum*, *C. chinense*, and *C. frutescens*). Several PIs were

clearly misidentified as the PIs clustered tightly by species. For all *C. annuum*-specific analyses (PCoA, STRUCTURE, heritability, and GWAS), we used all PIs that fell within the *C. annuum* cluster of this PCoA. The relationship between genetic distance and country of collection of the PI was examined through a PCoA of all accessions that formed the *C. annuum* cluster. PCoAs were calculated in TASSEL 5.2.35 (Bradbury et al. 2007) using genetic distance matrices generated from polymorphic markers specific to each data set. To more closely examine the fine-scale population stratification of *C. annuum* PIs, they were clustered into populations with the program STRUCTURE version 2.3.4 using the admixture model with correlated allele frequencies (Falush et al. 2003, 2007; Hubisz et al. 2009; Pritchard et al. 2000). SNPs polymorphic within the *C. annuum* PIs were filtered by linkage disequilibrium (LD) to meet the assumptions of unlinked SNPs for population structure analyses. LD pruning was completed in SVS version 8.7.0 (Golden Helix Inc., Bozeman, MT; <http://www.goldenhelix.com/>) using default settings.

Population numbers (K) of 1 to 9 were run 10 times each with 35,000 burn-in iterations and 35,000 Markov chain Monte Carlo repetitions. Summaries and figures of the STRUCTURE results were generated with the analysis pipeline CLUMPAK version 1.1 (Kopelman et al. 2015), which includes the software packages CLUMPP version 1.1 (Jakobsson and Rosenberg 2007), MCL version 14-137 (Enright et al. 2002; Van Dongen 2000), and DISTRUCT version 1.1 (Rosenberg 2004). Values for multiple runs of each K value were compared using 2,000 iterations of the largeKGreedy algorithm of CLUMPP and distinct solutions for each K separated with MCL. The optimal K value was determined using the delta-K method described in Evanno et al. (2005). Membership coefficients for each *C. annuum* PI were graphically displayed by geographic region using DISTRUCT.

GWAS. Mixed linear model (MLM) analysis of the adjusted means of defoliation and disease severity in the *C. annuum* accessions ($n = 107$; owing to a lack of germ for one PI) was performed with SNP and Variation Suite version 8.7.0 software (Golden Helix Inc.). An additional GWAS using a binary descriptor of the sequencing plate was done to search for sequencing bias introduced by the different laboratories that performed the sequencing. SNPs significantly associated with the sequencing plate were removed from all analyses. Associations were detected with a variance component approach utilizing EMMAX (Kang et al. 2010) and P3D (Zhang et al. 2010). Population structure and relatedness were controlled by inclusion of an identity-by-state matrix and five principal coordinates in the MLM. A Bonferroni correction was applied to the GWAS *P* values to determine a genome-wide significance threshold of 0.05. Manhattan and quantile-quantile (QQ) plots of association values were generated with the qqman package in R (Turner 2014). The pepper reference genome annotation (version 1.55) of Mexican landrace CM334 (Kim et al. 2014) was used to generate a list of candidate genes within ± 150 kb of the significant SNP. The distance of 150 kb was chosen to capture the most likely genes in LD with our significant SNPs, as previous genome-wide estimates of LD in *C. annuum* using GBS data were 139 kb (Nimmakayala et al. 2016) and 100 kb (Taranto et al. 2016).

Data archiving. All phenotypic and genotypic data associated with this research are available in Supplementary Tables S1, S2, and S3.

RESULTS

Bacterial spot disease screening in pepper accessions. A highly virulent strain of *X. gardneri* (USVLXG1) was selected to screen 287 pepper genotypes belonging to *C. annuum*, *C. baccatum*, *C. chacoense*, *C. chinense*, *C. frutescens*, *C. galapagoense*, *C. pubescens*, and *Capsicum* spp. Phenotyping of the control lines, ECW (highly susceptible), and ECW-56R (moderately susceptible) conformed to

the expected disease reactions. ECW-20R and ECW-30R carried *Bs2* and *Bs3* resistance alleles, respectively. Both lines were moderately resistant based on disease severity ratings of 3.9 and 3.7, respectively. This could be attributed to low levels of observed segregation in our replicated experiments. We observed a wide range of disease resistance among the PIs, with 40% of the genotypes exhibiting high susceptibility (disease ratings of >6 to 9), 33.8% genotypes showing moderate susceptibility (disease ratings of >4 to 6), 21% exhibiting moderate resistance (disease ratings of >2 to 4), and 5.2% exhibiting high resistance (disease ratings of 0 to 2) (Fig. 1). It is important to note here that these categories were based on the average disease severity ratings and that 29.3% of the accessions were segregating. Significant differences ($P < 0.0001$) were observed among the 287 PIs based on both disease severity rating as well as defoliation rating according to the Kruskal-Wallis test. Among the different *Capsicum* spp. screened here, *C. baccatum* contained the highest proportion of resistant PIs, with ~10% of the phenotyped *C. baccatum* var. *pendulum* PIs ($n = 35$) being highly resistant and 60% of the phenotyped *C. baccatum* PIs ($n = 5$) being moderately resistant based on disease severity (Fig. 1). The *C. chacoense* PI 260435, the original source of the resistance allele of the *Bs2* gene (Tai et al. 1999), displayed a high level of resistance against *X. gardneri* in our phenotyping screen. *C. baccatum* and *C. chacoense* genotypes also displayed lower percentages of defoliation. Fifty-five percent of the PIs in our screen belonged to *C. annuum*, 20% of which were classified as highly or moderately resistant (Fig. 1). The original source of the *Bs3*

resistance allele, PI 271322 (*C. annuum*), showed moderate resistance with disease severity ratings ranging from >2 to 4 and 1.6% defoliation. We further screened highly resistant, moderately resistant, and moderately susceptible PIs ($n = 172$) for the presence of known bacterial spot disease resistance alleles for *Bs2* and *Bs3*, using primers developed by Troung et al. (2011) and Romer et al. (2010), respectively. Of these, 13 PIs (10 PIs of *C. annuum*, two of *C. frutescens*, and one of *C. chinense*) contained *Bs3* resistance alleles and three PIs (two belonging to *C. pubescens* and one belonging to *C. chacoense*) contained *Bs2* resistance alleles. The average disease severity rating for the PIs with *Bs3* resistance alleles was 3.65, although six of the PIs were segregating for resistance. The average disease severity for the PIs with *Bs2* resistance alleles was 3.70, with two of three PIs segregating for disease resistance.

Mixed model analysis of resistance traits in the GWAS panel ($n = 107$ *C. annuum* PIs) revealed differences in the sources of variation contributing to the overall variance observed in defoliation and disease severity (Tables 1 and 2). Broad-sense heritability of disease severity ($H^2 = 0.62$) was much higher than that of defoliation ($H^2 = 0.37$). The main environmental component that explained the decrease in genetic contribution for defoliation was the high proportion of the overall phenotypic variation attributed to blocks (36.9%). Even though the resistance traits were rated on the same plants at the same time, block only explained 3.9% of the variation in disease severity.

Genotyping. We selected 187 PIs for sequencing that represented resistant, moderately resistant, and susceptible phenotypes, which included three PIs with *Bs3* resistance alleles (PI 271322, PI653619, and Grif 9271) along with the control lines including ECW and near-isogenic lines derived from ECW containing known resistance alleles for *Bs2* (20R), *Bs3* (30R), and *Bs5/6* (56R). Sequencing produced >438 million high-quality barcoded reads, which grouped into 1,195,192 unique tags and generated 388,971 SNPs. Per sample mean read depth ranged from 4.9 to 30.4 across the pepper data set, with the exception of a single poorly sequenced (mean depth of 0.38) *C. frutescens* accession (PI 14015). The pepper genotypes were used only for the purpose of verifying the species identity of the accessions; therefore, this sample was retained and was not reclassified. Mean read depth across the *Capsicum* PIs was 15.8 with a standard deviation of 5.4. The mean read depth per sample for the *C. annuum* data set ranged from 9.5 to 30.4, with an overall mean of 18.7 and a standard deviation of 4.8. Filtering of sequencing errors (MAF < 0.01 and/or >90% missing calls) within each data set (all genotyped peppers,

Fig. 1. Phenotyping of the U.S. Department of Agriculture collection of 287 *Capsicum* accessions inoculated with a representative strain of *Xanthomonas gardneri* (USVLXG1). **A**, Bar graph of mean disease severity (dissev) and mean defoliation rating (def) across the collection after artificial inoculation with USVLXG1. The error bars indicate standard error. **B**, Proportion of accessions displaying each category of reaction after inoculation with USVLXG1: highly resistant (disease ratings of 0 to 2), moderately resistant (>2 to 4), moderately susceptible (>4 to 6), and highly susceptible (>6 to 9).

TABLE 1. Variance components of defoliation in the U.S. Department of Agriculture *Capsicum annuum* plant introduction collection ($n = 107$)

Variance component	Variance	%V _p ^a	Standard error	P value ^b
Accession (A)	3.1×10^{-2}	37.4	4.7×10^{-3}	0
Block (B)	3.1×10^{-2}	36.9	2.3×10^{-2}	5.9×10^{-4}
Replicate within B	2.1×10^{-9}	2.6×10^{-6}	9.2×10^{-11}	1
Plant within A	2.6×10^{-9}	3.1×10^{-6}	1.1×10^{-10}	1
Residual	2.1×10^{-2}	25.6	9.1×10^{-4}	NA

^a Percentage of the phenotypic variation explained by the variance component.
^b NA = not applicable.

TABLE 2. Variance components of disease severity in the U.S. Department of Agriculture *Capsicum annuum* plant introduction collection ($n = 107$)

Variance component	Variance	%V _p ^a	Standard error	P value ^b
Accession (A)	3.7	62.2	0.54	0
Block (B)	0.23	3.9	0.40	0.44
Replicate within B	0.065	1.1	0.045	9.9×10^{-7}
Plant within A	0.36	6.1	0.041	0
Residual	1.6	26.7	0.23	NA

^a Percentage of the phenotypic variation explained by the variance component.
^b NA = not applicable.

$n = 187$; PIs excluding *C. baccatum*, $n = 161$; and only *C. annuum*, $n = 108$, as determined by genetic distance) reduced the number of markers to 367,549 SNPs, 293,160 SNPs, and 92,281 SNPs, respectively. After removal of SNPs with significant plate ascertainment bias (65 SNPs), a final set of 90,794 high-quality SNPs with a MAF > 0.05 was used for all association tests. Imputation of the *C. annuum* genotypes with BEAGLE reduced the missing data to 0%. Mean SNP spacing varied from 25 to 40 kb per chromosome, while the maximum distance ranged from 1.1 to 2.7 Mb. Additional filtering by LD to remove linked SNPs reduced the data set to 34,648 SNPs for *C. annuum* PIs, which were used for STRUCTURE analysis.

Genetic diversity and population structure. We visualized genetic diversity among 187 accessions of pepper, representing four species of *Capsicum* (namely *C. annuum*, *C. baccatum*, *C. chinense*, and *C. frutescens*) and undefined *Capsicum* species, using PCoA (Fig. 2A). The first principal coordinate explained 55.5% of the genetic variation and separated the PIs into three groups. *C. baccatum* was genetically distant from the other species. *C. chinense* and *C. frutescens*, while readily distinguishable from one another, formed a combined cluster. To improve the separation of the accessions of these two species, a separate PCoA was generated, excluding the distant *C. baccatum* (Fig. 2B). Seventy-nine percent of the genetic variation was explained by the first

Fig. 2. Principal coordinates (PCo) analysis of the genotyped U.S. Department of Agriculture pepper collection. Scatterplot of the first two coordinates of genetic distance between **A**, all pepper accessions ($n = 187$), **B**, pepper accession excluding the most distant species (*C. baccatum*, $n = 161$), and **C**, the *Capsicum annuum* subset ($n = 108$) of the collection.

principal coordinate, which separated *C. annuum* from *C. frutescens* and *C. chinense*. The distance between *C. frutescens* and *C. chinense* was responsible for the second coordinate (16.2% of the genetic variation), showing that these species are the most closely related of the set. With the improved separation, after exclusion of *C. baccatum*, it became clear that nine individuals were likely misidentified in the Germplasm Resources Information Network (GRIN), as they tightly cluster with different species groups than their current GRIN descriptors. The two PIs that were originally described as unidentified *Capsicum* spp. both cluster with *C. chinense*.

Population structure among the *C. annuum* PIs was examined with PCoA and STRUCTURE analysis. The diverse PIs were spread widely across the first two principal coordinates of the PCoA plot, with a few noticeable geographic trends (Fig. 2C). Several U.S. cultivars formed a tight cluster in the upper-right quadrant, indicating low genetic diversity among them. The European and Central American PIs were distant from one another, as they fell in the extremes of the plot. The PIs collected from Asia have wide genetic diversity, as they were spread across all four quadrants. STRUCTURE analysis separated the *C. annuum* PIs into two

clusters (Fig. 3). Most individuals were admixed with membership coefficients in both clusters (71%), while fewer individuals were derived from only the dark (22%) or light clusters (7%). Accessions from the United States, Europe, and Turkey had higher representation from the dark cluster, Central America from the light cluster, and Asia, Mexico, and South America were admixed.

Genome-wide association analysis. We used GWAS to identify SNPs associated with resistance to *X. gardneri* in *C. annuum* ($n = 107$). The GWAS models chosen used both principal components and a kinship matrix to correct for population structure and relatedness. This model was appropriate for disease severity (Fig. 4B), but a QQ plot of the observed versus expected P values for defoliation showed a slight inflation of the observed value (Fig. 4A), indicating a higher likelihood of residual false-positive associations. Therefore, the highly conservative Bonferroni correction was used to control for false-positive associations. We identified 20 significant SNPs associated with defoliation, which collocated within 150 kb of 92 unique genes on chromosomes 1, 2, 3, 8, and 10 (Fig. 5A). There were no significant SNPs associated with disease severity (Fig. 5B).

DISCUSSION

Screening of the USDA pepper collection for resistance to *X. gardneri* revealed several highly resistant genotypes. Use of a dip-inoculation technique for germplasm screening allowed identification of non-hypersensitive response-type resistance sources that are often missed using typical infiltration inoculation techniques. Dip inoculation also enabled evaluation of defoliation. Bacterial spot xanthomonads, especially *X. gardneri*, cause severe defoliation in highly susceptible varieties as a part of their pathogenesis. Future studies of *X. gardneri* resistance, however, should concentrate on disease severity because it is a better indicator of a resistant genotype owing to much higher heritability than that of defoliation. Despite the high heritability of disease severity, no QTLs were significantly associated with this trait. The large genome size of *C. annuum* combined with the low coverage of

Fig. 3. DISTRUCT plot of *Capsicum annuum* STRUCTURE results using $K = 2$ as determined by the Evanno delta- K method. Accessions are displayed as horizontal bars and black lines separate the accessions into a priori groupings based on geographic origin. The horizontal bars are shaded according to their proportional membership coefficient for each cluster (genetic grouping).

Fig. 4. Quantile-quantile plots of the observed distribution of P values plotted against the distribution expected under the null hypothesis. P values were obtained from genome-wide association analysis of **A**, defoliation and **B**, disease severity.

GBS led to little to no SNP coverage in some areas of the genome (up to 2.7 Mb). It is likely that lack of coverage caused false-negative associations in the GWAS of disease severity. GWAS of defoliation revealed significant QTLs associated with resistance to bacterial spot disease caused by *X. gardneri*. Candidate genes based on defoliation encoded products that have been previously shown to function in disease resistance against bacterial, fungal, and viral diseases as well as in hormone signaling and in regulating susceptibility. Here we discuss significant QTLs that collocated with candidate genes whose homologs have been identified in other crops to be involved in resistance against pathogens.

Plants have evolved programmed cell death (PCD) to limit pathogen invasion and restrict pathogen spread. We identified several SNPs associated with defoliation that were in close proximity to or were within PCD-related genes, encoding caspases (CA03g14020), the tBRCA2 homolog (CA02g06790), the ubiquitin protein ligase (CA01g19270), and the RING-H2 finger protein coding gene (CA01g19300). The RING-H2 finger protein (belonging to the RING-UBox superfamily) coding gene (CA01g19300) contained three SNPs associated with defoliation. Caspases have been implicated in innate immune response in plants as regulators of cell death. A BRCA2 homolog in *Arabidopsis* species was involved in systemic acquired resistance by transcriptional regulation of pathogenesis-related genes through interaction with the strand-exchange protein RAD51 (Wang et al. 2010). Previous studies have shown the role of ubiquitin protein ligases and RING finger proteins in modulating plant innate immunity, broad-spectrum disease resistance, as well as biotic and abiotic stress response (Joo et al. 2017; Lee et al. 2011; You et al. 2016). XA21-binding protein 3 (XB3), a RING finger containing E3 ubiquitin ligase, was shown to regulate bacterial blight resistance gene encoding receptor kinase XA21 in rice (Huang et al. 2013). Interestingly, ubiquitin protein ligases and RING finger proteins were shown to be targets of pathogen effectors, suggesting their involvement in disease susceptibility (Janjusevic et al. 2006; Park et al. 2012; Üstün and Börnke 2014).

The ethylene response factor, ERF5 (CA03g13530), collocated with SNPs associated with defoliation. The role of ethylene response factors in immunity against *Xanthomonas* species has been investigated in tomato. The *Xanthomonas* effector, XopD, targets ethylene responsive factor in tomato, which suppresses ethylene production in the host plant, thereby inhibiting ethylene-stimulated immunity (Kim et al. 2013).

Although ECW20R, ECW30R, and PIs containing resistant alleles of the *Bs2* and *Bs3* R genes (based on PCR screening) were included in this study and had moderately resistant disease severity ratings (ranging from >2 to 4) and relatively low defoliation scores, such resistant varieties were not considered in the highly resistant category because of the stringent cutoff of 0 to 2. Thus, QTLs responsible for *Bs2/Bs3*-mediated disease resistance were not identified in this analysis. Screening for *Bs2/Bs3* resistance gene sequences in the CM334 genome revealed a 100% identical homolog on chromosome 2 for the *Bs3* gene, whereas several homologs annotated as a *Bs2* resistance protein with 82 to 89% identity were on chromosomes 5, 6, 7, 9, and 10. QTLs associated with strong resistance in this study did not collocate (within ± 150 kb) with the *Bs3* gene or any of the paralogs of the *Bs2* gene.

Race shifts in bacterial spot pathogen populations are common (Horvath et al. 2012; Stall et al. 2009) and have been a challenge for pepper breeding programs by decreasing the durability of resistance (Gassmann et al. 2000). Thus, identifying novel sources of resistance against these races is necessary. The *X. gardneri* resistance germplasm and QTLs identified here are of considerable importance for breeders and researchers for introgression into elite pepper lines and gene pyramiding efforts against different species of bacterial spot xanthomonads. The novel sources of resistance identified in this study will serve as a primer for further work in breeding of disease-resistant varieties resilient to changing pathogen population structure.

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Fig. 5. Genome-wide association mapping results for **A**, defoliation and **B**, disease severity across the genotyped U.S. Department of Agriculture *Capsicum annuum* plant introductions ($n = 107$) after inoculation with *Xanthomonas gardneri*. The x axis indicates the physical position along each of the 12 pepper chromosomes, which are differentiated by grayscale shading. Single nucleotide polymorphisms exceeding the genome-wide significance level of 0.05 after Bonferroni correction are above $-\log_{10}(p)$ of 6.2.

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