Resistance in *Lycopersicon esculentum* Intraspecific Crosses to Race T1 Strains of *Xanthomonas campestris* pv. *vesicatoria* Causing Bacterial Spot of Tomato

Wencai Yang, Erik J. Sacks, Melanie L. Lewis Ivey, Sally A. Miller, and David M. Francis

First, second, and fifth authors: Department of Horticulture and Crop Science, and third and fourth authors: Department of Plant Pathology, The Ohio State University, Ohio Agricultural Research and Development Center, 1680 Madison Ave., Wooster.
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ABSTRACT

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We used molecular markers to identify quantitative trait loci (QTL) that confer resistance in the field to *Xanthomonas campestris* pv. *vesicatoria* race T1, a causal agent of bacterial spot of tomato. An F_2 population derived from a cross between Hawaii 7998 (H 7998) and an elite breeding line, Ohio 88119, was used for the initial identification of an association between molecular markers and resistance as measured by bacterial populations in individual plants in the greenhouse. Polymorphism in this cross between a *Lycopersicon esculentum* donor of resistance and an elite *L. esculentum* parent was limited. The targeted use of a core set of 148 polymerase chain reaction-based markers that were identified as polymorphic in *L. esculentum* × *L. esculentum* crosses resulted in the identification of 37 markers that were polymorphic for the cross of interest. Previous studies using an H 7998 × *L. pennellii* wide cross implicated three loci, *Rx1*, *Rx2*, and *Rx3*, in the hypersensitive response to T1 strains. Markers that we identified were linked to the *Rx1* and *Rx3* loci, but no markers were identified in the region of chromosome 1 where *Rx2* is located. Single marker-trait analysis suggested that chromosome 5, near the *Rx3* locus, contributed to reduced bacterial populations in lines carrying the locus from H 7998. The locus on chromosome 5 explained 25% of the phenotypic variation in bacterial populations developing in infected plants. An advanced backcross population and subsequent inbred backcross lines developed using Ohio 88119 as a recurrent parent were used to confirm QTL associations detected in the F_2 population. Markers on chromosome 5 explained 41% of the phenotypic variation for resistance in replicated field trials. In contrast, the *Rx1* locus on chromosome 1 did not play a role in resistance to *X. campestris* pv. *vesicatoria* race T1 strains as measured by bacterial populations in the greenhouse or symptoms in the field. A locus from H 7998 on chromosome 4 was associated with susceptibility to disease and explained 11% of the total phenotypic variation. Additional variation in resistance was explained by plant maturity (6%), with early maturing families expressing lower levels of resistance, and plant habit (6%), with indeterminate plants displaying more resistance. The markers linked to *Rx3* will be useful in selection for resistance in elite × elite crosses.

Additional keywords: advanced backcross breeding method.

Control of bacterial spot in tomatoes (*Lycopersicon esculentum* Mill.) is difficult due to several factors including marginal efficacy of commonly applied chemicals, the rapid development of resistance to these chemicals in bacterial populations, the involvement of races from multiple closely related but distinct species of the pathogen, and a lack of available disease resistance in commercial cultivars. In addition, the bacterial spot pathogens can be seedborne and may persist as epiphytic populations on asymptomatic seedlings and mature plants. Ethylene-bis-dithiocarbamate fungicides, which are commonly used to increase the efficacy of copper-containing compounds applied to reduce bacterial populations on tomatoes, have raised public health questions and have been voluntarily banned by tomato processors.

The taxonomy and etiology of species causing bacterial spot is complex. Previously, the causal agent was considered to be *X. campestris* pv. *vesicatoria*, in which five races causing disease on tomatoes have been identified (T1, T2, T3, T4, and T5). However, at least two species, *Xanthomonas campestris* pv. *vesicatoria* and *X. vesicatoria*, corresponding to races T1 and T2, respectively, are now known to cause bacterial spot in tomato (11), and two additional species have been proposed (12). These are *X. perforator*, corresponding to *X. campestris* pv. *vesicatoria* races T3, T4, and T5, and *X. gardneri*. Jones et al. (12) have also proposed renaming *X. campestris* pv. *vesicatoria* as *X. euvesicatoria*. Pending acceptance of the proposed taxonomy, we refer in this paper to species causing bacterial spot by their currently accepted names and the recognized races when necessary for clarification. Of the four proposed species causing bacterial spot on tomato, three have been found in the Midwest. Strains of *X. campestris* pv. *vesicatoria* race T1 predominate followed by *X. vesicatoria* (race T2) and *X. campestris* pv. *vesicatoria* race T3 (*X. perforator*) strains (21). Race T3 strains have not dominated the population despite their occurrence in the region for several years, and race T4 strains have only been identified in Florida and Central America.

The challenge of breeding for multiple species causing bacterial spot is formidable. Sources of resistance have been identified and confirmed for *X. campestris* pv. *vesicatoria* races T1 and T3 and *X. vesicatoria* (race T2) (24,26,28). The *L. esculentum* breeding line, Hawaii 7998 (H 7998), is considered the most reliable source of resistance to *X. campestris* pv. *vesicatoria* race T1 (13). In a wide cross involving H 7998 × *L. pennellii* LA716, hypersensitivity involves at least three loci (35,39). These genetic studies utilized a cross to a susceptible wild species due to the limited genetic polymorphism found within *L. esculentum*. The three quantitative trait loci (QTL), *Rx1*, *Rx2*, and *Rx3*, were

Corresponding author: D. M. Francis; E-mail address: francis.77@osu.edu

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identified based on progression of the hypersensitive response (HR). Unfortunately, the polymorphic markers identified in the H 7998 × *L. pennellii* cross are inherited from the wild parent, are linked to susceptibility, and are of limited use for breeding purposes. Furthermore, the HR does not predict resistance in the field with *X. campestris* pv. *vesicatoria* race T1 strains (30). Resistance to *X. vesicatoria* (race T2) and *X. campestris* pv. *vesicatoria* (race T3) has been characterized using statistical approaches that estimate gene numbers and genetic effects. Segregation for *X. vesicatoria* resistance in F_2 and inbred backcross (IBC) populations derived from PI 114490 suggest that genetic control is conferred by a minimum of two loci (23). The poor correlation between *X. vesicatoria* (race T2) and *X. campestris* pv. *vesicatoria* race T3 resistance in the IBC population suggests that resistance to all species and races is not controlled by the same genes in PI 114490. The inheritance of *X. campestris* pv. *vesicatoria* race T3 resistance in Hawaii 7981 (H 7981) is conferred by an incompletely dominant hypersensitivity gene (29) and modifiers (27). Resistance to race T4 was reported in *L. pennellii* accession LA716 (1) but the efficacy of this resistance has not been demonstrated in the field nor has the reported map position been supported by subsequent studies.

Assuming no new species or races are discovered, a long-term breeding strategy will require that between five and seven loci from several sources of resistance be combined. Resistance that is controlled by more than one gene or is environmentally influenced may be difficult to work with using traditional breeding approaches. A strategy that combines marker-assisted selection (MAS) with populations that facilitate replicated field trials offers the best opportunity to pyramid resistance in a single line or hybrid. Cycles of field selection alternated with cycles of MAS could facilitate the development of resistant germplasm on an accelerated time-scale. As a prerequisite, it will be necessary to discover markers linked to the most important genes or loci and combine these into parental lines. Hybrids will offer an opportunity to further pyramid resistance for those loci with dominant action. For this approach to be successful, it will require selection for desirable recombination events and the selection for unlinked loci. A MAS strategy is complicated for bacterial spot resistance because the three lines (H 7998, H 7981, and PI 114490) are either *L. esculentum* or *L. esculentum* var. *cerasiformae*. The majority of markers available for wide cross mapping are not polymorphic in crosses between elite breeding materials and *L. esculentum* sources of resistance.

The work described here was initiated to identify markers that are polymorphic within *L. esculentum* and linked to QTL controlling resistance from H 7998 in order to facilitate the transfer of resistance to *X. campestris* pv. *vesicatoria* race T1 into processing tomato varieties. Our efforts focused on resistance to *X. campestris* pv. *vesicatoria* race T1 because these strains are the most common in the Midwest. The experience gained in this study will help refine strategies for developing cultivars resistant to multiple species and races of bacterial spot pathogens.

MATERIALS AND METHODS

Plant materials and experimental design. Population development for these studies began in July 1995 by crossing H 7998 with Ohio 88119. The initial hybrid was self-pollinated to develop a segregating F_2 population. A BC_3 advanced backcross (AB) population consisting of 86 families was developed through backcrossing the hybrid using Ohio 88119 as the recurrent parent. Genetic structure was maintained with BC_2 and BC_3 crosses to Ohio 88119 tracing back to individual BC_1 plants through single seed descent. The recurrent parent was chosen due to its concentrated early set, use as an elite line in commercial tomato hybrids (2,7), and susceptibility to bacterial spot. The AB population was converted to a BC_3S_5 IBC population through subsequent self-pollination. Inbred lines 9816 and 9834 were selected from the IBC population through further self-pollination.

Tomato seeds for all studies were sown in 288 cell flats filled with MetroMix-360 growing medium (Premier Horticulture Inc., Red Hill, PA) in a greenhouse. Greenhouse temperatures ranged from 25 to 35°C. At the first true leaf stage, a fertilization regimen of 50 ppm of N-P-K (Peters Professional All-Purpose Fertilizer, 20-20-20, Scotts-Sierra Horticultural Products Co., Marysville, OH) was applied daily.

Greenhouse evaluations were established using F_2 plants with resistant and susceptible parents as controls. Seedlings were transplanted into 10.2-cm plastic pots filled with MetroMix-510 growing medium (Premier Horticulture Inc.) 4 weeks after germination, and the daily fertilization regimen of 50 ppm of N-P-K was continued.

Evaluation of the AB population for response to bacterial spot was performed in replicated field trials. Seedlings were transplanted approximately 6 weeks after germination; production practices, plant spacing, and row spacing were as recommended for commercial growers (20). Field trials were established using randomized complete block designs with two blocks containing each line plus resistant and susceptible controls. Plots of each line consisted of 10 plants. Tests of inbred families originating from AB individuals were performed as a confirming generation.

Inoculum preparation and inoculation. Four *X. campestris* pv. *vesicatoria* race T1 strains (*Xcv*110c, *Xcv*118, *Xcv*89, and *Xcv*767) were used in the study. Inoculum was produced by growing the bacteria on yeast, dextrose, calcium carbonate (YDC) agar medium (15) at 28°C for 48 to 72 h. Bacterial cells were removed from the agar plates and suspended in 10 mM $MgSO_4 \cdot 7H_2O$, and the suspensions were standardized to $A_{600} = 0.15$ (a concentration of approximately 3×10^8 CFU/ml). For evaluations of bacterial populations in the greenhouse, inoculations were performed by dipping plants in a suspension of *Xcv*110c containing the surfactant Silwet L-77 as described by Salmeron et al. (22). In the field, inoculum was applied to plants with a backpack sprayer in the late evening approximately 2 weeks posttransplant of seedlings. Plants were spray-inoculated with the strain *Xcv*110c for the initial evaluation of the AB population. Subsequent evaluations for resistance in inbred families and lines were done with a mixture of strains *Xcv*118, *Xcv*89, and *Xcv*767.

Scoring for disease response. Greenhouse evaluations were based on bacterial populations present in inoculated tissue determined by a dilution plate method because bacterial population levels correlate well with resistance to bacterial spot in H 7998 (17,34). Three leaves from each F_2 individual were harvested 2 weeks after inoculation, surface-sterilized with 70% ethanol, homogenized in 1 ml of 100 mM potassium phosphate buffer (pH 7.4), and subjected to 10-fold serial dilutions. Twenty-five microliters of each serial dilution was plated onto YDC medium. Agar plates were incubated at 25°C for 4 days prior to counting colonies. Analysis of variance (ANOVA) and mean separations were performed on log-transformed colony forming units ($\log_{10}$CFU) per gram of tissue using the general linear model (GLM) procedure of SAS (SAS Institute, Cary, NC). Mean separations were based on least significant difference (LSD) after a significant F test in the ANOVA.

In the field, plants were rated for disease severity on the scale of Horsfall and Barratt (9) modified as follows. This scale translates percentage of diseased tissue to numbers, where 1 = 0%, 2 = 0 to 3%, 3 = 3 to 6%, 4 = 6 to 12%, 5 = 12 to 25%, 6 = 25 to 50%, 7 = 50 to 75%, 8 = 75 to 87%, 9 = 87 to 94%, 10 = 94 to 97%, 11 = 97 to 100%, and 12 = 100% diseased tissue. Because the AB families segregate (heterozygous for resistance and homozygous for susceptible), three ratings were recorded per plot based on the worst and best plants and the plot average. Plots were rated twice, with the early rating timed to correspond to when 80% of plots had reached the mature green stage of fruit

ripeness. The late rating was timed to correspond to when 80% of the plots had reached harvest maturity of greater than 80% red ripe fruit.

Molecular markers. A total of 148 simple sequence repeat (SSR) and single nucleotide polymorphism (SNP) markers were used to detect polymorphisms between H 7998 and Ohio 88119. These 148 primer pairs represented a core set of publicly available markers previously described (31,38, provided online by Cornell University), shown to be polymorphic within the *L. esculentum* germplasm pool (38; W. Yang and D. M. Francis, *unpublished data*), and covering 90% of the mapped tomato genome within ≈10 cM. Sequences of primers and map positions of SSR markers were obtained from the Solanaceae Genome Network (SGN; provided online by Cornell University) with marker name prefixed by "SSR" or Suliman-Pollatschek et al. (31) with marker name prefixed by "TOM". The M-13 primer (CACGACGTTGTAAAACGAC) was added to the 5′-end of each SSR forward primer to facilitate automated genotyping. Primers for SNP marker Rx3-L1 were developed based on conserved domains of protein kinases, and primers for SNP marker Cf9 were designed according to the sequence of the cloned resistance gene (10). CosOH73 is a marker designed based on conserved ortholog set (T1584, SGN website provided by Cornell University). The remaining SNP markers are described in a previous study (38) and represent 44 genes out of 1,245 that were shown to be polymorphic within *L. esculentum*.

Because resistance to *X. campestris* pv. *vesicatoria* race T1 is inherited as a quantitative trait in early generations (34,35,39), identification of molecular markers linked to resistance was accomplished in two steps. First, the SNP markers Rx3-L1 and Cf9, linked to the regions of the genome containing *Rx3* and *Rx1*, respectively, were used to screen the F_2 population to detect if there was a relationship between these markers and resistance. A more comprehensive analysis of markers showing polymorphisms between H 7998 and Ohio 88119 was performed using the AB population to detect other possible associations between markers and resistance.

DNA isolation and marker analysis. Young leaves were collected from individual plants of the F_2 population. Due to segregation in each AB family, tissue was bulked from eight plants such that there was a greater than 99% probability of detecting both loci at a given marker in segregating families. Genomic DNA was isolated using the modified CTAB method described by Kabelka et al. (14).

Genotyping using SNP markers was conducted using restriction digestion of amplified products according to the methods described previously (38). SSR analysis was conducted with an IR^2 DNA Analyzer (Li-Cor Inc., Lincoln, NE). For each AB family or IBC line, three pairs of primers were pooled based on the polymerase chain reaction (PCR) product size. PCRs were conducted in a 20-µl reaction volume. Each reaction consisted of 10 mM Tris-HCl (pH 9.0 at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$, 100 µM each of dNTP, 10 nM IRD 700 dye-labeled M-13 forward primer (Li-Cor), 0.1 µM each of M-13 tailed forward primer, 0.1 µM of each reverse primer, 2 µl of genomic DNA template, and 1 unit of *Taq* DNA polymerase. Reactions were heated at 94°C for 2 min followed by 36 cycles of 45 s at 94°C, 45 s at suitable annealing temperature (Table 1), and a 45-s extension at 72°C. Final reactions were extended at 72°C for 5 min. Amplification was performed in a programmable thermal controller (PTC-100; MJ Research, Inc., Watertown, MA). Following the amplification reactions, 14 µl of the amplification reaction products was mixed with 3 µl of formamide loading buffer (95% formamide, 20 mM EDTA, pH 8.0, and bromo phenol blue), denatured at 95°C for 3 min, and then chilled immediately to 4°C. Each sample, 0.8 µl, was loaded onto a 7% denaturing gel (7.0 M urea, 7% acrylamide, and 1× Tris-borate-EDTA) using an 8-channel Hamilton syringe (Hamilton Company, Reno, NV). IRD-labeled molecular markers (Li-Cor) were loaded in two-end lanes as a standard. Electrophoresis was performed at constant power (40 W). Data were collected using the Saga Generation 2 software package (Li-Cor). Automated band calling and size determination were performed using the same software.

Statistical analysis. In the F_2 population, genotypic data from molecular markers were scored as homozygous for H 7998, homozygous for Ohio 88119, and heterozygote. The AB population was scored as heterozygous or homozygous. Segregation ratios of marker genotypes were analyzed by chi-square analysis. Independence and normality of residuals for phenotypic data were evaluated by the Shapiro-Wilks test, and normal probability plots as well as summary statistics were calculated using the UNIVARIATE procedure of SAS (version 8.0, SAS Institute). Residuals were found to be independent and normally distributed with constant variance.

The association between response to bacterial spot and the genotype of 24 polymorphic markers scored in the AB population was determined using mixed linear model analysis. Statistical models are described below. In all cases, degrees of freedom for the *F* statistics were calculated via Satterthwaite's approximation (19) due to the unbalanced data sets. Significant *F* tests ($P < 0.05$) provided evidence for linkage between a marker and resistance. Calculations for mixed linear models were performed using either the GLM or the MIXED procedure of SAS.

ANOVA using GLM was performed to determine an association between disease response and marker genotype in the F_2 populations derived from Ohio 88119 × H 7998. The statistical model was $X_i = \mu + M_i + \varepsilon_i$ (model 1), where X_i is the trait value of the ith marker class, µ is the population mean, M_i is the effect of the ith marker class, and ε_i is the experimental error. For these studies, the experimental error (ε_i) is the effect of the genotype within the marker class and the appropriate *F* test for marker was equal to M_i/ε_i.

For field experiments using AB and IBC populations, marker was considered a fixed effect while block and genotype within marker were considered random effects. The statistical model was $X_{ijk} = \mu + B_i + M_j + G_k(M_j) + \varepsilon_{ijk}$ (model 2), where X_{ijk} is the trait value of the kth genotype of the jth marker class in the ith block, µ is the population mean, B_i is the effect of the ith block, M_j is the effect of the jth marker class, $G_k(M_j)$ is the effect of the kth genotype within the jth marker class, and ε_{ijk} is the experimental error. The appropriate *F* test for marker was equal to $M_i/G_k(M_j)$.

Total phenotypic variation explained by marker loci was estimated from the F_2 population based on the R^2 value and calculated for replicated trials by estimating variance components by restricted maximum likelihood (REML) using the VARCOMP procedure of SAS. In replicated experiments, the phenotypic variation explained by the QTL is expressed as the variation due to marker divided by the total variation (V_m/V_p).

RESULTS

Detection and mapping of markers polymorphic in *L. esculentum* crosses. A total of 37 of the 148 markers we tested detected polymorphisms between Ohio 88119 and H 7998 for an apparent polymorphism rate of 25% (Table 1). The markers we used represent a collection that was screened for polymorphism between multiple *L. esculentum* parents, and the rate of polymorphism detected between Ohio 88119 and H 7998 represents a maximum estimation. Of 114 SSR sequences generated by Suliman-Pollatschek et al. (31), only seven were polymorphic between H 7998 and Ohio 88119 for a minimum estimate of 7/114 or 6.14% polymorphism. Similarly, of 609 potential SSR sequences available through SGN, only 115 have been mapped based on their ability to distinguish polymorphisms in several wide crosses. Of these 115 SSR markers, only 20 are polymorphic between Ohio 88119 and H 7998, and the variation we detected

therefore represents a minimum estimation of 20/609 or 3.3% polymorphism. Similarly, the 44 SNP markers we screened represent 44 polymorphic genes out of 1,245 expressed sequence tags (ESTs) screened (38). The polymorphism rate based on SNPs in ESTs is therefore as low as 10/1,245 or 0.8% in the Ohio 88119 × H 7998 cross.

We attempted to develop or identify markers linked to the HR-inducing loci *Rx1*, *Rx2*, and *Rx3* that were polymorphic within *L. esculentum* germplasm in order to determine their role in resistance in the field. Primers based on the *Cf9* resistance gene and the SNP marker LEOH36 are linked to the region of chromosome 1 containing *Rx1*. No polymorphic markers were identified for the region of chromosome 1 below the centromere, and *Rx2* was not tagged by the markers screened. The markers TOM49, Rx3-L1, and CosOH73 are linked to *Rx3* (39). The marker Rx3-L1, was detected using primers that were based on domains conserved in protein kinases. The polymorphic allele from Ohio 88119 was sequenced to design specific primers (data not shown). Subsequent BLAST searches revealed homology to the Institute for Genomics Research (TIGR) tomato EST, TC124705, and an open reading frame found within the restriction fragment length polymorphism probe TG23. TG23 also detected a polymorphism between H 7998 and Ohio 88119, and this polymorphism co-segregated with the Rx3-L1 polymorphism (data not shown).

Map positions of other SNP and SSR markers (Fig. 1) were based on segregation in the *L. esculentum* × *L. pennellii* segmental substitution population (5) and an *L. esculentum* × *L. pimpinellifolium* cross (32) as previously described (38), or from the mapping resources available on the SGN website (provided online by Cornell University). Several of the polymorphic markers clustered, and regions of the genome were not covered. There was a clustering of markers on chromosomes 4 and a paucity of markers in other portions of the genome suggesting that polymorphisms are not evenly distributed between Ohio 88119 and H 7998.

Detection of QTL from H 7998 for resistance to race T1. Marker genotypes of F_2 individuals were determined and the segregation of marker classes in the F_2 population followed the expected 1:2:1 ratio (homozygous for H 7998, heterozygotes, and homozygous for Ohio 88119). Based on single-marker ANOVA using the $\log_{10}$CFU of bacterial populations, marker Rx3-L1 on chromosome 5 was significantly (P = 0.005) associated with resistance (Table 2). This locus explained 25% of the variation of resistance based on the R^2 value from model 1. There was no significant difference between the means of $\log_{10}$CFU for heterozygotes (6.92) and $\log_{10}$CFU for individuals homozygous for the H 7998 allele (6.88); both were significantly different from the mean $\log_{10}$CFU (7.65) for individuals with the Ohio 88119 allele ($LSD_{0.05}$ = 0.6649). In contrast, the marker Cf9 on chromosome 1 was not significantly associated with resistance (P = 0.49), suggesting that HR conferred by *Rx1* does not effectively reduce bacterial populations in the leaf.

The AB families were used to confirm the association between marker Rx3-L1 and disease resistance detected in the greenhouse study. The AB population also provided an opportunity to explore other loci that may contribute to resistance using a population that was amenable to replicated testing in the field. Due to the clus-

TABLE 1. Markers detecting polymorphisms between H 7998 and Ohio 88119

Locus[w]	Marker	Chrom.[x]	Forward primer	Reverse primer	Temp.[y]	Enzyme[z]
LEOH36	SNP	1	TCACAAAAATGGCGATGAGA	CCACCTGTGGATCCTTGACT	56	*Bcl*I
Cf9	SNP	1	CAGGCACAGAGTTACATGGG	CAACCAGTGAAGGAAGGGAG	60	*Hae*III
LEOH23	SNP	2	CTATGCGTTTGTCGGTCGT	CAAGGTAGTTGAAGGTATGACCA	54	*Tsp*509 I
SSR96	SSR	2	GGGTTATCAATGATGCAATGG	CCTTTATGTCAGCCGGTGTT	45	...
TOM11	SSR	2	ATTGTAATGGTGATGCTCTTCC	CAGTTACTACCAAAAATAGTCAAACAC	45	...
SSR111	SSR	3	TTCTTCCCTTCCATCAGTTCT	TTTGCTGCTATACTGCTGACA	45	...
TOM59	SSR	3	TAACACATGAACATTAGTTTGA	CACGTAAAATAAAGAAGGAAT	45	...
LEOH37	SNP	4	TTGATATATTCCATGTGTGTCTC	ACTACAAATTAACAAACTTAAATGG	51	*Nmu*CI
SSR43	SSR	4	CTCCAAATTGGGCAATAACA	TTAGGAAGTTGCATTAGGCCA	45	...
SSR310	SSR	4	GCGATGAGGATGACATTGAG	TTTACAGGCTGTCGCTTCCT	45	...
SSR146	SSR	4	TATGGCCATGGCTGAACC	CGAACGCCACCACTATACCT	45	...
TOM49	SSR	5	AAGAAACTTTTTGAATGTTGC	ATTACAATTTAGAGAGTCAAGG	45	...
TOM152	SSR	5	ATTCAAGGAACTTTTAGCTCC	TGCATTAAGGTTCATAAATGA	45	...
Rx3-L1	SNP	5	CTCCGAGCGAAGAGTCTAGAGTC	GAAGGCAAAAGGAAAAGGAGAAGGATGG	60	*Bsr*BI
CosOH73	SNP	5	CTTCCCGACAAGCACAAAAA	CGAATGCTCTGTACCATTTCC	56	*Alu*I
LEOH8	SNP	9	CCACTGATCAATGTGGTGGA	CAACCACAAATGGCTCCTAAA	55	*Hae*III
SSR69	SSR	9	TTGGCTGGATTATTCCTGTTG	GCATTTGATAGAAGGCCAGC	45	...
SSR318	SSR	10	GCAGAGGATATTGCATTCGC	CAAACCGAACTCATCAAGGG	52	...
TOM196	SSR	11	CCTCCAAATCCCAAAACTCT	TGTTTCATCCACTATCACGA	45	...
SSR20	SSR	12	GAGGACGACAACAACAACGA	GACATGCCACTTAGATCCACAA	45	...
LEOH9	SNP	Unknown	GGCAATGCCACTGACTTACA	CTCTCTGCTGCTTCGGCTAC	55	*Aci*I
LEOH20	SNP	Unknown	CAGACCTAACAAGACAGGCAAA	ATCAGGCATGACCATGGAAG	55	*Hae*III
LEOH26	SNP	Unknown	GAAGATTCGGAGGTCAAACG	AACTCCTCAACTGCCTCAGC	56	*Fok*I
SSR71	SSR	Unknown	AAATGGCATGGAGAATGGAA	CATCCACTGAGAGCCCAAAG	45	...
SSR104	SSR	2	TTCCATTTGAATTCCAACCC	CCCACTGCACATCAACTGAC	45	...
SSR231	SSR	3	TGCCAATCCACTCAGACAAA	TGGATTCACCAAGGCTTCTT	45	...
SSR603	SSR	4	GAAGGGACAATTCACAGAGTTTG	CCTTCAACTTCACCACCACC	45	...
SSR306	SSR	4	ACATGAGCCCAATGAACCTC	AACCATTCCGCACGTACATA	45	...
SSR450	SSR	4	AATGAAGAACCATTCCGCAC	ACATGAGCCCAATGAACCTC	45	...
SSR638	SSR	4	TGTTGGTTGGAGAAACTCCC	AGGCATTTAAACCAATAGGTAGC	45	...
SSR627	SSR	4	TACAGAATAGGGTTTGCCATA	GTTTTAGTGGGTTGTGTTGAA	52	...
TOM210	SSR	4	CGTTGGATTACTGAGAGGTTTA	ACAAAAATTCACCCACATCG	45	...
TOM236	SSR	9	GTTTTTTCAACATCAAAGAGCT	GGATAGGTTTCGTTAGTGAACT	45	...
SSR70	SSR	9	TTTAGGGTGTCTGTGGGTCC	GGAGTGCGCAGAGGATAGAG	45	...
SSR248	SSR	10	GCATTCGCTGTAGCTCGTTT	GGGAGCTTCATCATAGTAACG	45	...
SSR637	SSR	11	AATGTAACAACGTGTCATGATTC	AAGTCACAAACTAAGTTAGGG	48	...
TOM144	SSR	11	CTGTTTACTTCAAGAAGGCTG	ACTTTAACTTTATTATTGCGACG	45	...

[w] Markers used for genetic analysis of F_2 and advanced backcross populations derived from the cross of Ohio 88119 × H 7998 are underlined.
[x] Chromosome (Chrom.), forward and reverse polymerase chain reaction (PCR) primers.
[y] Optimum annealing temperature (Temp. [°C]) for PCR amplification.
[z] Restriction endonuclease used to detect single nucleotide polymorphisms (SNP) in amplified products.

tering of several polymorphic markers, 24 were used to analyze the AB population (Table 2). Two marker classes, heterozygous and homozygous for Ohio 88119, were observed among the AB population. Since AB families segregate, plot ratings were based on the most susceptible plant, the most resistant plant, and the plot average. Results of single marker-trait analysis were consistent across ratings, and only plot average is presented for convenience (Table 2). Of the 24 markers analyzed, four were significantly ($P < 0.05$) associated with resistance based on either the early rating or late rating (Table 2). These four markers corresponded to two H 7998 chromosomal regions. The region covered by markers TOM49, Rx3-L1, and CosOH73 on chromosome 5 was associated with resistance to disease. Estimates of variance components revealed that markers Rx3-L1 and CosOH73 explained about 12 and 14% of the total phenotypic variation for the early rating, respectively. Markers TOM49, Rx3-L1, and CosOH73 were associated with 15, 41, and 38% of the total phenotypic variation for the late rating, respectively. A region on chromosome 4 inherited from H 7998 and linked to marker SSR43 was associated with susceptibility to disease in the late rating and explained 11% of the phenotypic variation. There was no interaction detected between these two QTL. Markers Cf9 and

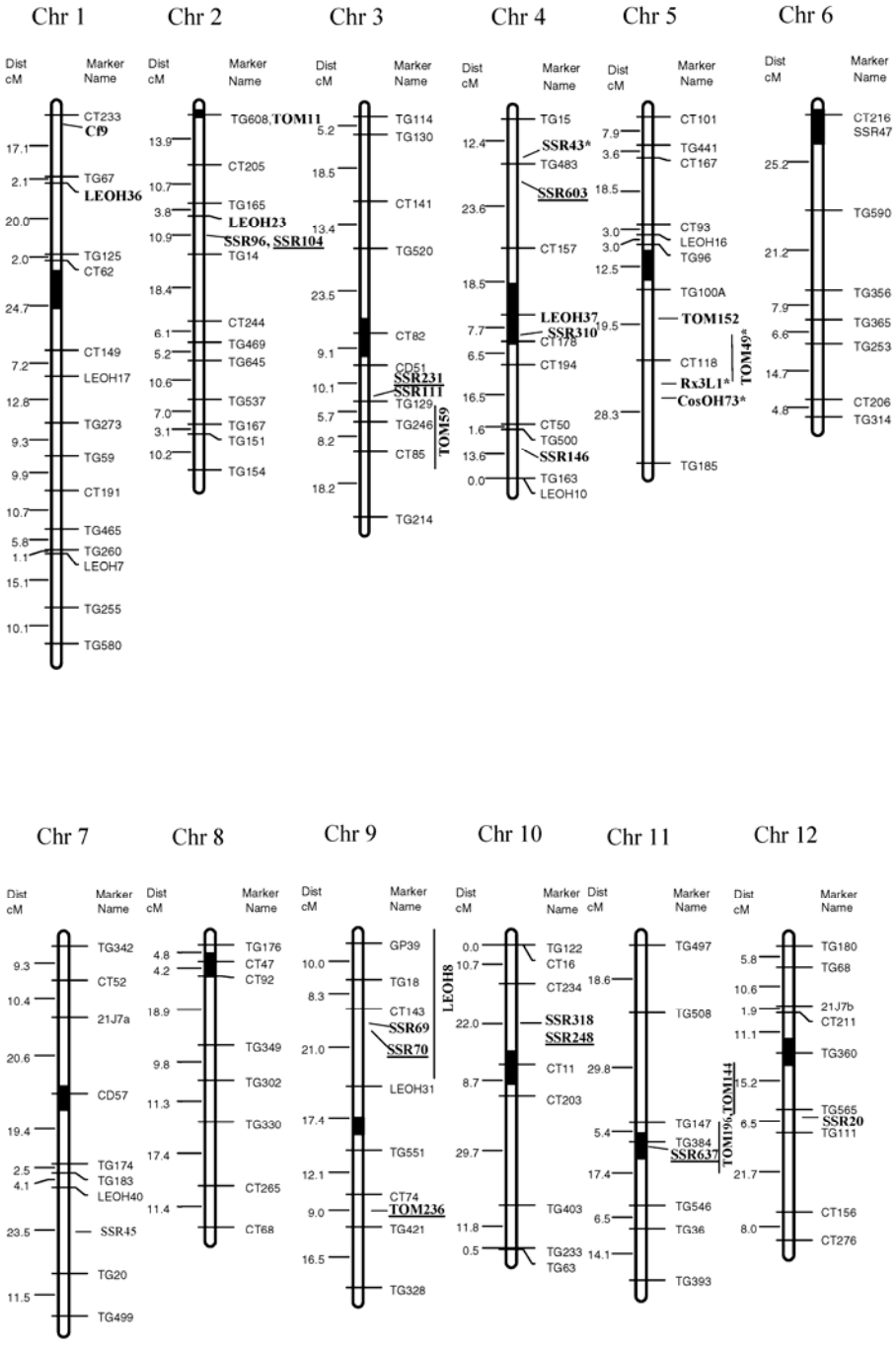


Fig. 1. Approximate map positions of markers (in bold and large font) used in this study. The framework map was adapted from Yang et al. (38). Dark region indicates centromere of chromosome. Map positions of simple sequence repeat (SSR) markers were obtained from Solanaceae Genome Network (provided online by Cornell University) or Suliman-Pollatschek et al. (31). Markers significantly associated with *Xanthomonas campestris* pv. *vesicatoria* race T1 resistance are marked by an * and markers showing polymorphisms between H 7998 and Ohio 88119 but not used to analyze the population are underlined, except SSR306, SSR450, and SSR638 are clustered at the region of SSR310 on chromosome 4, and TOM210 and SSR627 are clustered at the region of SSR146 on chromosome 4. These markers are not shown on the map.

LEOH36 were not significantly associated with the resistance for both early (P = 0.76 to 0.37) and late (P = 0.38 to 0.65) ratings, again suggesting that the HR-inducing locus *Rx1* does not confer resistance under field conditions. These results were consistent with the greenhouse study.

Phenotypic effects of QTL. H 7998 had an average score of 1.5 for the early rating period and 2.5 for the late rating, whereas Ohio 88119 had an average rating of 3.5 for the early rating and 5.5 for the late rating. None of the AB families were as resistant as H 7998 (Fig. 2). Approximately 30 and 18% of AB families were significantly ($P < 0.05$) more resistant than Ohio 88119 for early and late rating periods, respectively. These numbers are consistent with minimum gene number estimates of three to five (6,25). All of the superior AB families from the late rating were homozygous for the Ohio 88119 allele for marker SSR43 (chromosome 4) and 86% of these families were heterozygous for the H 7998 alleles near the *Rx3* region. The average effects of H 7998 QTL in AB lines were estimated by the proportion of phenotypic variance explained by the linked markers (Table 2) or by comparison of the means of the genotypic classes (Table 3).

TABLE 2. Single marker-trait analysis of resistance to *Xanthomonas campestris* pv. *vesicatoria* race T1 in segregating populations derived from Ohio 88119 × H 7998

			$Log_{10}CFU$[v]		Early evaluation[w]		Late evaluation		
Marker	Population[u]	Chrom.	P (M/error)[x]	R^2[y]	P (M/Gen(M))[x]	V_m/V_p[y]	P (M/Gen(M))	V_m/V_p	QTL effect[z]
Cf9	F_2	1	0.490	0.01					NS
Rx3-L1	F_2	5	0.005	0.25					+
Cf9	AB	1			0.7621	0.00	0.3757	0.00	NS
LEOH36	AB	1			0.3727	0.00	0.6452	0.00	NS
TOM11	AB	2			0.1144	0.02	0.117	0.04	NS
LEOH23	AB	2			0.5932	0.00	0.5782	0.00	NS
SSR96	AB	2			0.9164	0.00	0.7919	0.00	NS
SSR111	AB	3			0.2833	0.00	0.3888	0.00	NS
TOM59	AB	3			0.4647	0.00	0.3167	0.00	NS
SSR43	AB	4			0.3743	0.00	0.0379	0.11	–
LEOH37	AB	4			0.5307	0.00	0.8478	0.00	NS
SSR310	AB	4			0.8619	0.00	0.6949	0.00	NS
SSR146	AB	4			0.1942	0.01	0.3186	0.00	NS
TOM152	AB	5			0.2462	0.00	0.2141	0.01	NS
TOM49	AB	5			0.1529	0.01	0.005	0.15	+
Rx3-L1 (TG23)	AB	5			0.0006	0.12	<0.0001	0.41	+
CosOH73	AB	5			0.0001	0.14	<0.0001	0.38	+
LEOH8	AB	9			0.9420	0.00	0.781	0.00	NS
SSR69	AB	9			0.8197	0.00	0.8803	0.00	NS
SSR318	AB	10			0.6688	0.00	0.818	0.00	NS
TOM196	AB	11			0.6357	0.00	0.3583	0.00	NS
SSR20	AB	12			0.0642	0.05	0.0915	0.07	NS
LEOH9	AB	Unknown			0.4019	0.00	0.0903	0.04	NS
LEOH20	AB	Unknown			0.4238	0.00	0.0856	0.04	NS
LEOH26	AB	Unknown			0.4157	0.00	0.5455	0.00	NS
SSR71	AB	Unknown			0.5616	0.00	0.8772	0.00	NS

[u] Two populations were used for single marker-trait analysis, an F_2 population and an advanced backcross (AB) population.
[v] The F_2 population was scored based on bacterial populations ($log_{10}CFU$).
[w] The AB population was evaluated early and late in the season using a Horsfall-Barratt (9) rating system in which 1 = 0%, 2 = 0 to 3%, 3 = 3 to 6%, 4 = 6 to 12%, 5 = 12 to 25%, 6 = 25 to 50%, 7 = 50 to 75%, 8 = 75 to 87%, 9 = 87 to 94%, 10 = 94 to 97%, 11 = 97 to 100%, and 12 = 100% diseased tissue.
[x] Significance of the single marker-trait analysis was based on analysis of variance with the P value for the appropriate F test indicated, where M indicates the marker effect.
[y] The proportion of variance explained by the quantitative trait loci (QTL) based on regression (R^2) for the F_2 where individuals are not replicated, and variance due to marker divided by total phenotypic variance (V_m/V_p) for the AB population where plots were replicated.
[z] The effect of significant associations relative to the H 7998 allele is indicated by + if the locus contributes resistance and – if it contributes to susceptibility and NS if there is no significant effect.

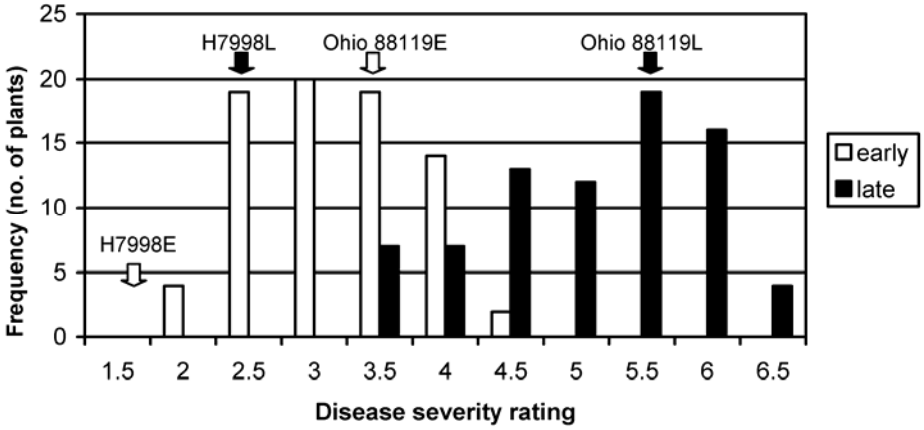


Fig. 2. Bacterial spot disease rating frequency distribution for the advanced backcross population derived from Ohio 88119 × H 7998. H 7998E and H 7998L indicate early and late ratings for H 7998, respectively. Ohio 88119E and Ohio 88119L indicate early and late ratings for H 7998, respectively.

There were no significant differences for disease ratings for plots that were heterozygous or homozygous for chromosome 1 alleles at markers Cf9 and LEOH36. For markers TOM49, Rx3-L1, and CosOH73 on chromosome 5, families with the H 7998 allele tended to have lower disease ratings.

MAS for *Rx3*. In order to exploit the apparent linkage of molecular markers and resistance on chromosome 5, we alternated off-season greenhouse MAS with inoculated field trials. Map positions of the four chromosome 5 markers favor the order TOM152, TOM49, Rx3-L1, and CosOH73 with the most likely position of the *Rx3* QTL in the TOM49-CosOH73 interval. We therefore used the Rx3-L1 primers to accelerate the selection of inbred breeding lines that were homozygous for the resistance QTL. Selection for horticultural characteristics was imposed in the disease nursery, and single plants were advanced from field trials. Inbred backcross lines (IBLs) were entered into test-cross evaluation at BC_3S_6, and two lines, 9816 and 9834, were advanced to replicated multi-year evaluations. Single plant selections were continued from the replicated multi-year trials. Both lines performed well in disease evaluations as inbred lines and as parents for hybrid varieties (Table 4).

Correlation with maturity. Previous studies using classical genetic approaches indicate that as many as three to five genes contribute to field resistance to bacterial spot in H 7998 (25,35,39). In these studies, the HR was a poor predictor of field performance, explaining less than 14% of the variation. In addition to the two loci we detected, our data suggest that plant maturity explains approximately 6.3% of the phenotypic variance for resistance with late maturing plants showing more resistance (P = 0.0427). It is possible that early maturation and early senescence increase symptom expression. This hypothesis is consistent with the observation that reduced expression of ethylene receptors enhances HR and resistance in tomato to *X. campestris* pv. *vesicatoria* (3). In the field, plant habit also affects the scoring of resistance, with indeterminate plants appearing more resistant than determinate plants (P = 0.040; R^2 = 0.0665).

TABLE 3. Mean bacterial spot disease ratings for two genotypes inoculated with *Xanthomonas campestris* pv. *vesicatoria* race T1 strains based on markers in advanced backcross population derived from Ohio 88119 × H 7998

Marker		Chrom.	Early evaluation[y]	Late evaluation[y]
Cf9		1		
	OO[z]		3.18 a	5.03 a
	HO		3.13 a	5.23 a
LSD 0.05			0.25	0.31
LEOH36		1		
	OO		3.21 a	5.13 a
	HO		3.07 a	5.02 a
LSD 0.05			0.25	0.3
SSR43		4		
	OO		3.14 a	5.01 b
	HO		3.31 a	5.54 a
LSD 0.05			0.29	0.35
TOM49		5		
	OO		3.22 a	5.25 a
	HO		3.00 b	4.67 b
LSD 0.05			0.2	0.29
Rx3-L1 (TG23)		5		
	OO		3.32 a	5.38 a
	HO		2.81 b	4.46 b
LSD 0.05			0.24	0.28
CosOH73		5		
	OO		3.31 a	5.35 a
	HO		2.76 b	4.46 b
LSD 0.05			0.24	0.28

[y] Means followed by the same letter are not significantly different at the 0.05 level.

[z] OO = homozygous for the Ohio 88119 allele; HO = heterozygote.

DISCUSSION

Genetic polymorphisms within *L. esculentum* are limited (18, 37). The paucity of markers has prevented a detailed study of many economically important traits in cultivated tomato. Due to the lack of genetic polymorphism, previous genetic analyses of resistance in H 7998 were conducted using a cross between H 7998 and a susceptible wild species *L. pennellii* accession LA716 (35,39). The polymorphic markers identified in that cross are not useful for breeding because they are inherited from the wild parent and linked to susceptibility. Publicly available SSR markers and newly developed SNP markers were prescreened for polymorphism in *L. esculentum* and used to detect polymorphisms between H 7998 and Ohio 88119. The polymorphism rate ranged from 3.3 to 6.14% for SSR marker sets and was 0.8% for SNPs in ESTs. The polymorphic markers clustered and only 37.5% of the genome was covered within 10 to 20 cM of a marker although the core set of 148 polymorphic markers covered over 90% of the tomato genome. This result suggests an uneven distribution of polymorphisms between the two varieties and highlights the need for a concerted effort to develop more molecular markers that detect polymorphisms between *L. esculentum* varieties and breeding lines.

The development of an HR to race T1 is controlled by two (36) to three loci in H 7998 × *L. pennellii* LA716 crosses (34,35,39). The loci *Rx1* and *Rx2* are located on opposite sides of the chromosome 1 centromere; *Rx3* is located on chromosome 5 between TG23 and TG60. The three loci act independently and have additive effects (39). Our results support a role for the HR-inducing *Rx3* locus in resistance measured in the field, though we have not formally demonstrated that the same locus is responsible for both field resistance and HR. The SNP markers TG23 and Rx3-L1 co-segregated and detected a significant association with disease resistance in the Ohio 88119 × H 7998 populations. Two flanking markers, TOM49 and CosOH73, also showed a significant association with the resistance. Both flanking markers explained a lower proportion of the variance relative to Rx3-L1. Our results also suggest that the HR-inducing *Rx1* locus is not important for reducing either bacterial populations in greenhouse inoculations or symptoms in the field. The SNP markers Cf9 and LEOH36 are linked to the region where the *Rx1* locus is mapped, but these markers did not detect an association with reduced bacterial populations in the F_2 population nor reduced disease in AB populations. We were unable to identify a polymorphic marker in the *Rx2* region, despite considerable effort.

TABLE 4. Bacterial spot disease ratings from field trials inoculated with *Xanthomonas campestris* pv. *vesicatoria* race T1 strains *Xcv*110c, *Xcv*118, *Xcv*89, and *Xcv*767

Genotype	Rating[z] 2 Year	Rating[z] 3 Year	Comment
Ohio 981205	8.0	–	Ohio 88119 (*Pto*)
Ohio 88119	7.3	7.7	Susceptible check
PS 696	6.0	–	Elite hybrid check
Ohio E3259	5.7	5.1	Δ open pollinated (OP) partially resistant
Ohio 8245	4.8	–	Δ OP partially resistant
Ohio 9834 × E3259	4.5	–	*Rx3*/wt F_1
Ohio 9816 × E3259	4.3	–	*Rx3*/wt F_1
Ohio 9834	4.2	4.6	*Rx3*
Ohio 9816	3.7	4.3	*Rx3*
Mean	5.45	5.43	
P	0.0062	0.0089	
LSD 0.05	2.18	2.45	

[z] Ratings based on a modified Horsfall-Barratt (9) rating system in which 1 = 0%, 2 = 0 to 3%, 3 = 3 to 6%, 4 = 6 to 12%, 5 = 12 to 25%, 6 = 25 to 50%, 7 = 50 to 75%, 8 = 75 to 87%, 9 = 87 to 94%, 10 = 94 to 97%, 11 = 97 to 100%, and 12 = 100% diseased tissue.

The lack of a role in resistance for *Rx1* is not surprising given that the role of HR in conferring resistance in the field is not well established. The correlation coefficient for regression between HR and field symptoms was only 0.37 in the H 7998 × LA716 progeny (33). In crosses between two *L. esculentum* lines, H 7998 and cv. Walter, HR had a correlation with the field reaction of $r = 0.31$ (30). It is therefore likely that loci other than those reported for HR play a role in resistance to bacterial spot in the field. For example, a locus on chromosome 4 linked to SSR43 explained 11% of the phenotypic variation of resistance, with the allele from H 7998 being associated with susceptibility to disease. Maturity and plant habit each explained a significant, though small (6%), portion of variation.

We postulate that a QTL, *Rx3*, explains as much as 41% of the phenotypic variance for resistance and resides in the interval flanked by TOM49 and CosOH73 (Table 2). This QTL has the potential to reduce bacterial infection by 20% (Table 3). Further, our ability to detect this QTL in an AB population suggests that resistance is additive to dominant in action. This observation is consistent with the report that disease severity in the F_1 derived from a cross between H 7998 and cv. Walter was slightly skewed toward resistance (25). The detection of a QTL from H 7998 that contributes to susceptibility was not expected. AB families that were heterozygous marker SSR43 had higher disease ratings, with an average increase in symptoms of 10%. The failure to recover families and lines with H 7998 levels of resistance is not unexpected given that the AB families were either heterozygous for H 7998 alleles or homozygous for Ohio 88119 alleles. In addition, the AB population structure separates QTL and minimizes the positive interaction of H 7998 alleles.

The existence of multiple races of bacterial spot and a lack of available disease resistance in commercial cultivars makes it difficult to control this disease in humid growing environments. Breeding for resistance using traditional approaches is limited due to the quantitative nature of resistance and the existence of multiple races. Identification of molecular markers linked to the resistance QTL and using MAS offer an opportunity to pyramid resistance in a breeding line or hybrid. Likewise, the development of breeding populations that permit replicated testing will also be an essential feature of strategies to develop resistance to multiple races. In this study, we successfully identified a major QTL conferring resistance to *X. campestris* pv. *vesicatoria* race T1 from H 7998 and transferred resistance into an elite breeding line with MAS. IBLs 9816 and 9834 contain partial resistance and provide a resource to pyramid resistance from other resistant germplasm.

As part of a gene pyramiding strategy for bacterial resistance, it will be necessary to consider a wider complex of organisms. Bacterial speck of tomato, caused by *Pseudomonas syringae* pv. *tomato*, is an economically important disease worldwide (8). Bacterial canker is a systemic disease of tomato caused by the gram-positive bacterium, *Clavibacter michiganensis* subsp. *michiganensis*. Bacterial spot, bacterial speck, and bacterial canker can occur simultaneously in humid growing environments. Developing varieties with resistance is an important approach to control these diseases, and it is worth noting that a gene, *Pto*, conferring resistance to bacterial speck of tomato and a QTL, Rcm 5.1, conferring resistance to bacterial canker also map to chromosome 5 (4,16). To effectively pyramid bacterial spot resistance with resistance to other pathogens, it will be necessary to select for desirable recombination events in addition to bringing unlinked genes together. Molecular markers will facilitate this process.

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