Identification of a Candidate Gene in Solanum habrochaites for Resistance to a Race 1 Strain of Pseudomonas syringae pv. tomato

Zhilong Bao, Fanhong Meng, Susan R. Strickler, Diane M. Dunham, Kathy R. Munkvold, and Gregory B. Martin*

Abstract
Bacterial speck disease caused by Pseudomonas syringae pv. tomato (Pst) is a persistent problem on tomato (Solanum lycopersicum L.). Resistance against race 0 Pst strains is conferred by the Pto protein, which recognizes either of two pathogen effectors: AvrPto or AvrPtoB. However, current tomato varieties do not have resistance to the increasingly common race 1 strains, which lack these effectors. We identified accessions of Solanum habrochaites S. Knapp & D. M. Spooner that are resistant to the race 1 strain T1. Genome sequence comparisons of T1 and two Pst strains that are virulent on these accessions suggested that known microbe-associated molecular patterns (MAMPs) or effectors are not involved in the resistance. We developed an F2 population from a cross between one T1-resistant accession, LA2109, and a susceptible tomato cultivar to investigate the genetic basis of this resistance. Linkage analysis using whole-genome sequence of 58 F2 plants identified quantitative trait loci (QTL), qRph1, in a 5.8-Mb region on chromosome 2, and qRph2, in a 52.4-Mb region on chromosome 8, which account for 24 and 26% of the phenotypic variability, respectively. High-resolution mapping of qRph1 confirmed it contributed to T1 resistance and delimited it to a 1060-kb region containing 139 genes, including three encoding receptor-like proteins (RLPs) and 17 encoding receptor-like protein kinases (RLKs). One RLK gene, Solyc02g072470, is a promising candidate for qRph1, as it is highly expressed in LA2109 and induced on treatment with MAMPs. qRph1 might be useful for enhancing resistance to race 1 strains and its future characterization could provide insights into the plant immune system.

Bacterial speck disease of tomato is caused by Pst and can be a problem in production areas throughout the world where moist, cool conditions occur (Pedley and Martin, 2003; Young et al., 1986). The disease manifests as necrotic lesions (specks) on all aerial parts of the plant and can result in decreased fruit quality and yield, resulting in significant economic losses (Jones, 1991). The experimental tractability of both Pst and tomato has facilitated the study of their interaction and led to many insights into the molecular basis of bacterial pathogenesis and the plant immune system (Martin, 2012; Velasquez and Martin, 2013).

Two races of Pst are defined based on the ability of the host resistance (R) protein Pto to recognize and confer resistance to them (Jones, 1991; Pedley and Martin, 2003). Race 0 strains translocate the type III effectors AvrPto and AvrPtoB into the plant cell where they are recognized by Pto, whereas race 1 strains either lack these effectors, do not accumulate the proteins, or have variants that are not recognized by Pto (Kunkeaw et al., 2010; Lin et al., 2006). Genome sequences are available for the widely studied Pst strain DC3000 (race 0) and five...
other \textit{Pst} strains, including two race 1 strains, T1 and NY-T1 (Almeida et al., 2009; Buell et al., 2003; Jones et al., 2015) (http://pseudomonas-syringae.org). Recently, the tomato genome has been sequenced, further enhancing the use of this species for understanding the molecular basis of many phenotypes including plant immunity (Tomato Genome Consortium, 2012).

Plants employ two interrelated immune systems to combat pathogen attack (Dodds and Rathjen, 2010). In the first, the extracellular domains of host transmembrane pattern recognition receptors (PRRs) detect MAMPs, which are typically conserved molecules required for the microbial lifestyle (Zipfel, 2014). Subsequent signaling events activate pattern-triggered immunity (PTI). Pathogens adapted to a particular host deliver virulence proteins (effectors) into the plant cell where they act in a variety of ways to defeat PTI (Anderson and Frank, 2012; Dou and Zhou, 2012). At this stage, a second immune system can be deployed in which intracellular host receptors (R proteins) recognize, either directly or indirectly, the presence of a pathogen effector to activate effector-triggered immunity (ETI) (Maekawa et al., 2011). In a further cycle of this molecular “arms race,” some pathogens have evolved effectors that interfere with ETI (Guo et al., 2009; Rosebrock et al., 2007; Wei et al., 2015). Numerous details about the mechanisms underlying each of these steps are known, and their study constitutes an active area of research (Boller and Felix, 2009).

In tomato, the PRRs FLS2 and FLS3 detect the presence of the \textit{Pst} flagellin-derived MAMPs Flg22 and FlgII-28, respectively (Cai et al., 2011a; Clarke et al., 2013; G. Martin, unpublished data, 2015; Robatzek and Wirthmueller, 2013; Veluchamy et al., 2014). Tomato also responds to bacterial Csp22, derived from cold shock protein (Felix and Boller, 2003; Veluchamy et al., 2014), although its cognate PRR has not yet been reported. FLS2 and FLS3 act with the coreceptor BAK1 to activate PTI, which can be monitored on MAMP treatment through the generation of reactive oxygen species (ROS), activation of mitogen-activated protein kinases, defense gene expression, and ultimately inhibition of bacterial population growth (Chakravarthy et al., 2009; Chinchilla et al., 2007; Heese et al., 2007; Nguyen et al., 2010; G. Martin, unpublished data, 2015). The \textit{Pst} effectors AvrPto and AvrPtoB interfere with the FLS2–BAK1 and FLS3–BAK1 complexes and thereby impede PTI, allowing progression of bacterial speck disease (Cheng et al., 2011; Martin, 2012; G. Martin, unpublished data, 2015; Shan et al., 2008; Xiang et al., 2008).

The resistance protein Pto, a serine–threonine protein kinase, binds to either AvrPto or AvrPtoB and acts with the Prf nucleotide-binding–leucine-rich-repeat (LRR) protein to activate ETI, which is associated with transcriptional reprogramming, localized programmed cell death, and inhibition of pathogen growth, among other responses (Oh and Martin, 2011; Pombo et al., 2014; Salmeron et al., 1996; Xing et al., 2007). In addition to its effectiveness against race 0 strains of \textit{Pst}, Pto/Prf-mediated ETI can also potentially suppress infection of tomato by diverse \textit{P. syringae} pathovars that express \textit{avrPto} or \textit{avrPtoB} (Lin and Martin, 2007).

Extensive natural variation is observed among tomato germplasm for fruit and leaf morphology, plant architecture, and other traits (Goldman, 2008; Male, 1999). A recent screen of 14 heirloom varieties (also called heritage varieties) for generation of ROS on treatment with Flg22, FlgII-28, or Csp22 identified natural variation for responses to each of these MAMPs (Veluchamy et al., 2014). The twelve wild relatives of tomato (in \textit{Solanum} sect. \textit{Lycopersicon}) also provide a valuable resource for the identification of genes contributing to plant immunity (Granillo et al., 2011; Peralta et al., 2006). Many of these wild relatives can be readily crossed with cultivated tomato allowing such genes to be introgressed into existing varieties. For example, \textit{Pto} was identified in \textit{Solanum pimpinellifolium} L. and has been introduced into many processing tomatoes (Pedley and Martin, 2003). At least three other wild tomato species are also known to have the \textit{Pto} gene (Riely and Martin, 2001; Rose et al., 2005).

The \textit{Pto} gene has been reported to be present in 14 of the top 20 processing varieties in California and to be used on >60% of the state acreage to protect the crop from bacterial speck disease (Pedley and Martin, 2003). The gene has been remarkably effective for over 20 yr, possibly because \textit{Pst} strains that lack AvrPto or AvrPtoB are less virulent (Lin and Martin, 2005). Nevertheless, race 1 strains now predominate in many tomato-growing regions and there have been an increasing number of reports of the breakdown of \textit{Pto}-mediated resistance (Arredondo and Davis, 2000; Buonaurio et al., 1996; Cai et al., 2011a; Kunkeaw et al., 2010). It would therefore be useful to identify and characterize sources of resistance to race 1 strains to both combat the disease and possibly lead to novel insights into the plant immune system. There have been earlier reports of resistance to a race 1 strain in some wild relatives of tomato, but the genetic basis for this resistance was not clear (Rose et al., 2005; Stamova et al., 1990). Recently, resistance to \textit{Pst} race 1 strain A9 was identified in \textit{S. habrochaites} accession LA1777 and four QTL were shown to contribute to this resistance (Thapa et al., 2015). Here we report the discovery that \textit{S. habrochaites} LA2109 and other accessions from the same geographical area exhibit resistance to the race 1 strain T1 and the use of a mapping-by-sequencing approach to determine genomic regions associated with this phenotype.

### Materials and Methods

#### Plant Material and Growth Conditions

Wild tomato accessions were obtained from the Tomato Genetics Resource Center (tgrc.ucdavis.edu). The wild tomato accessions, \textit{F}_1, and \textit{F}_2 plants generated from a cross between LA2109 and ‘Rio Grande’ (RG)-PtoS were grown in Sunshine MVP soil mix (Sun Gro Horticulture). The RG-PtoS and RG-PtoR plants were grown in Cornell Plus mix, consisting of 0.16 m³ peat moss, 0.34
m³ vermiculite, 2.27 kg lime, 2.27 kg Osmocote Plus 15-9-12 and 0.54 kg Uni-Mix 11-5-11 (Everris, Israeli Chemicals Ltd). Plants were grown in the greenhouse and moved to a growth chamber with 25°C, 75% humidity, and 16-h light after pathogen inoculation.

**Microbe-Associated Molecular Pattern Treatments, Pathogen Inoculation, and Bacterial Growth Assays**

Two lateral leaflets on the second leaf of 3-wk old tomato plants were syringe-infiltrated with 1 μg mL⁻¹ FlgII-28 (EST-NILQRMRRELAVQSNNSATDREA) (Cai et al., 2011a) or 10 μg Csp22 (AVGTVKWFNAEKGFGRIT-PDDG) (Felix and Boller, 2003) and harvested 6 h later. Tissue samples were immediately frozen in liquid nitrogen and kept at −80°C until use. Six-week-old plants were inoculated with 10⁵ colony-forming units (CFU) mL⁻¹ Pst T1 by vacuum infiltration for the visual monitoring of disease symptoms and 10⁴ CFU mL⁻¹ for the measurement of bacterial populations. For each replicate, three leaf discs were collected and combined; each experiment involved replicates from three independent plants.

**Preparation of DNA Libraries and Illumina Sequencing**

Genomic DNA was extracted using a DNeasy Plant Mini kit (Qiagen) and used to make individual libraries as described previously (Zhong et al., 2011). Briefly, 700 ng of DNA from each plant was fragmented using NEBNext dsDNA Fragmentase (New England Biolabs) for 25 min at 37°C before stopping the reaction with ethylenediaminetetraacetic acid (EDTA) at a final concentration of 125 mM. Genomic DNA enriched for fragments between 300 and 500 bp was purified using AMPure XP beads (Beckman Coulter) and eluted in water. End-repair, dA-tailing, Y-shape adaptor ligation, triple-SPI purification and size selection, and PCR enrichment were performed as described (Zhong et al., 2011). A total of 60 uniquely barcoded libraries were developed (22 for resistant plants, eight for moderately-resistant plants, 28 for susceptible plants, and two for RG-PtoS) and subdivided into four pools of 15 libraries. Each of the four pools was sequenced in one lane by the Genomics Resources core facility at Weill Cornell Medical College using Illumina HiSeq 2500 paired-end sequencing (2 × 100 bp) technology. LA2109 was sequenced separately in one lane using Illumina HiSeq 2000 paired-end sequencing (2 × 100 bp). All genome sequence data (fastq files) are available in the Genbank SRA (Leinonen et al., 2011) as Bioproject ID: PRJNA289640.

**Genotyping and Linkage Analysis**

Illumina sequence reads from F₂ plants, LA2109, and RG-PtoS were mapped to the S. lycopersicum reference genome Heinz 1706 SL2.40 (Tomato Genome Consortium, 2012) with Bowtie 2 (Langmead and Salzberg, 2012). Duplicate reads were removed with Picard (http://picard.sourceforge.net) and local realignment around insertions or deletions was performed using GATK (DePristo et al., 2011). The alignments were converted to mpileup format and single nucleotide polymorphism (SNPs) called with SAMtools and bcftools respectively (Li, 2011; Li et al., 2009). Biallelic SNPs unique to the LA2109 parent were selected as markers. Single nucleotide polymorphisms at Hardy–Weinberg equilibrium with a minimum read depth of 2, maximum read depth of 10, genotype quality score of at least 10, and a quality of at least 950 were selected for further analysis using vcftools (Danecek et al., 2011). A Perl script, snp_window.pl (https://github.com/srs218/manuscripts) was developed to bin the SNPs into windows of 100,000 bp with at least 10 SNPs per window and to calculate a consensus genotype per individual per bin to result in 1852 markers. An additional 2681 markers were generated in 1-kb bins with at least five SNPs per bin around the linked region on chromosome 2. Missing genotypes were inferred by using the no-double-crossover method in r/QTL (Broman et al., 2003). A linkage map was constructed using the marker physical location from S. lycopersicum Heinz 1706 SL2.40 (Tomato Genome Consortium, 2012). A single QTL genome scan using extended Haley–Knott regression was performed to identify candidate QTL loci. A two-dimensional, two-QTL scan was performed to determine interaction and heritability. All QTL computations were performed using rQTL software (Broman et al., 2003).

**High-Resolution Mapping with Molecular Markers**

Molecular markers in the qRph1 region were generated on the basis of sequence gaps in the LA2109 genome by comparison with the reference Heinz 1706 reference genome sequence SL2.40. These gaps represent potential DNA insertions or deletions in the LA2109 genome. Primers were designed to span these gap regions and to amplify a fragment from both LA2109 and RG-PtoS genomic DNA. DNA from two young leaves of each resistant F₂ plant was isolated using an extraction buffer (200 mM Tris-HCl, pH 8.0; 0.5% w/v sodium dodecyl sulfate) and dissolved in 100 μL distilled water. One micro-liter of DNA was used for PCR amplification. All resistant plants were genotyped with 11 insertion–deletion molecular markers and plants with a recombination event in the candidate region were used as informative recombinants to delimit the region conferring T1 resistance.

**RNA Extraction, Reverse Transcription—Polymerase Chain Reaction, and Quantitative Reverse Transcription—Polymerase Chain Reaction**

Leaf tissue was ground in liquid nitrogen and total RNA was extracted with TRIzol reagent following the manufacturer’s instructions (Life Technologies, cat. #15596–018). The concentration of total RNA was determined with a Nanodrop spectrophotometer (Thermo Scientific Co.). RNA (1 μg) was treated with TURBO DNA-free Kit (Life Technologies, cat. AM1907M) and tested for DNA contamination by amplifying SICBL1 (Solyc12g015870) for 25 cycles; no PCR product was detected. The RNA
was then used for RT-PCR. First strand complementary DNA (cDNA) was synthesized following instructions provided with the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific Co., cat. #K1622). One microliter and 0.4 µL of cDNA templates were used for RT-PCR and qRT-PCR, respectively. SlCBL1 was used as an internal control for RT-PCR and as a reference gene for qRT-PCR data normalization (Pombo et al., 2014).

Phylogenetic Analysis

Tomato genes similar to the qRph1 candidate Solyc02g072470 at the nucleotide and amino acid levels were identified from a BLAST search of the Heinz ITAG2.3 gene predictions (Tomato Genome Consortium, 2012) and used for phylogenetic analyses along with Solyc02g072470, Solyc02g070890 (SIFSLS2.1) and Solyc02g070910 (SIFSLS2.2). PhyML in SeaView software was used for all analyses with 100 bootstraps under default settings of the GTR model for nucleotide analysis and the JTT model for amino acid analysis (Gouy et al., 2010). Phylogenetic trees were optimized by FigTree software (http://tree.bio.ed.ac.uk/software/figtree/).

Results

Solanum habrochaites Accession LA2109 Exhibits Resistance to Pseudomonas syringae pv. tomato Race 1 Strain T1

In an initial screen of germplasm from the Tomato Genetics Resource Center core collection we discovered that Solanum habrochaites accession LA2109 developed no symptoms of bacterial speck disease after inoculation with the Pst race 1 strain T1 (Fig. 1). This accession was collected in Loja province in southern Ecuador in 1980 by a team led by Charles Rick from the University of California–Davis. Photographs of the accession in its native site are available along with notes describing LA2109 as a “single very large plant in the village ... vigorous and scrambling over a low stone wall” (grc.ucdavis.edu). We subsequently tested an expanded set of 35 accesses from S. habrochaites including many that were collected from the same geographical area as LA2109 (Fig. 1; Table 1; Supplemental Table S1). Fourteen additional accesses were found to be resistant to T1 with 13 being from the same region (Loja province) as LA2109, indicating these accesses might share a common genetic basis for their resistance to T1. To ensure that the glabrous leaf morphology of LA2109 did not interfere with our inoculation protocol, we tested the highly virulent Pst race 1 strain NY-T1 on LA2109 (Jones et al., 2015) and found that this strain caused severe bacterial speck disease (Supplemental Fig. S1).

LA2109 has both Pto-Dependent and Pto-Independent Resistance to Pseudomonas syringae pv. tomato

Pto-mediated resistance, which involves recognition of the effector proteins AvrPto or AvrPtoB, has been previously reported in S. habrochaites, so we tested if LA2109 also carries this gene (Riely and Martin, 2001). Upon inoculation of LA2109 with Pst DC3000, a strain which carries both avrPto and avrPtoB, we observed no signs of bacterial speck disease (Fig. 2A). However, inoculation with a mutant of DC3000, which lacks both effector genes, resulted in the appearance of numerous specks and chlorosis starting after 3 to 4 d, indicating that LA2109 does indeed have a functional Pto gene (Fig. 2B). Consistent with these results, populations of the DC3000 mutant reached significantly higher levels than T1 in leaves of LA2109 3 d after inoculation (Fig. 2C). Like other race 1 strains, T1 is fully virulent on Pto-expressing tomato varieties (Shan et al., 2000). Together, these data suggest that LA2109 has both Pto-mediated disease resistance and Pto-independent resistance to T1.

LA2109 Response to Known Peptide Microbe-Associated Molecular Patterns, Type III Effectors, or Coronatine does not Appear to Play a Role in its Resistance to T1

The available genome sequences of T1, NY-T1, and DC3000 allowed us to investigate whether specific features unique to T1 might explain LA2109 resistance to this strain. The Flg22 region in NY-T1 flagellin has a phenylalanine at position 16 whereas T1 has a serine (Jones et al., 2006). Nevertheless, we wished to examine the possibility that LA2109 detects AvrPtoB in T1, thus explaining its resistance to this strain. A T1 strain carrying a deletion in avrPtoB is not available, so we relied instead on a T1-like strain Pst19, which also has an avrPtoB gene that does not appear to be expressed; importantly, an avrPtoB mutant is available for Pst19 (Kunkeaw et al., 2010). Inoculation of LA2109 with Pst19 or Pst19ΔavrPtoB resulted in similar symptoms of speck disease (Fig. 3A), and the two strains reached similar population sizes in leaves (Fig. 3B). Thus, the Pto gene in LA2109 and the avrPtoB homolog in Pst19, and likely in T1, do not contribute to the resistance to T1 that we observed in LA2109.
This substitution is known to compromise FlgII-28 PTI-elicitation activity and it could possibly explain the hypervirulence of NY-T1 (Cai et al., 2011a). However, the FlgII-28 region in strains T1 and DC3000 differs by just two amino acids and synthetic peptides of both are fully active in eliciting PTI (Supplemental Fig. S2, Fig. S3; Cai et al., 2011a). Thus it seems unlikely that the variation in FlgII-28 plays a role in the differential response of LA2109 to these three Pst strains.

We next considered the possibility that a specific type III effector in T1 is recognized by LA2109 thereby activating ETI. However, the repertoires of effectors in T1 and NY-T1 were reported recently to be identical except that NY-T1 also has an effector candidate with limited similarity to HopAO1 (Jones et al., 2015). It is possible that NY-T1 evades ETI because HopAO1 interferes with the recognition of an effector by LA2109. To test this possibility, we cloned the HopAO1 candidate effector gene from NY-T1 and transformed it into T1. Inoculation of the T1(hopAO1) strain onto LA2109 revealed no change in T1 virulence (Supplemental Fig. S3). Together these results suggest that LA2109 does not recognize a unique effector in T1.

Finally, we considered whether LA2109 might respond differently to coronatine as NY-T1 and DC3000 produce this phytotoxin, but T1 does not (Almeida et al., 2009; Jones et al., 2015). Infiltration of 1.25 nM of coronatine into leaves of LA2109 and Moneymaker (susceptible to T1) resulted in a similar amount of chlorosis (Supplemental Fig. S3). This observation suggests that coronatine probably does not play a role in the LA2109 response to T1 and it supports an earlier report that production of coronatine does not correlate with Pst virulence (Kunkeaw et al., 2010).

Resistance of LA2109 is Effective Against Other Pseudomonas syringae Pathovars

To determine if LA2109 is resistant to other strains of P. syringae, we chose a subset of diverse strains from a region of southern Ecuador that has many T1-resistant S. habrochaites accessions. The map and close-up show the collection sites of 36 accessions of S. habrochaites (also see Table 1; Supplemental Table S1). Triangle, rectangle, and red circle symbols indicate accessions that were susceptible, moderately resistant, or resistant to T1, respectively. The photograph and inset show LA2109 4 d after inoculation with T1 (no symptoms of disease were observed; see Supplemental Figure S1 for inoculation of LA2109 with NY-T1).
recent study (Cai et al., 2011b) and tested them first on a Pto-expressing tomato cultivar (RG-PtoR) to determine whether they lacked avrPto and avrPtoB and were virulent on tomato. Two strains of *P. syringae* pv. *apii* (a pathogen of celery [*Apium graveolens* L.]) and two from *P. syringae* pv. *persicae* (a pathogen of peach [*Prunus persica* (L.) Batsch]) that met these criteria were inoculated onto LA2109 (Supplemental Fig. S4). Each of these strains caused numerous necrotic lesions on RG-PtoR and little or no symptoms of disease on LA2109. Thus, LA2109 might recognize a feature present in a diverse range of *P. syringae* pathovars.

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**Table 1. *Solanum habrochaites* accessions that were screened for resistance to *Pseudomonas syringae* pv. *tomato* T1.**

<table>
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<tr>
<th><em>S. habrochaites</em> accession†</th>
<th>Collection site, province, and country</th>
<th>Disease index‡</th>
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<td>Alausi, Chimborazo, Ecuador</td>
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<td>Loja (Piedestal district), Loja, Ecuador</td>
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<td>Pallatanga, Chimborazo, Ecuador</td>
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<tr>
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† Accessions were obtained from the Tomato Genetics Resource Center (tgrc.ucdavis.edu).

‡ Disease index is described in the Results section. Index ranges from 1, for no symptoms of bacterial speck disease, to 3 for extensive symptoms of the disease.

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Figure 2. *Solanum habrochaites* accession LA2109 has both Pto-dependent and Pto-independent resistance to *Pseudomonas syringae* pv. *tomato*. Bacterial speck disease on LA2109 4 d after inoculation with (A) DC3000 or (B) DC3000ΔavrPtoΔavrPtoB. Insets show close up of a representative leaflet. (C) Bacterial populations in leaves of LA2109 after inoculation with T1 or DC3000ΔavrPtoΔavrPtoB. Error bars indicate standard deviation. The asterisk indicates a statistically significant difference as determined by a Student's *t*-test (*P* < 0.05).
**F₂ Population Development to Investigate the Genetic Basis of LA2109 T1 Resistance**

The analyses described above provided no clues about features that are unique to T1 which might explain the resistance in LA2109 (genome sequences are not available for the other four *P. syringae* strains). We therefore initiated a genetic approach by crossing LA2109 to RG-PtoS (female parent), a variety fully susceptible to T1. An F₁ plant, which was verified based on its morphology and later by DNA markers, successfully set seeds for an F₂ population. Newly emerged branches from the F₁ and LA2109 plants were excised, rooted, and grown for 6 wk, and seedlings were inoculated with T1. Leaves of F₁ plants developed a few specks 7 d after inoculation, whereas no disease symptoms were observed on LA2109 (data not shown). As expected, RG-PtoS developed severe symptoms of speck disease.

**Identification of Quantitative Trait Loci on Chromosomes 2 and 8**

A total of 423 F₂ plants, along with the parents (LA2109 and RG-PtoS), were inoculated with T1 and disease symptoms were scored 7 d later using a system in which LA2109, which developed no or very few specks was scored as a 1 and RG-PtoS, with many dead leaves, was scored as a 3. The F₂ plants were similarly scored and classified into three groups: resistant (scored as a 1), moderately resistant (2), and susceptible (3) (Fig. 4). Plants scored as moderately resistant encompassed a range of disease symptoms and the numbers of plants in each of the three groups did not fit any simple segregation ratio suggesting multiple loci might be involved in T1 resistance. For further analysis, 22 resistant, eight moderately resistant, and 28 susceptible F₂ plants were identified and used for low coverage (3×) whole-genome sequencing (Fig. 5A; Supplemental Table S2). Parental accessions LA2109 and RG-PtoS were sequenced at...
approximately 53× and 6×, respectively (Supplemental Table S2). The low coverage of the sequence data from the individual F2 plants made accurate genotyping difficult, so a consensus genotype was called in bins along the reference genome chromosome (Supplemental Table S3). Linkage analysis detected major QTLs on chromosomes 2 and 8 (Fig. 5B). The QTL on chromosome 2, accounting for 23.7% of the phenotypic variation and referred to as \( qRph1 \) (quantitative resistance to \( Pst \) T1 in \( S. habrochaites \)), spanned genome coordinates 35,128,470 to 40,887,114 bp (5.8 Mb) with a maximum logarithm of odds (LOD) score of 4.2 found at 38,157,470 bp of the reference tomato genome sequence SL2.40 (Tomato Genome Consortium, 2012; Fig. 5B). The QTL on chromosome 8, accounting for 25.9% of the phenotypic variation and referred to as \( qRph2 \), spanned genome coordinates 2,089,886 to 54,513,866 bp (52.4 Mb) and had a maximum LOD score of 4.5. This QTL was not investigated further because of the lack of recombination on chromosome 8, which precluded fine mapping of the region.

Segregation distortion was detected on chromosome 1 between approximately 2.5 and 70.3 Mb and chromosome 6 between 0 and 34.3 Mb, where no RG-PtoS homozygotes were detected in the F2 plants. Both of these chromosomes are known to have loci involved in unilateral incompatibility, where pistils of self-incompatible species reject the pollen of self-compatible species (ui1.1, on chromosome 1 which encodes an S-locus F-box protein, maps to approximately 33.3–49.4 Mb, near the self-incompatibility locus [Li and Chetelat, 2015] and ui6.1, which encodes a Cullin protein, is located on chromosome 6 at 49.6 Mb [Li and Chetelat, 2010]).

**High-Resolution Mapping of \( qRph1 \)**

To further test whether the candidate \( qRph1 \) is associated with resistance to T1, we designed one cleaved amplified polymorphic sequence marker (ZB3), four codominant insertion–deletion markers (ZB19, ZB8, ZB14, and ZB5), and three markers (ZB11, ZB12 and ZB16) with a large deletion in LA2109 that need two pairs of primers to genotype both parents at the same location. We used these eight markers to genotype 49 T1-resistant F2 plants (selected from the population of 423 F2 plants) (Fig. 6A; Supplemental Table S4). If the \( qRph1 \) region confers resistance to T1, then we expected a statistically significant variance from a 1:2:1 ratio for markers closely linked to the \( qRph1 \) locus. Genotyping data indicated that, depending on the marker used, 35–39% of resistant plants were homozygous for LA2109 DNA, 45–53% were heterozygous (LA2109/RG-PtoS), and 10–18% were homozygous for RG-PtoS (Fig. 6A). Chi-square tests of a 1:2:1 ratio for each marker revealed \( p \)-values of: ZB3 \( (p = 0.03 < 0.05) \), ZB19 \( (p = 0.08) \), ZB8 \( (p = 0.05) \), ZB14 \( (p = 0.03 < 0.05) \), ZB11 \( (p = 0.15) \), ZB12 \( (p = 0.03 < 0.05) \), ZB16 \( (p = 0.08) \), and ZB5 \( (p = 0.13) \) indicating that the DNA region associated with markers ZB3, ZB8, ZB14, and ZB12 is not segregating randomly (Fig. 6A). These data support the linkage of this region to \( qRph1 \) as identified from our QTL analysis.

To further delimit the region containing \( qRph1 \), we combined our analysis of the 49 plants above with an additional 36 T1-resistant plants derived from the 423 F2 plants.
The Region Encompassing \( qRph1 \) has Potential Immunity-Related Genes with Some Encoding Receptor-Like Proteins and Receptor-Like Protein Kinases

By inspection of the tomato reference genome sequence, we found that 139 genes are annotated in the 1060-kb region encompassing \( qRph1 \) (Fig. 6C). These genes include receptor-like protein kinases (RLKs) and receptor-like proteins (RLPs). The locations of \( SIFLS2.1 \) and \( SIFLS2.2 \) are also shown.

The coordinates of this region are based on the Heinz 1706 SL2.40 reference genome. We are currently developing a larger \( F_2 \) population to allow further fine mapping of this region.
region defined above (Supplemental Table S5). It is possible that LA2109 encodes additional genes in this region, but a high-quality *S. habrochaites* genome sequence is not available, and our reads of LA2109 were insufficient for generating a de novo targeted assembly of this region. Among the 139 genes are some in classes that have been implicated in plant immunity, including two CBL-interacting kinases (Cipk1 and Cipk16; Cipk6 plays a role in *Pst* immunity [de la Torre et al., 2013]), RPW8.2 (confering broad-spectrum powdery mildew resistance [Xiao et al., 2001]), as well as WRKY and bZIP transcription factors (Tsuda and Somssich, 2015).

Perhaps of greatest interest is the presence in this region of numerous genes encoding RLPs and RLKs (Fig. 6C; Supplemental Table S5). Such proteins have well-established functions in recognition of MAMPs and subsequent immune signaling. One of the RLK genes in the region, *SERK2*, has been implicated in plant immunity (Chen et al., 2014; Roux et al., 2011); the annotation for the *SERK2* tomato gene (Solyc02g072320), however, indicates only a 288 bp open-reading frame, and, for now, we excluded it from further analysis. We also excluded two RLK genes for which we could not detect a transcript in leaves (Solyc02g071800 and Solyc02g071820) and two RLP genes, one of which seemed incorrectly annotated (Solyc02g072380) and the other for which we could not detect a transcript in leaves (Solyc02g072390).

**One Receptor-Like-Protein-Encoding Gene is Highly Expressed in LA2109 and Other T1-Resistant Accessions but not in Rio Grande**

For the remaining one RLP gene and 14 RLK genes, we developed primers and examined the abundance of their transcripts in leaves of LA2109 and a pair of Rio Grande lines (RG-PtoR and RG-PtoS) that are near isogenic for the *Pto* region and which were expected to be identical in the 1060-kb segment (Fig. 7; Supplemental Table S6). *SIFLS2.1* and a gene encoding a calcineurin B-like protein were included as controls. After verifying there was no DNA contamination of our RNA samples, we used semiquantitative RT-PCR to measure transcript abundance. Receptor-like protein genes Solyc02g071810 and Solyc02g071820 have highly similar nucleotide sequences, and we could only design primers to detect transcripts of both genes. These two genes and 11 of the other ones examined had similar transcript abundance in all three of the tomato lines (Fig. 7). However, two, Solyc02g072470 and Solyc02g072480, had higher transcript abundance in LA2109 than in RG-PtoR and RG-PtoS. The lower transcript abundance in the latter two lines was not due to DNA differences, as the primers successfully amplified the gene sequences from RG-PtoS and LA2109 genomic DNA (Supplemental Fig. S5). We focused on Solyc02g072470 for subsequent experiments because it is highly expressed in LA2109, whereas Solyc02g072480 is not (Fig. 7). Six additional *S. habrochaites* accessions, which originate from the same area as LA2109 and are resistant to T1, were also found to have increased abundance of Solyc02g072470 transcripts. However, little can
be concluded about this possible correlation until more *S. habrochaites* accessions are examined that are both susceptible and resistant to T1 and derived from different geographical areas (Supplemental Fig. S6).

**Solyc02g072470 Transcript Abundance is Increased in Leaves on Treatment with Microbe-Associated Molecular Patterns**

The expression of genes encoding PRRs and PTI-related proteins is often induced on treatment with MAMPs (Rosli et al., 2013; Zipfel et al., 2006). We therefore used qRT-PCR to analyze expression of Solyc02g072470 6 h after treatment of leaves with FlgII-28 (Cai et al., 2011a; Clarke et al., 2013) or Csp22 (Felix and Boller, 2003). Transcript abundance of this gene was increased in response to FlgII-28 in both RG-PtoS (fourfold) and LA2109 (1.5-fold), although the transcript abundance in LA2109 was much higher in uninduced leaves than RG-PtoS (Fig. 8). After treatment of leaves with Csp22, Solyc02g072470 was also significantly induced in LA2109; Csp22 treatment had no effect on transcript abundance in RG-PtoS.

**Solyc02g072470 has Eighteen Predicted Leucine-Rich Repeats and is a Class XII Leucine-Rich Repeat Receptor-like Kinase**

The Solyc02g072470 gene is annotated as having four exons, and its protein is predicted to have 18 leucine-rich repeats (LRRs) and an intracellular kinase domain (Supplemental Fig. S7). Its predicted amino acid sequence in LA2109 is 97% identical with its ortholog in Heinz 1706. By using its DNA and protein sequences in a BLAST search, we identified 14 and 18 tomato genes, respectively, that are similar to Solyc02g072470 (Supplemental Fig. S8). Like FLS2, the protein is an LRR XII RLK, although its amino acid sequence is very different from that PRR, and it has been placed in a distinct clade that contains several of the closely-related genes we examined in Fig. 7 (Sakamoto et al., 2012).

**Discussion**

We initially discovered that *S. habrochaites* accession LA2109 showed no signs of speck disease after being inoculated with *Pst* T1 and subsequently found that such resistance is common among *S. habrochaites* accessions collected in a localized region in southern Ecuador. This finding raised the possibility that these accessions have a common genetic basis for this phenotype. To investigate the underlying mechanism involved, we focused on LA2109 as representative of these accessions and found that it also expressed a Pto-like activity. This was not unexpected as another *S. habrochaites* accession, and other wild relatives of tomato have been reported to have Pto (Riely and Martin, 2001; Rose et al., 2005).

We observed that LA2109 is susceptible to two other sequenced *Pst* strains (NY-T1 and DC3000ΔavrPtoΔavrPtoB), but a comparison of factors involved in host interactions among these three strains did not reveal any obvious explanations for the differential responses. This prompted us to use a mapping-by-sequencing approach that ultimately defined QTLs on chromosomes 2 (qRph1) and 8 (qRph2) that are associated with T1 resistance. Recombination suppression on chromosome 8 impeded the ability to further delineate the boundaries of qRph2. The region containing qRph1 on chromosome 2 was further delimited by molecular markers and found to contain 139 genes. To help determine which of these genes is responsible for T1 resistance we are developing a population with a more uniform genetic background.
background by backcrossing plants carrying the qRph1 region to RG-PtoS for several generations. This should facilitate more accurate assessment of T1 resistance and higher-resolution mapping in this region. In the meantime, one gene appears to be a promising candidate for qRph1, as it is highly expressed in LA2109 and other T1-resistant S. habrochaites accessions but not in RG-PtoS. These results set the stage for future identification of a gene (or genes), which might give insight into the molecular basis for the resistance of LA2109 to T1 and potentially shed light on a novel aspect of the plant immune system.

Our consideration of whether specific MAMPs might potentially explain the differential resistance of LA2109 to T1, DC3000ΔavrPtoΔavrPtoB, and NY-T1 did not produce any likely candidates among the three known peptide MAMPs of Pst. It remains possible that another MAMP present in T1—but missing from the other two strains—is recognized by LA2109 and activates a strong PTI response. Consistent with this hypothesis, the essentially identical repertoire of type III effectors in T1 and NY-T1 suggests ETI is not involved in LA2109 resistance to T1. It is possible that amino acid differences in one or more effectors in T1, compared with NY-T1, allows it or them to be detected by LA2109. However, the lack of any R-protein-like encoding genes in our candidate region also suggests ETI is not involved in the phenotype. Finally, we considered the possible involvement in LA2109 resistance of coronatine, which is produced by NY-T1 and DC3000 but not by T1. However, we saw no difference in the sensitivity to this phytotoxin between LA2109 and another tomato cultivar susceptible to T1. A previous study also reported that production of coronatine does not correlate with Pst virulence (Kunkeaw et al., 2010). Collectively, the known differences among these three Pst strains did not suggest any factor that might explain the specific resistance in LA2109 to T1.

We discovered that although LA2109 is susceptible to NY-T1 and DC3000ΔavrPtoΔavrPtoB, it is resistant to four diverse P. syringae pathovars (pathogens of celery and peach) that are able to cause disease on the Pto-expressing tomato line RG-PtoR. If genome sequences become available for these four strains it might be possible to investigate what features they have in common with T1 that differ from other P. syringae strains that cause disease on LA2109. To date, we have identified just three strains that lack avrPto and avrPtoB and cause disease on LA2109: DC3000ΔavrPtoΔavrPtoB, NY-T1, and Pst19 (a genome sequence is not available for Pst19). In the future, a broadened screen is needed to identify additional strains that are able to cause disease on tomato, generally, and either do or do not cause disease on LA2109. Genome sequence comparisons of strains in these two classes might then identify candidate features explaining the differential response of LA2109 to them.

Our initial analysis to understand the genetic basis of LA2109 resistance indicated that the phenotype was not segregating in a simple fashion and might be quantitative. By using a mapping-by-sequencing approach involving low-coverage, whole-genome sequencing of F1 plants and higher coverage data from the parental accessions, we were able to identify two QTLs and, in the case of qRph1, identify markers to define candidate loci for conferring resistance. Strict filtering of F2 SNPs was necessary mainly due to divergence between the two parental accessions, which resulted in uncertainty in the true mapping location of many reads derived from the LA2109 parent. While low-coverage data cannot easily be used to accurately determine genotype at individual loci, by pooling loci and calling a consensus genotype for linked loci in the F2 population, we were able to make genotype calls. As a result of the relatively small number of F2 individuals and divergence between the parental accessions, whole genome sequencing data was not sufficient to allow the fine mapping of QTL regions, particularly on chromosome 8 where recombination was found to be reduced with respect to other chromosomes. Reduced recombination for chromosome 8 was also found in another study involving a cross between S. lycopersicum and S. habrochaites (Zhang et al., 2002), and further analysis of this region might require development of a population derived from a cross between LA2109 and a T1-susceptible S. habrochaites accession (Grandillo et al., 2011; Scott, 1997).

Despite the complications resulting from low sequence coverage in the F2 plants, by using additional DNA marker-based mapping in a large F2 population, we were able to delimit a 1060-kb region that is associated with resistance to T1. The genome sequence surrounding and including this region contains many genes encoding RLKs, including the two FLS2 genes present in tomato (FLS2.1 and FLS2.2). We observed no difference in the transcript abundance of FLS2.1 in LA2109 and RG-PtoS and data from our QTL analysis and high-resolution mapping placed both genes outside of the region conferring T1 resistance. Within the 1060-kb region, 139 genes have been annotated in the most recent version of the tomato gene predictions (ITAG release SL2.4 [Fernandez-Pozo et al., 2015]). None of these genes encode nucleotide-binding-LRR proteins, although there are several other genes that belong to classes that are implicated in defense responses. Future high-resolution mapping using a large F2 population derived from the backcrossing, which we have underway, should help narrow down the list of candidate genes.

Although further mapping is required to definitively identify qRph1, we chose to investigate the genes encoding RLKs and RLPs in the 1060-kb region because of the well-known role of these types of proteins in PTI. Our initial intention was to determine if some of these genes are not expressed in leaves so as to exclude them from being qRph1. However, of the 18 genes we examined, a transcript was detected for 15 (Solyc02g071800, Solyc02g07870 and Solyc02g072390, Solyc02g07870 were not expressed in leaves). Interestingly, four of the RLK genes appear to be paralogs; they are clustered within a 57-kb region and likely arose by duplication events (Solyc02g072400, Solyc02g072440, Solyc02g072470, and Solyc02g072480).
Two of these genes, Solyc02g072470 and Solyc02g072480, had higher transcript abundance in LA2109 than in RG-PtoS. Solyc02g072470 is especially striking in this regard and we found its transcript is also highly abundant in the other T1-resistant accessions that were collected near LA2109. However, we found that transcript abundance of Solyc02g072470 was increased in leaves exposed to FlgII-28 or Csp22. Such induced expression is a characteristic of genes that encode PRRs (Rosli et al., 2013). A recent study reported that LA2109 has a higher ROS response to Csp22 than RG-PtoS (Veluchamy et al., 2014). However, in an analysis of 139 RG-PtoS × LA2109 F2 plants, we saw no correlation between response to Csp22 and resistance to T1 (R² = 0.01274). Together, these observations make Solyc02g072470 a strong candidate for qRph1, but additional work is needed, including the development of transgenic tomato lines overexpressing this gene to determine if they acquire resistance to T1.

A screen of 278 accessions of tomato wild species for resistance to two race 1 Pst strains (A9 and 407) was reported recently (Thapa et al., 2015). Five accessions were resistant to these strains with two of them being from S. habrochaites (LA2869 and LA1777). Using a series of LA1777 introgression lines, four QTLs were identified as contributing to A9 resistance, each of which explained 10 to 12% of the phenotypic variation. Of potential interest is one of these QTLs, bsRrl-2, located on chromosome 2 at position 95 cm of the genetic map for the LA1777 population. Although it is not possible to determine precisely how this position corresponds to the tomato reference genome coordinates, based on the positions of the markers used in the LA1777 map, it appears that bsRrl-2 lies about 8100 kb away from the 1060-kb region we have identified. Nevertheless, it is possible that our locus and the ones described in that study contribute to a common pathway enhancing resistance to race 1 Pst strains. Regardless of whether they contribute to the same pathway or to different mechanisms, the introgression into tomato varieties of the qRph1 region and the four LA1777 QTLs have the potential to provide some level of resistance to the increasingly prevalent race 1 strains of Pst. In addition, in light of the fact that LA2109 is resistant to some diverse P. syringae pathovars, it is possible that qRph1 might provide protection to a broader range of bacterial pathogens, as has been reported for EFR and Xa21 (Lacombe et al., 2010; Mendes et al., 2010; Tripathi et al., 2014).

Acknowledgments

We thank Paige Reeves for assistance in the greenhouse; Dr. Hernan Rosli, Dr. Marina Pombo, Dr. Sarah Hind, Simon Schwizer, and Christine Kraus for technical advice; Dr. Magdalen Lindeberg for the MAMP comparisons; Dr. Naama Menda for reviewing the Perl script; and Christine Kraus and Simon Schwizer for helpful comments on the manuscript. We thank the UC–Davis C. M. Rick Tomato Genetics Resource Center for providing tomato seeds. This research was supported, in part, by National Science Foundation grant IOS-1025642 (GBM).

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