Two Loci from *Lycopersicon hirsutum* LA407 Confer Resistance to Strains of *Clavibacter michiganensis* subsp. *michiganensis*

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ABSTRACT

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We used molecular markers to identify quantitative trait loci (QTL) that contribute to resistance to bacterial canker of tomato caused by *Clavibacter michiganensis* subsp. *michiganensis*. Resistance was first identified as a marker-trait association in an inbred backcross (IBC) population derived from crossing *Lycopersicon hirsutum* accession (LA407) with *L. esculentum*. Single-marker QTL analysis suggested that at least two loci originating from *L. hirsutum* LA407, *Rcm* 2.0 on chromosome 2 and *Rcm* 5.1 on chromosome 5, contribute to resistance in replicated trials. Two segregating F_2 populations were developed by crossing resistant inbred backcross lines (IBLs) to elite *L. esculentum* lines and used to confirm QTL associations detected in the IBC

population. In these populations, realized heritability estimates were higher for selection based on maximal disease than for selection based on disease progression. Realized heritability in the population carrying *Rcm* 2.0 was 0.63 and 0.14, respectively, for each selection criteria. Realized heritability estimates were 0.85 for selection based on maximal disease and 0.37 for selection based on disease progression in a population carrying *Rcm* 5.1. The disease response of F_3 families selected for resistance suggested that both *Rcm* 2.0 and *Rcm* 5.1 confer resistance to bacterial strains in the repetitive sequence-based polymerase chain reaction DNA fingerprint classes A and C. Markers linked to *Rcm* 2.0 explained up to 56% of the total phenotypic variation for resistance in one population, and markers linked to *Rcm* 5.1 explained up to 73% of the total phenotypic variation for resistance in a separate population.

Additional keywords: inbred backcross breeding method.

Bacterial canker of tomato occurs on tomatoes cultivated in humid environments and is increasing where greenhouse-grown transplants are used to establish field stands. In the Midwestern United States and Canada, yield loss attributed to bacterial canker has been as high as 84% for commercial growers (10,19). Bacterial canker is caused by the gram-positive pathogen *Clavibacter michiganensis* subsp. *michiganensis*. Pathogenicity traits of *C. michiganensis* subsp. *michiganensis* include systemic infection, wilt, stunting, canker, leaf necrosis, and symptoms known as "bird's-eye" spots on fruit (13). In North America, four distinct genetically characterized strains of *C. michiganensis* subsp. *michiganensis*, classified as A through D, have been identified by repetitive sequence-based polymerase chain reaction (rep-PCR) genomic fingerprinting (16).

Sources of resistance to *C. michiganensis* subsp. *michiganensis* have been identified in several wild relatives of the cultivated tomato (*Lycopersicon esculentum* Mill.), including *L. pimpinellifolium* (Just.) Mill., *L. hirsutum* Humb. and Bonpl., and *L. peruvianum* (L.) Mill. (3,7,21,28). The best characterized source of resistance is that found in *L. peruvianum* LA2157. Genetic analysis of an intraspecific BC_1 population revealed five possible quantitative trait loci (QTLs) associated with resistance (21). A second segregating F_2 population derived from the interspecific hybridization between *L. peruvianum* LA2157 and a susceptible *L. esculentum* revealed only three QTLs linked with resistance (29). It is not known how bacterial strains used in these studies fit into the rep-PCR classification or how representative they are of the popula-

tions found in North America. Furthermore, crosses between the cultivated tomato and *L. peruvianum* require embryo or ovule rescue in early generations; therefore, the LA2157 source of resistance to *C. michiganensis* subsp. *michiganensis* may have limitations for use in breeding programs if multiple loci are to be introgressed.

Recently, a source of resistance to *C. michiganensis* subsp. *michiganensis* was identified in *L. hirsutum* accession LA407 (7). Resistance was recovered against strains in the A and C fingerprint classes in progeny from an inbred backcross (IBC) population derived from the hybridization of *L. hirsutum* LA407 to susceptible *L. esculentum*. Individual inbred backcross lines (IBLs) were identified that performed significantly better than resistant or susceptible controls in both greenhouse and field trials. Using classical genetic analysis, estimates of the number of genes conferring resistance fell in the range of one to three and estimates of broad-sense heritability were moderate.

IBC populations (30) and populations created by the advanced backcross (AB) method (27) have advantages for breeding and introgression. Populations created by the IBC breeding method have been used successfully to introgress beneficial alleles from unadapted or wild germ plasm into elite breeding lines. Examples include enhanced seed protein content in common bean, *Phaseolus vulgaris* L. (24), fruit mass in cucumber, *Cucumis sativus* L. (18), and insect resistance in tomato (11). Recent studies suggest that, when combined with molecular marker analysis, such populations also allow the discovery and characterization of beneficial alleles from unadapted or wild germ plasm (4,27). The application of molecular marker analysis to IBC populations of *Brassica napus* L. has led to an enriched composite genetic map of oilseed rape and the identification and characterization of individual loci that control quantitatively inherited traits (4). The strategy of AB-QTL analysis (27) has been tested in populations involving crosses with four wild *Lycopersicon* spp. (2,8,9,26). It appears that IBC populations and populations created by AB-QTL

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analysis have advantages for breeding and introgression, allow discovery and characterization of beneficial alleles from unadapted germ plasm, and permit linkage analysis of molecular markers with traits of agronomic importance. In these populations, the discovery of QTLs occurs while advancing material in a breeding context.

There remains a need for research in the use of IBC and AB populations for breeding and QTL discovery. For example, unequal allele frequencies that result in unequal marker class sizes (unbalanced data) during QTL analysis is a characteristic of both populations. Unless corrections are made to the statistical models used for analysis, the significance of QTLs may be overestimated. In this study, we analyzed resistance to *C. michiganensis* subsp. *michiganensis* in the LA407 IBC population using molecular markers. We developed appropriate statistical models to facilitate marker analysis of unbalanced data sets in replicated trials. This analysis identified loci from *L. hirsutum* LA407 that contributed to resistance. Significant marker-QTL associations were subsequently confirmed in populations developed to verify the linkages detected in the IBC population.

MATERIALS AND METHODS

Plant material. An IBC population consisting of 64 BC₂S₅ lines (IBLs), derived from a cross between *L. hirsutum* accession LA407 and *L. esculentum*, was used for molecular marker analysis. The recurrent parents in this population were *L. esculentum* 'Hunt 100' (F₁ and BC₁) and 'Peto 95-43' (BC₂). Individual IBLs were developed through five generations of selfing with single-seed descent. Each line within the LA407 IBC population has a theoretical *L. hirsutum* LA407 genome content of 12.5%.

Populations used to confirm the association of DNA-based markers with resistance were developed by crossing selected IBLs to a susceptible *L. esculentum* breeding line. IBLs designated '2353' and '2361' were homozygous at introgressed *L. hirsutum* LA407 chromosomal regions on chromosome 2 and 5, respectively, and were crossed with the susceptible line Ohio 86120. Hybrids of each cross were allowed to self-pollinate to produce the F₂ generation. The F₃ families were produced by clonally propagating F₂ individuals and saving self-pollinated seed from a single clone. The replicate clones were inoculated and evaluated for resistance.

Tomato seed for all studies were sown in 288-cell flats filled with MetroMix-360 growing medium (Premier Horticulture, Inc., Red Hill, PA). At the first true leaf stage, a fertilization regimen of N-P-K at 50 ppm (Peters Professional All-Purpose Fertilizer, 20-20-20; Scotts-Sierra Horticultural Products Co., Marysville, OH) was applied daily. Greenhouse temperatures ranged from 25 to 35°C. For greenhouse studies, seedlings and rooted cuttings were transplanted into 10.2-cm plastic pots filled with MetroMix-510 growing medium (Premier Horticulture, Inc.) 4 weeks following germination or rooting and the daily fertilization regimen of N-P-K at 50 ppm continued. For field trials, the fertilization regimen was discontinued following inoculation. Seedlings were held in the greenhouse an additional week following inoculation and prior to field transplant. Production practices for field trials were as recommended for commercial growers (20).

***C. michiganensis* subsp. *michiganensis* inoculation.** *C. michiganensis* subsp. *michiganensis* strains in the A and C PCR fingerprint class, A300 and C290, were used for inoculation. Culture conditions, greenhouse inoculations, and field inoculations were performed as described previously (7).

Experimental design. Evaluation of the LA407 IBC population for response to *C. michiganensis* subsp. *michiganensis* was performed in separate greenhouse and field trials. Greenhouse evaluations were established using completely randomized designs with two replicates of each line plus resistant and susceptible controls. Evaluation of the IBC population to petiole-clip inoculation of *C. michiganensis* subsp. *michiganensis* C290 was performed in 1997,

while response to petiole-clip inoculation of *C. michiganensis* subsp. *michiganensis* A300 was performed in 1998. Field trials were established using randomized complete block designs with two (1997) or three (1998) blocks containing each IBL plus resistant and susceptible controls spaced at 30 cm. Plots of each line consisted of 10 plants. Field evaluation of the response to spray inoculation using strain C290 was performed in 1997, while response to spray inoculation of strain A300 was performed in 1998.

Evaluations of the IBL 2353 × Ohio 86120 and IBL 2361 × Ohio 86120 F₂ populations were performed in the greenhouse using completely randomized designs. In all, 160 F₂ individuals derived from IBL 2353 and Ohio 86120 plus 5 individual plants of each parental control were petiole clip inoculated with strain A300. In a separate experiment, 159 F₂ individuals derived from IBL 2361 and Ohio 86120 plus 5 individual plants of each parental control were petiole clip inoculated with strain C290.

Tests of F₃ families originating from F₂ individuals were performed as a confirming generation. Three separate experiments were performed using F₃ families. Families that genotyped as homozygous resistant and homozygous susceptible at each relevant marker locus were evaluated. First, 10 F₃ families derived from IBL 2361 and Ohio 86120 were petiole clip inoculated with strain C290 and evaluated in the greenhouse. All plants were arranged in a completely randomized design. Second, a total of 16 F₃ families derived from IBL 2353 and Ohio 86120 were spray inoculated with strain A300 and evaluated in a field trial. Eight individual plants per family plus eight individual plants of each parental control, spaced at 30 cm, were arranged in a randomized complete block design of two blocks. Finally, to determine if loci conferring resistance to *C. michiganensis* subsp. *michiganensis* were specific to strains in the A or C DNA fingerprint classes, six F₃ families derived from resistant F₂ individuals were evaluated for response to petiole clip inoculation in a greenhouse study. One set of F₃ families was inoculated with A300 while a duplicate set was inoculated with C290.

Scoring for disease response. Scoring for response to petiole clip or spray inoculation was based on visual assessment of symptom severity using an additive disease rating scale of zero to five. One point was assigned for each characteristic symptom of *C. michiganensis* subsp. *michiganensis*, such as wilting, leaf necrosis, or canker. A half-point was subtracted or added if symptoms were mild or severe. Symptom-free plants were scored as zero and dead plants were scored as five.

As a method of measuring resistance to *C. michiganensis* subsp. *michiganensis*, weekly evaluations were recorded for analysis of individual disease rating scores (IDRS) and for calculation of an index based on percent of maximal disease (PMD). The IDRS reported represented the time point at which the population reached maximum disease. PMD is similar to measuring an area under the disease progress curve (AUDPC), with the exception that PMD permits comparison of genotype or genotypic classes regardless of the disease rating scale and represents the final calculated value as a percent. A detailed description of our evaluation methods and scoring for disease response was published previously (7,12).

DNA isolation for restriction fragment length polymorphism and PCR analysis. DNA for restriction fragment length polymorphism (RFLP) analysis was isolated from 1 g of fresh frozen young leaf tissue macerated in 3 ml of extraction buffer (0.35 M sorbitol, 0.1 M Tris(hydroxymethyl)aminomethane [Tris], 5 mM ethylene-diaminetetraacetic acid [EDTA], and 25 mM sodium bisulfite at pH 7.5) with a mortar and pestle. Cells and nuclei were lysed by the addition of 3 ml of hexadecyltrimethylammonium bromide (CTAB) nuclei lysis buffer (0.2 M Tris, 0.05 M EDTA, 2 M NaCl, and 2% CTAB, at pH 7.5) and 1.5 ml of 5% *N*-lauryl-sarcosine. The homogenate was incubated at 65°C for 10 min. Following incubation, 7 ml of chloroform/isoamyl alcohol (24:1) was added and the homogenate centrifuged for 15 to 20 min at 2,000 rpm. The aqueous phase was removed and

0.8 to 1 volume of isopropanol was added to precipitate the DNA. The DNA was resuspended in TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, pH 8.0).

DNA for PCR analysis was isolated from single 1-cm² leaflets macerated in 1 ml of extraction buffer using small polyethylene sample bags and gentle rubbing. Lysis, extraction, and precipitation were scaled proportionately to the large-scale preparation (described previously). The DNA was resuspended in T 1/10 E buffer (10 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA, pH 8.0). DNA concentration for both RFLP and PCR analysis was calibrated on the basis of ethidium bromide fluorescence using defined amounts of DNA as a standard.

RFLP analysis. For RFLP analysis, 5 µg of restriction enzyme digested DNA were separated on a 0.8% agarose gel (Ultrapure, BRL Life Technologies, Grand Island, NY) in 1× TBE (0.09 M Tris, 0.09 M boric acid, and 2 mM EDTA). Restriction endonucleases used were *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III, and *Xba*I. Gels were blotted onto GeneScreen Plus (NEN, Life Sciences, Boston) according to manufacturer's recommendations. Hybridizations were carried out overnight at 60°C in 5× HSB (4.4 M NaCl, 0.1 M PIPES, and 20 mM EDTA at pH 6.8), 5× Denhardt's solution, 5% Lauryl sulfate (SDS), and denatured salmon sperm DNA at 50 µg/ml. Tomato genomic and cDNA clones (25) were labeled with (³²P)-dCTP (NEN, Life Sciences) by Klenow extension (6) using random primers (Ambion Inc., Austin, TX). Blots were washed (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% SDS and 0.2× SSC, 0.1% SDS at 60°C), exposed to X-ray film (Kodak, New York) at -80°C, and developed.

PCR analysis. PCR reactions were performed in a total volume of 50 µl with each reaction containing 5 to 10 ng of template DNA, 50 pmol of each oligonucleotide primer, 0.2 mM each

dNTP, 2 units of *Taq* polymerase (Fisher Scientific Co., Pittsburgh) in buffer containing 100 mM Tris-HCl, pH 8.3, 500 mM KCl, 1.5 mM MgCl₂, and 0.01% (wt/vol) gelatin under a drop of oil. Amplifications were performed in a DNA thermal cycler (MJ Research, Inc., Watertown, MA) programmed for 94°C for 2 min followed by 36 cycles of 1 min at 94°C, 1 min at 60 to 62°C, and 2 min at 72°C. After cycling, the reaction was completed with 5 min at 72°C. Following PCR and restriction enzyme digestion, fragments were separated on 2% agarose, stained with ethidium bromide, resolved under UV light, and photographed.

The PCR-based markers used in this study were selected from previously published data, developed from GenBank sequence data, or designed from conserved domains of resistance genes. The resistance gene analog (RGA) used was based on kinase domains found in resistance genes. Primers for TG091 are from Alpert and Tanksley (1). Primers for the RGA were RGA-F, 5'-CTACCCACAATGAGCATGAGC-3' and RGA-R, 5'-GTGCAT-CTCCAGTTTCCAC-3', and primers for CT202 were CT202F, 5'-TAATCCGAGAAGGTGATCCG-3' and CT202R, 5'-GGCTT-ATAACCCATGCCAAAAG-3'. Polymorphisms were detected after digestion of the PCR products with *Dra*I for TG091, *Rsa*I for the RGA, and *Tsp*45I for CT202.

Statistical analysis. Genotypic data from RFLP and PCR-based markers were scored as homozygous *L. hirsutum* LA407, homozygous *L. esculentum*, and heterozygote. Segregation ratios of marker genotypes were analyzed by χ^2 analysis. Independence and normality of residuals for phenotypic data were evaluated by using the Shapiro-Wilks test and normal probability plots as well as summary statistics calculated using the UNIVARIATE procedure of SAS (version 7.0; SAS Institute, Cary, NC). Residuals were found to be independent and normally distributed with constant variance.

TABLE 1. Marker loci significantly ($P < 0.05$) associated with response to *Clavibacter michiganensis* subsp. *michiganensis* in the LA407 inbred backcross population within four environments

Trial ^v	Response ^w	Chromosome ^x	Marker ^y	Genotypic means of marker classes ^u			P value	H allele ^z
				HH	HE	EE		
1997 GH C290	IDRS	2L	TG620	0.50	...	2.82	0.0175	+
	PMD	2L	TG620	25.00	...	43.09	0.0118	+
		10L	TG420	54.06	46.25	41.96	0.0094	-
1997 Field C290	IDRS	2L	TG620	1.88	...	2.88	0.0067	+
	PMD	5L	RGA	2.25	2.00	2.88	0.0138	+
		10S	TG230	3.38	...	2.84	0.0457	-
		1S	TG125	62.88	66.73	49.21	0.0246	-
		2L	TG620	30.90	...	49.99	0.0189	+
		10S	TG230	62.37	...	49.29	0.0243	-
1998 GH A300	IDRS	2L	TG620	1.50	...	4.49	0.0001	+
	PMD	5L	RGA	1.00	4.25	4.50	0.0001	+
		10S	TG230	3.13	...	4.49	0.0188	+
		10L	TG408	3.13	...	4.49	0.0188	+
		2L	TG620	20.42	...	68.82	0.0004	+
		5L	RGA	10.00	67.08	69.03	0.0001	+
		7S	TG418	53.44	...	69.06	0.0318	+
		7L	TG572	53.44	...	69.06	0.0318	+
1998 Field A300	IDRS	2L	TG091	3.54	3.83	4.05	0.0432	+
	PMD	2L	TG492	3.56	...	4.04	0.0473	+
		2L	TG620	3.17	...	4.03	0.0370	+
		4L	TG427	4.75	4.00	3.99	0.0342	-
		1S	CP046	63.67	48.75	43.44	0.0032	-
		1S	TG125	51.12	58.11	43.59	0.0420	-
		2L	TG091	37.60	37.29	44.60	0.0354	+
		2L	TG620	26.61	...	44.21	0.0060	+
		4L	TG427	58.66	44.14	43.44	0.0033	-
		9S	TG018	51.06	44.54	42.91	0.0035	-

^u HH = homozygous *Lycopersicon hirsutum* LA407; HE = heterozygote; EE = homozygous *L. esculentum*.

^v *C. michiganensis* subsp. *michiganensis* strains C290 and A300 in greenhouse (GH) and field trials.

^w Disease response: IDRS = individual disease rating score corresponding to maximal disease and PMD = percentage of maximal disease.

^x L = long arm of chromosome; S = short arm of chromosome.

^y RGA = resistance gene analog; CP046, RGA, and TG091 are polymerase chain reaction-based markers; the remainder are restriction fragment length polymorphism markers.

^z Effect of *L. hirsutum* LA407 (H) allele on resistance (+) or susceptibility (-) to *C. michiganensis* subsp. *michiganensis*.

The association between response to *C. michiganensis* subsp. *michiganensis* and the genotype of 63 polymorphic markers scored in the LA407 IBC 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 (17) due to the unbalanced data sets. Significant *F* tests ($P < 0.05$) provided evidence for linkage between a marker and resistance. All calculations for mixed linear models were performed using the MIXED procedure of SAS.

For greenhouse experiments, marker was considered as a fixed effect while genotype within marker was considered as a random effect. The statistical model was $X_{ij} = \mu + M_i + G_j(M_i) + \epsilon_{ij}$ (equation 1), where X_{ij} is the trait value of the j^{th} genotype of the i^{th} marker class, μ is the population mean, M_i is the effect of the i^{th} marker class, $G_j(M_i)$ is the effect of the j^{th} genotype within the i^{th} marker class, and ϵ_{ij} is the experimental error. For greenhouse experiments, the appropriate *F* test for marker was equal to $M_i/G_j(M_i)$.

For field experiments, marker was considered as a fixed effect while block and genotype within marker were considered random effects. The statistical model was $X_{ijk} = \mu + B_i + M_j + G_k(M_j) + \epsilon_{ijk}$ (equation 2), where X_{ijk} is the trait value of the k^{th} genotype of the j^{th} marker class in the i^{th} block, μ is the population mean, B_i is the effect of the i^{th} block, M_j is the effect of the j^{th} marker class, $G_k(M_j)$ is the effect of the k^{th} genotype within the j^{th} marker class, and ϵ_{ijk} is the experimental error. The appropriate *F* test for marker was equal to $M_j/G_k(M_j)$.

Analysis of variance (ANOVA) using general linear models was performed with the GLM procedure of SAS to determine an association between disease response and marker genotype in the *F*₂ populations derived from IBL 2353 × Ohio 86120 and IBL 2361 × Ohio 86120. The statistical model was $X_i = \mu + M_i + \epsilon_i$ (equation 3), where X_i is the trait value of the i^{th} marker class, μ is the population mean, M_i is the effect of the i^{th} 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 .

The association between disease response and marker genotype of the IBL 2361 × Ohio 86120 *F*₃ progeny was obtained with the statistical model as described in equation 1. Analysis to determine associations between disease severity response and marker genotypes in the IBL 2353 × Ohio 86120 *F*₃ progeny was obtained with the statistical model as described in equation 2. Tests to determine specificity of marker loci to different strains of *C. michiganensis* subsp. *michiganensis* were analyzed using the statistical model as described in equation 1. Fisher's least significant difference, performed after a significant ANOVA, provided pair-wise comparison of means. Total phenotypic variation explained by marker loci was calculated by estimating variance components by restricted maximum likelihood (REML) using the VARCOMP procedure of SAS. Linkage relationships between marker loci were determined by maximum likelihood using Linkage 1 version 3.5 (23). Realized heritability was estimated based on indirect selection of

marker genotype for susceptibility and resistance to *C. michiganensis* subsp. *michiganensis* within the *F*₂ populations. Realized heritability was calculated as the difference in the mean performance of susceptible and resistant progeny in the *F*₃ generation divided by the difference in the mean of the *F*₂ generation (5).

RESULTS

Genotypic analysis of the LA407 IBC population. The LA407 IBC population was genotyped with 58 RFLP and 5 PCR-based markers of known chromosome location. The 63 markers were polymorphic between parental lines and provided coverage of the tomato genome with at least 2 markers per chromosome arm based on the map of Tanksley et al. (25). In general, lower levels of heterozygosity were observed than expected. In several IBLs, however, heterozygosity was observed at multiple unlinked loci, suggesting outcrossing or a heterozygous advantage in these lines during population development. In addition, a lower than expected proportion (4.2%) of the *L. hirsutum* LA407 genome was recovered in each line. These results suggest that selection during population development or segregation distortion due to fertility biased the population away from theoretical expectations, as has been noted for other crosses to *L. hirsutum* accessions (2). Eighty-six percent of the *L. hirsutum* LA407 genome is represented in the IBC population.

Major loci from *L. hirsutum* LA407 that contribute to resistance. The 63 polymorphic markers were analyzed for significant associations with response to disease as evaluated in replicated greenhouse and field studies. Of the 63 markers analyzed, 14 were significantly associated ($P < 0.05$) with resistance when evaluated at the maximal IDRS or PMD in at least one greenhouse or field study (Table 1). The 14 markers corresponded to nine *L. hirsutum* LA407 chromosomal regions. Four of the *L. hirsutum* LA407 chromosomal regions were associated with susceptibility to disease, whereas five regions were associated with resistance. Regions on chromosome 2, linked to TG620, and chromosome 5, linked to an RGA marker, exhibited the highest level of significant association with resistance. These markers also showed significant association in multiple disease evaluations. Within the LA407 IBC population, IBL 2353, homozygous at TG620 on chromosome 2, and IBL 2361, homozygous at the RGA on chromosome 5, were selected to confirm marker association with resistance.

Confirmation of markers associated with resistance. Greenhouse studies were conducted to evaluate response to petiole clip inoculation of *F*₂ individuals derived from IBL 2353 and Ohio 86120 with A300 and *F*₂ individuals derived from IBL 2361 and Ohio 86120 with C290. Symptoms developed approximately 14 days postinoculation and scoring for response was recorded at 7-day intervals for analysis of an IDRS and for calculation of PMD.

Marker genotypes of individuals were determined for RFLP marker TG620 and an additional PCR-based marker, TG091, on chromosome 2 in the IBL 2353 × Ohio 86120 population (Table 2).

TABLE 2. Molecular markers significantly ($P < 0.05$) associated with *Clavibacter michiganensis* subsp. *michiganensis* resistance in *F*₂ progeny derived from crossing inbred backcross lines (IBLs) 2353 and 2361 (resistant) with Ohio 86120 (susceptible)

Marker ^w	RE ^x	Chromosome	IBL	No. of <i>F</i> ₂ individuals per progeny class ^y			<i>P</i> value IDRS ^y	PMD ^z
				HH	HE	EE		
TG620	<i>DraI</i>	2	2353	35	62	35	0.0003	0.0050
TG091	<i>DraI</i>	2	2353	42	74	32	0.0110	0.0540
RGA	<i>RsaI</i>	5	2361	43	72	43	0.0029	0.0031
CT202	<i>Tsp45I</i>	5	2361	42	75	38	0.0066	0.0075

^v HH = homozygous *Lycopersicon hirsutum*, HE = heterozygous; EE = homozygous *L. esculentum*. Each marker loci segregated as expected in a 1:2:1 ratio (TG620 $\chi^2 = 0.48$; TG091 $\chi^2 = 1.36$; RGA $\chi^2 = 1.24$; CT202 $\chi^2 = 0.37$).

^w Marker distance between TG620 and TG091 = 5.5 cM and between RGA and CT202 = 1.3 cM.

^x RE = restriction enzyme-producing polymorphism between parental lines.

^y IDRS = individual disease rating score corresponding to maximal disease.

^z PMD = percentage of maximal disease.

The PCR-based marker RGA and CT202 (both from chromosome 5) were scored in the IBL 2361 × Ohio 86120 population (Table 2). Three marker classes were observed among the F₂ progeny in both populations for all four markers and segregation followed the expected 1:2:1 ratio (Table 2). Based on single-marker analysis, TG620, TG091, the RGA, and CT202 were significantly associated with resistance when evaluated at the IDRS corresponding to maximal disease or PMD (Table 2). The linkage distance between TG620 and TG091 was estimated to be 5.5 cM and the distance between the RGA and CT202 was estimated to be 1.3 cM.

To further confirm linkage between markers and resistance, F₃ families originating from F₂ individuals were selected and evaluated for response to inoculation with *C. michiganensis* subsp. *michiganensis* in greenhouse and field studies. A significant association was confirmed between resistance with TG620 and TG091 on chromosome 2 and the RGA and CT202 on chromosome 5 when evaluated at maximal disease or PMD (Table 3). The ability to replicate F₃ families allowed estimates of variance components for the introgressed regions from chromosome 2 and chromosome 5. Markers from chromosome 2 were associated with 56% of the total phenotypic variation for resistance when evaluated using maximal disease and 37% when evaluated using PMD. Markers in the introgressed region from chromosome 5 accounted for 68 to 73% of the total phenotypic variation for resistance when evaluated using maximal disease and 39 to 51% when evaluated using PMD (Table 3). Realized heritability for resistance from IBL 2353 based on genotypic selection was estimated at 0.63 when evaluated using maximal disease and 0.14 using PMD. For IBL 2361, realized heritability based on genotypic selection was estimated at 0.85 when evaluated using maximal disease and 0.37 using PMD.

To determine if marker-defined loci conferring resistance to *C. michiganensis* subsp. *michiganensis* were specific to strains in the A or C DNA fingerprint classes, F₃ families were evaluated for response to petiole clip inoculation in a greenhouse study (Table 4). One set of F₃ families was inoculated with A300, while a second set was inoculated with C290. Based on response to inoculation, a clear delineation between the F₃ families (resistant) and Ohio 86120 (susceptible) was evident, suggesting that both introgressed regions (chromosome 2 and chromosome 5) confer resistance to strains in the A and C fingerprint classes of *C. michiganensis* subsp. *michiganensis*.

DISCUSSION

Identifying and introgressing QTL from wild species often presents a conflict between evaluating plant material that has acceptable agronomic characteristics and sufficient genetic diversity. Advanced backcross populations and IBC populations may offer a compromise that allows for genetic analysis within more uniform genetic backgrounds. These populations have the advantage that resulting germ plasm is more readily used in breeding programs

due to the higher proportion of the cultivated genome. In this study, we detected QTL for resistance using a small IBC population and a limited number of markers. Although the small population may limit our ability to identify all QTL that affect resistance, we have confirmed the presence of two loci from *L. hirsutum* LA407 that contribute resistance to strains of *C. michiganensis* subsp. *michiganensis* in the A and C fingerprint classes. The verification of these QTL in large confirming populations demonstrates the utility of QTL analysis using a small IBC population as a hypothesis-forming generation. Further generations and the markers identified in this study will allow us to minimize deleterious linkage and combine both resistance loci into elite *L. esculentum* backgrounds through marker-assisted selection.

The possibility of identifying beneficial QTLs from unadapted germ plasm while simultaneously introgressing them into a desirable genetic background makes the IBC breeding method an attractive tool. The IBC population structure provides a pedigree that preserves genetic structure, allows replication to reduce the effect of environmental variation, and retains a high proportion of the adapted genome. We have also shown that the IBC breeding method, even with its characteristic unequal allele frequencies, permits molecular marker analysis. For IBC populations, statistical models for detecting QTLs by association with single molecular markers follows well-established methods based on a comparison between marker genotypic means that are tested by ANOVA and *F* statistics (15). If models for single-marker QTL analyses include random effects of genotypes, locations, and blocks, appropriate estimates of the denominator degree of freedom used to

TABLE 4. Mean separation for response to inoculation with *Clavibacter michiganensis* subsp. *michiganensis* strains A300 and C290 of F₃ families originating from resistant F₂ individuals

Parent or progeny ^x	A300		C290	
	IDRS ^y	PMD ^z	IDRS	PMD
Ohio 86120 (S)	4.4 a	58.1 a	3.5 a	46.5 a
IBL 2353 (R)	1.4 b,c	22.3 b,c	0.8 b	11.5 c
IBL 2361 (R)	1.4 b,c	20.5 b,c	1.5 b	21.3 b,c
F ₃ 61151 (R)	2.6 b	31.4 b	1.2 b	18.8 b,c
F ₃ 53060 (R)	2.0 b,c	29.0 b,c	2.4 b	31.9 b,c
F ₃ 53177 (R)	1.8 b,c	25.1 b,c	1.9 b	22.3 b,c
F ₃ 53025 (R)	1.8 b,c	22.4 b,c	1.6 b	21.8 b,c
F ₃ 61037 (R)	1.8 b,c	25.0 b,c	2.1 b	25.7 b,c
F ₃ 61094 (R)	1.6 b,c	23.6 b,c	1.2 b	20.4 b,c
F ₃ 53125 (R)	1.4 b,c	19.7 b,c	1.4 b	19.5 b,c
F ₃ 53166 (R)	1.2 c	18.4 c	1.1 b	15.3 c
LSD (0.05)	0.95	12.04	1.06	12.16

^x F₃ families with prefix of 53 were derived from self-pollination of resistant F₂ individuals of inbred backcross line (IBL) 2353 × Ohio 86120. F₃ families with prefix of 61 were derived from self-pollination of resistant F₂ individuals of IBL 2361 × Ohio 86120. Tests of F₃ families included 10 plants per family. R = resistant; S = susceptible; LSD = least significant difference.

^y IDRS = individual disease rating score corresponding to maximal disease.

^z PMD = percentage of maximal disease.

TABLE 3. Molecular markers significantly (*P* < 0.05) associated with *Clavibacter michiganensis* subsp. *michiganensis* resistance in F₃ families originating from F₂ individuals genotyped as homozygous *Lycopersicon hirsutum* LA407 (resistant) and homozygous *L. esculentum* (susceptible) at each marker loci

Marker	RE ^w	F ₃ families ^x	Chromosome	<i>P</i> value		Vp ^v	
				IDRS ^y	PMD ^z	IDRS	PMD
TG620	<i>Dra</i> I	2353	2	0.0013	0.0106	0.56	0.37
TG091	<i>Dra</i> I	2353	2	0.0013	0.0106	0.56	0.37
RGA	<i>Rsa</i> I	2361	5	0.0003	0.0022	0.73	0.51
CT202	<i>Tsp</i> 45I	2361	5	0.0015	0.0120	0.68	0.39

^v Proportion of total phenotypic variation explained by marker locus.

^w RE = restriction enzyme-producing polymorphism between parental lines.

^x F₃ families were produced by self-pollination of F₂ individuals. Tests of F₃ families included 8 to 10 plants per family.

^y IDRS = individual disease rating score corresponding to maximal disease.

^z PMD = percentage of maximal disease.

determine *F* statistics for marker analyses may require complex computation (22). Estimates of the denominator degree of freedom for IBC populations can be calculated using Satterthwaite's approximation.

In genetic analysis, more power is gained by increasing population size than by replication (14). However, in plant breeding, we want to select the best genotypes across environments. These conflicting goals are further complicated by testing the statistical robustness of putative QTL. In QTL analysis involving unbalanced data, we must consider the appropriate statistical model in addition to an appropriate significance threshold. In populations characterized by unequal class sizes, appropriate single-marker analyses require estimating denominator degrees of freedom. Although the estimation of denominator degrees of freedom and the use of mixed linear models results in test statistics that are only approximate, the approach is more robust than the alternative of using degrees of freedom based on the assumption of a balanced data set. In our analysis, QTLs that were significant at $P = 0.05$ were significant at $P < 0.0001$ using standard ANOVA models which assume a balanced data set and where the denominator degree of freedom was not appropriately estimated. Assuming that the proper statistical model has been applied, significance thresholds must then be chosen to balance type I and type II errors (15). In the process of QTL identification and introgression using AB-QTL analysis and IBC breeding designs, it is possible to compensate for either inappropriate statistical models or significance thresholds by pursuing subsequent generations during the breeding process. As such, AB-QTL analysis and the IBC breeding method offer a solution to the conflicting goals of breeding and genetic analysis (26,27).

Three loci on chromosomes 5, 7, and 9 originating from the wild tomato accession *L. peruvianum* LA2157 have been identified as contributing to resistance (29). Markers associated with the *L. peruvianum* LA2157 resistances on chromosomes 7 and 9 were not found associated with resistance in our population. Although the QTLs on chromosomes 7 and 9 may be unique to the *L. peruvianum* genome, it is also possible that the *L. peruvianum* QTLs are specific to the bacterial strains used. Likewise, although *Rcm* 2.0 from *L. hirsutum* LA407 may be unique to this wild species genome, it may be specific to the A and C strains used in our analysis. As the genetics of *C. michiganensis* subsp. *michiganensis*-tomato interactions become better studied, it will be important to work with pathogen strains that are genetically characterized. Currently, the rep-PCR classification of Louws et al. (16) provides the best method to classify *C. michiganensis* subsp. *michiganensis* strains at a subspecies level.

QTLs on chromosome 5 are common to *L. hirsutum* LA407 and *L. peruvianum* LA2157 and were associated with resistance to *C. michiganensis* subsp. *michiganensis*. In our analysis, the *L. hirsutum* LA407 QTL, *Rcm* 5.1, was detected by primers based on kinase domains found in resistance genes and maps to the same general region as the *Pto* locus that confers resistance to race 0 strains of *Pseudomonas syringae* pv. *tomato*. Although linkage to the *Pto* cluster does not prove the involvement of a classically defined "R" gene in resistance to *C. michiganensis* subsp. *michiganensis*, the occurrence of QTLs from *L. hirsutum* LA407 and *L. peruvianum* LA2157 in this region suggest that further studies to fine map the location of these loci may help determine their function.

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