The Immune Receptor Roq1 Confers Resistance to the Bacterial Pathogens Xanthomonas, Pseudomonas syringae, and Ralstonia in Tomato

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Xanthomonas species, Pseudomonas syringae and Ralstonia species are bacterial plant pathogens that cause significant yield loss in many crop species. Generating disease-resistant crop varieties can provide a more sustainable solution to control yield loss compared to chemical methods. Plant immune receptors encoded by nucleotide-binding, leucine-rich repeat (NLR) genes typically confer resistance to pathogens that produce a cognate elicitor, often an effector protein secreted by the pathogen to promote virulence. The diverse sequence and presence/absence variation of pathogen effector proteins within and between pathogen species usually limits the utility of a single NLR gene to protecting a plant from a single pathogen species or particular strains. The NLR protein Recognition of XopQ 1 (Roq1) was recently identified from the plant Nicotiana benthamiana and mediates perception of the effector proteins XopQ and HopQ1 from Xanthomonas and P. syringae respectively. Unlike most recognized effectors, alleles of XopQ/HopQ1 are highly conserved and present in most plant pathogenic strains of Xanthomonas and P. syringae. A homolog of XopQ/HopQ1, named RipB, is present in most Ralstonia strains. We found that Roq1 confers immunity to Xanthomonas, P. syringae, and Ralstonia when expressed in tomato. Strong resistance to Xanthomonas perforans was observed in three seasons of field trials with both natural and artificial inoculation. The Roq1 gene can therefore be used to provide safe, economical, and effective control of these pathogens in tomato and other crop species and reduce or eliminate the need for traditional chemical controls.

Keywords: plant immunity, Ralstonia, Xanthomonas, Pseudomonas, tomato

INTRODUCTION

Bacterial pathogens from the species Pseudomonas syringae and the genera Ralstonia and Xanthomonas can infect many different crop species and inflict significant yield losses when environmental conditions favor disease. Xanthomonas and P. syringae tend to enter plant stem, leaf, or flower tissue through wounds or natural openings, such as stomata or hydathodes, whereas...
**Ralstonia** is soilborne, entering roots through wounds and natural openings before colonizing xylem tissue (Vasse et al., 1995; Gudesblat et al., 2009). Once inside the host these bacteria manipulate host metabolism and suppress plant immunity using multiple strategies, including effector proteins delivered by the type III secretion system (Kay and Bonas, 2009; Peeters et al., 2013; Xin et al., 2018). This enables the pathogens to multiply to high titers while the plant tissue is still alive and showing few or no visual symptoms. Once the bacteria reach high populations, they typically cause necrosis of infected leaf tissue or wilting and eventual death of the plant.

Effective control measures for bacterial pathogens are relatively limited, particularly once plants become infected (Davis et al., 2013). Soil fumigation can reduce *Ralstonia* populations in the soil but this is expensive, potentially hazardous to workers and the environment, and of limited efficacy (Yuliar et al., 2015). Copper sulfate and antibiotics such as streptomycin have been used to control *Xanthomonas* species and *P. syringae* but have adverse environmental impacts and many strains have evolved tolerance to these chemicals (Kennelly et al., 2007; Griffin et al., 2017). Applying chemicals that induce systemic acquired resistance, such as acibenzolar-S-methyl, can provide partial control but increases production cost and can depress crop yields when used repeatedly (de Pontes et al., 2016).

The most effective, economical, and safe way to control bacterial pathogens is to plant crop varieties that are immune to the target pathogen (Jones et al., 2014; Vincelli, 2016). Such immunity is often mediated by plant immune receptor genes. Plants have large families of cell surface and intracellular immune receptor proteins that surveil for the presence of invading pathogens (Zipfel, 2014; Jones et al., 2016). Effector proteins delivered by the bacterial type III secretion system are common elicitors of intracellular plant immune receptors encoded by nucleotide-binding domain and leucine-rich repeat containing (NLR) genes (Li et al., 2015; Jones et al., 2016; Kapos et al., 2019). While effector proteins contribute to virulence on a susceptible host, an immune response is activated in the plant if that plant has the cognate receptor to recognize the effector. NLR genes typically confer strong, dominant resistance to pathogens that deliver the cognate recognized effector protein (Tai et al., 1999; Jones and Dangl, 2006; Boller and He, 2009; Deslandes and Rivas, 2012; Li et al., 2015). Disease-resistant plants can be generated by identifying the appropriate plant immune receptor genes and transferring them into the target crop species (Dangl et al., 2013). We recently identified the *Nicotiana benthamiana* immune receptor gene named Recognition of XopQ 1 (Roq1, which appears to be restricted to the genus *Nicotiana* and contributes to resistance against *Xanthomonas* spp. and *P. syringae* (Schultink et al., 2017). The Roq1 protein is a Toll/Interleukin-1 Receptor (TIR) NLR immune receptor that mediates recognition of the *Xanthomonas* effector protein XopQ and the homologous effector HopQ1 from *P. syringae*. XopQ is present in most species and strains of *Xanthomonas* (Ryan et al., 2011) and HopQ1 is present in 62% (290 of 467) sequenced putative pathogenic *P. syringae* strains (Dillon et al., 2019). XopQ/HopQ1 has homology to nucleoside hydrolases and has been shown to enhance virulence on susceptible hosts (Ferrante and Scortichini, 2009; Li et al., 2013), possibly by altering cytokinin levels or interfering with the activity of host 14-3-3 proteins (Giska et al., 2013; Li et al., 2013; Hann et al., 2014; Teper et al., 2014). The conservation of XopQ/HopQ1 and their importance in virulence suggests that Roq1 has widespread potential to confer resistance to these pathogens in diverse crop species. Indeed, transient expression assays demonstrated that Roq1 can recognize XopQ/HopQ1 alleles from *Xanthomonas* and *P. syringae* pathogens of tomato, pepper, rice, citrus, cassava, brassica, and bean (Schultink et al., 2017). However, it was not known if Roq1 can confer disease resistance when expressed in a crop plant.

Tomato is one of the most important vegetable crops and is highly susceptible to several bacterial diseases. Bacterial spot, bacterial speck, and bacterial wilt of tomato are caused by *Xanthomonas* species, *P. syringae* pv. *tomato* and *Ralstonia*, respectively. These diseases are difficult to control, especially if the pathogens become established in a field and environmental conditions favor disease (Rivard et al., 2012; Potnis et al., 2015). Tomato breeding germplasm has only limited resistance against these diseases and in some cases linkage drag has complicated introgression of resistance genes from wild relatives (Sharma and Bhattachary, 2019). *Ralstonia* contains a homolog of XopQ/HopQ1 called RipB. Roq1 is able to mediate the perception of RipB (Staskawicz and Schultink, 2019), and silencing Roq1 in *N. benthamiana* resulted in severe wilting phenotypes upon *Ralstonia* infection (Nakano and Mukaihara, 2019). This suggests that expressing Roq1 in tomato could also confer resistance to bacterial wilt. Like XopQ/HopQ1 in *Xanthomonas* and *P. syringae*, RipB is highly conserved and is present in approximately 90% of sequenced *Ralstonia* isolates (Sabbagh et al., 2019). Here we present data showing that expression of Roq1 in tomato confers resistance against *Xanthomonas*, *Pseudomonas*, and *Ralstonia* upon recognition of the cognate pathogen effector.

**MATERIALS AND METHODS**

**Generation of Tomato Expressing Roq1**

The Roq1 coding sequence was amplified from *N. benthamiana* cDNA and cloned into the pORE E4 binary plasmid (Coutu et al., 2007). The expression of Roq1 was driven by the constitutive PENTCUP2 promoter, which was derived from tobacco and has been reported to drive expression in leaf, root, and stem tissue (Malik et al., 2002). *Agrobacterium tumefaciens* co-cultivation was used to transform Roq1 into the tomato variety Fla. 8000 at the University of Nebraska Plant Transformation Core Research Facility. Transformed plants were selected by resistance to kanamycin, confirmed by genotyping, and selfed to obtain homozygous lines.

**Bacterial Leaf Spot and Leaf Speck Disease Assays**

*Xanthomonas* cultures were grown in NYG broth (0.5% peptone, 0.3% yeast extract, 2% glycerol) with rifampycin (100 µg/mL) overnight at 30°C. *P. syringae* cultures were grown in KB broth (1% peptone, 0.15% K2HPO4, 1.5% glycerol, 5 mM...
MgSO₄, pH 7.0) with rifampicin (100 µg/mL) overnight at 28°C. Bacterial cultures were spun down at 5200 g, washed once with 10 mM MgCl₂, and then diluted to the appropriate infiltration density with 10 mM MgCl₂. Leaf tissue of tomato plants (approximately 4 weeks old) was infiltrated with bacterial solution using a needleless syringe. To quantify bacterial growth, leaf punches were homogenized in water, serially diluted and plated on NYG (for Xanthomonas spp.) or KB (for P. syringae) plates supplemented with 100 µg/mL rifampicin and 50 µg/mL cycloheximide to measure colony forming units. Three biological replicates were performed for each condition and the reported results are representative of at least three independent experiments. Xanthomonas perforans strain 4B, Xanthomonas euvesicatoria strain 85-10, and P. syringae strain DC3000 and the corresponding XopQ/HopQ1 deletion mutants and complemented strains were described previously (Wei et al., 2007; Schwartz et al., 2015; Schultink et al., 2017). The P. syringae pv. tomato Race 1 strain was isolated from a field of tomatoes with the PTO resistance gene in 1993 in California.

**Transient Expression of RipB and XopQ**

Alleles of RipB from Ralstonia strains GMI1000 and MolK2 (NCBI Genbank accessions CAD13773.2 and WP_003278485) were synthesized and cloned into a BsaI-compatible version of the pORE E4 vector (Coutu et al., 2007). This plasmid was transformed into A. tumefaciens strain C58C1. A. tumefaciens cultures were grown on a shaker overnight at 30°C in LB broth with rifampicin (100 µg/mL), tetracycline (10 µg/mL), and kanamycin (50 µg/mL). The cells were collected by centrifugation and resuspended in infiltration buffer [10 mM 2-(N-morpholino)ethanesulfonic acid, 10 mM MgCl₂, pH 5.6], and diluted to an OD₆₀₀ of 0.5 for infiltration into Nicotiana tabacum leaf tissue. Each experiment was performed on multiple leaves and multiple plants with the selected images being representative of the observed result.

*N. tabacum roq1* Mutant Lines

Nicotiana tabacum roq1 mutant lines were generated by transforming *N. tabacum* with a construct coding for CAS9 and a guide RNA targeting the *Roq1* gene with the sequence GATGATAAGGAGTTAAAGAG. This construct was also used for the generation of *N. bethamiana roq1* mutants published in Qi et al. (2018). Transformed *N. tabacum* plants were generated by Agrobacterium co-cultivation and selected for using kanamycin. Transformed plants were genotyped for the presence of mutations at the target site by PCR and Sanger sequencing (Supplementary Table S1).

**Bacterial Wilt Virulence Assays**

*Ralstonia* virulence on tomato was measured as previously described (Kokhkhani et al., 2018). Briefly, cells of *Ralstonia* strains GMI1000 and UW551 grown overnight in CPG (0.1% casein hydrolysate, 1% peptone, 0.5% glucose, pH 7.0) at 28°C were collected by centrifugation and diluted to an OD₆₀₀ of 0.1 in water (1 × 10⁶ CFU/mL). 50 mL of this suspension was poured on the soil around 17-day old tomato plants. Disease was rated daily for two weeks on a 0–4 disease index scale, where 0 is no leaves wilted, 1 is 1–25% wilted, 2 is 26–50% wilted, 3 is 51–75% wilted, and 4 is 76–100% wilted. Data represent a total of four biological replicates with 10 plants per replicate. Virulence data were analyzed using repeated measures ANOVA (Kokhkhani et al., 2018). For petiole infection, the petiole of the first true leaf was cut with a razor blade horizontally approximately 1 cm from the stem. A drop of bacterial solution (2 µL; OD₆₀₀ = 0.001) was pipetted onto the exposed cut petiole surface.

**Field Trial Disease Assays**

Three field trials were conducted at the University of Florida Gulf Coast Research and Education Center in Balm during the spring seasons of 2018 and 2019 and the fall season of 2018 and under the notification process of the United States Department of Agriculture. Large-fruited, fresh market tomato lines were used in these trials and included the inbred line, Fla. 8000, and nearly isogenic lines containing either *Roq1* (event 316.4) or *Bs2* (Kunwar et al., 2018). The *Roq1* tomato line selected for the field trial was the same line used in the experiments shown in Figures 1, 2, 5. For each trial, seeds were sown directly into peat-lite soilless media (Speedling, Sun City, FL, United States) in 128-cell trays (38 cm³ cell size). Transplants were grown in a greenhouse until 5 or 6 weeks, then planted to field beds that had been fumigated and covered with reflective plastic mulch. Field trials were conducted using a randomized complete block design with four blocks and 10-plant plots. Field plants were staked and tied, and irrigation was applied through drip tape beneath the plastic mulch of each bed. A recommended fertilizer and pesticide program were followed throughout the growing season, excluding the use of plant defense inducers, copper, or other bactericides (Freeman et al., 2018). Fruits were harvested from the inner eight plants of each plot at the breaker stage and beyond and graded for marketability according to USDA specifications with block considered a random effect. Field trials were inoculated with *X. perforans* race T4 (strain mixture of GEV904, GEV917, GEV1001, and GEV1063). Bacterial strains were grown on nutrient agar medium (BBL, Becton Dickinson and Co., Cockeysville, MD, United States) and incubated at 28°C for 24 h. Bacterial cells were removed from the plates and suspended in a 10 mM MgSO₄ solution, and the suspension was adjusted to OD₆₀₀ = 0.3, which corresponds to 10⁸ CFU/mL. The suspension for each strain was then diluted to 10⁶ CFU/mL, mixed in equal volume, and applied along with polyoxyethylene sorbitan monolaurate (Tween 20; 0.05% [vol/vol]) for field inoculation. Field trial plants were inoculated approximately 3 weeks after transplanting.

Bacterial spot disease severity was recorded three to eight weeks after inoculation using the Horsfall-Barratt scale (Horsfall and Barrat, 1945), and ratings were converted to midpoint percentages for statistical analysis. Blocks were considered random effects.

**Generation of the *Ralstonia ΔripB* Mutant**

An unmarked ΔripB mutant was created using sacB selection with the vector pUFR80 (Castañeda et al., 2005). Briefly, the
Phylogenetic Analysis of XopQ, HopQ1, and RipB Alleles

RipB alleles were identified by BLAST search of the NCBI protein database. Clustal Omega (Sievers et al., 2011) was used to generate a multiple sequence alignment with XopQ and HopQ1 alleles. To span the diversity of RipB alleles without having many redundant sequences, only a single sequence was retained if there were multiple identical or nearly identical sequences identified. A maximum likelihood tree was generated using PhyML (Guindon et al., 2010). The phylotype calls of the strains were obtained from previously published worked (Liu et al., 2009; Mukaihara and Tamura, 2009; Safni et al., 2014).

RESULTS

Tomatoes Expressing Roq1 Are Resistant to Xanthomonas and P. syringae

We generated homozygous tomato plants expressing the Roq1 gene from N. benthamiana and tested them for resistance to Xanthomonas and P. syringae by measuring bacterial growth in planta. Population sizes of wild-type X. perforans strain 4B and X. euvesicatoria strain 85-10 were approximately 100-fold smaller in tomato expressing Roq1 compared to wild-type tomato at 6 days post inoculation (Figure 1). In contrast, XopQ deletion mutants multiplied equally well in leaves of both wild-type and Roq1 tomato. Disease symptoms begin as small water-soaked lesions and progress to necrosis of infected tissue. Wild-type X. perforans and X. euvesicatoria caused severe disease symptoms on wild-type tomato plants but failed to cause visible symptoms on Roq1 plants (Figure 2). The XopQ mutants caused similar disease symptoms on both wild-type and Roq1 tomato. Similar results were observed for P. syringae DC3000, and its HopQ1 mutant (Figures 1, 2) and a Race 1 isolate of P. syringae pv. tomato (Supplementary Figure S1). Tomatoes expressing Roq1 were resistant to Xanthomonas and Pseudomonas XopQ/HopQ1 mutants complemented with a wild-type copy of XopQ/HopQ1 (Supplementary Figure S2). A second tomato line expressing Roq1, derived from an independent transformation event, also showed resistance to wild-type X. euvesicatoria and X. perforans but not to the XopQ deletion strains (Supplementary Figure S3).

regions upstream and downstream of ripB were amplified using the primers ripBupF/R and ripBdwnF/R (Supplementary Table S1). These fragments were inserted into pUFR80 digested with HindIII and EcoRI using Gibson Assembly (Gibson et al., 2009) (New England Biolabs, Ipswitch, MA, United States) and this construct was incorporated into the genome of strain GMI1000 using natural transformation, with successful integrants selected on CPG + kanamycin (Coupat et al., 2008). Plasmid loss was then selected for on CPG plates containing 5% w/v sucrose. Correct deletions were confirmed using PCR and sequencing.
Expression of Roq1 Confers Resistance to Xanthomonas perforans in the Field

To determine if the resistance observed in growth chamber experiments would hold up under commercial tomato production conditions, we tested the ability of Roq1 tomatoes to resist X. perforans infection in the field. Roq1 tomatoes were grown along with the Fla. 8000 wild-type parent as well as a Fla. 8000 variety expressing the Bs2 gene from pepper as a resistant control (Kunwar et al., 2018). For each of the three growing seasons, both Roq1 and the resistant Bs2 control tomatoes showed significantly lower disease severity than the parental Fla. 8000 variety (Table 1) (p < 0.05). The total marketable yield of the Roq1 plants was not significantly different from that of the susceptible parent for any of the three seasons (p > 0.05). No obvious difference in growth morphology was observed between Roq1 and wild-type tomato plants (Supplementary Figure S4).

The Ralstonia Effector RipB, a Homolog of XopQ/HopQ1, Is Recognized by Roq1

RipB, considered a "core" Ralstonia effector, is present in approximately 90% of sequenced strains (Sabbagh et al., 2019) making it an attractive target ligand for engineering crop plants to be resistant to this pathogen. Roq1 perceives diverse alleles of XopQ and HopQ1 and we hypothesized that it can also recognize different alleles of RipB. We constructed a phylogenetic tree using a subset of RipB alleles identified by BLAST search to approximately span the diversity of the four phylotypes based on sequence analysis of the internal transcribed spacer region of the 16S–23S rRNA gene (Poussier et al., 2000; Prior and Fegan, 2004; Safni et al., 2014). We selected RipB alleles from Ralstonia strains GMI1000 and MolK2, from phylotypes I and II, respectively, for subsequent analysis. These two RipB alleles share 71% amino acid identity with each other and approximately 52% identity with XopQ excluding the divergent N terminus containing the putative type III secretion signal. An alignment of these two RipB proteins with XopQ and HopQ1 is shown in Supplementary Figure S5. To test for Roq1-dependent recognition of RipB, we used Agrobacterium to transiently express RipB from GMI1000 and Molk2 in leaf tissue of wild-type and roq1 mutant N. tabacum. The N. tabacum roq1-1 mutant was generated using a CRISPR/CAS9 construct targeting exon 1 of the Roq1 gene (Supplementary Figure S6). Both RipB alleles

**TABLE 1 | Field trial results.**

<table>
<thead>
<tr>
<th>Season/Genotype</th>
<th>Disease severity</th>
<th>Marketable yield (kg/ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Spring 2018</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fla. 8000</td>
<td>86 ± 5</td>
<td>54,655 ± 9,450</td>
</tr>
<tr>
<td>Fla. 8000 Roq1</td>
<td>1 ± 1</td>
<td>52,656 ± 3,810</td>
</tr>
<tr>
<td>Fla. 8000 Bs2</td>
<td>1 ± 1</td>
<td>66,270 ± 10,309</td>
</tr>
<tr>
<td><strong>Fall 2018</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fla. 8000</td>
<td>25 ± 7</td>
<td>19,576 ± 11,038</td>
</tr>
<tr>
<td>Fla. 8000 Roq1</td>
<td>5 ± 1</td>
<td>18,538 ± 5,901</td>
</tr>
<tr>
<td>Fla. 8000 Bs2</td>
<td>0 ± 0</td>
<td>33,770 ± 13,176</td>
</tr>
<tr>
<td><strong>Spring 2019</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fla. 8000</td>
<td>84 ± 2</td>
<td>73,009 ± 15,243</td>
</tr>
<tr>
<td>Fla. 8000 Roq1</td>
<td>11 ± 7</td>
<td>92,837 ± 11,072</td>
</tr>
<tr>
<td>Fla. 8000 Bs2</td>
<td>5 ± 1</td>
<td>80,516 ± 14,531</td>
</tr>
</tbody>
</table>

A field trial was conducted in Florida with disease pressure from Xanthomonas perforans. Disease severity presented as percent infected tissue, converted from field ratings that were scored using the Horsfall–Barratt scale. Harvested tomatoes were graded and sized by USDA specifications to calculate the total marketable yield. The values shown are means ± standard deviation from at least four replicate plots of 10 plants each. Tomato plants expressing the Bs2 immune receptor gene were included as a resistant control.
triggered a strong hypersensitive/cell death response in wild-type *N. tabacum*, indicating immune activation. This response was absent in the *roq1-1* mutant but could be restored by transiently expressing Roq1 along with XopQ, RipB<sub>GMI1000</sub>, or RipB<sub>MolK2</sub> (Figure 4).

**Roq1** Tomatoes Are Resistant to *Ralstonia* Containing RipB

Our observation that Roq1 can recognize RipB in leaf transient expression assays suggested that Roq1 can mediate resistance to bacterial wilt caused by *Ralstonia* in tomato. We tested this hypothesis by challenging wild-type and Roq1-expressing tomato plants with *Ralstonia* strain GMI1000 using a soil soak inoculation disease assay. Wild-type plants developed severe wilting approximately 7 days after inoculation, whereas Roq1 tomato plants remained mostly healthy over the 2-week time course (Figure 5A and Supplementary Figure S7). The *Roq1* tomato plants were susceptible to a deletion mutant lacking RipB (GMI1000 ΔripB). We also challenged plants by introducing bacteria directly to the xylem by placing bacteria on the surface of a cut petiole. Wild-type plants were wilted by eight days whereas *Roq1* plants remained healthy (Figure 5B). Tomatoes expressing *Roq1* were also resistant to *Ralstonia* strain UW551, which is a race 3 biovar 2 potato brown rot strain from phylotype II that has a RipB allele (Figure 3 and Supplementary Figure S8).

**Distribution of RipB Alleles in Ralstonia**

To investigate the potential for using *Roq1* to protect plants from *Ralstonia*, we investigated the occurrence of RipB alleles in select strains. Table 2 lists some *Ralstonia* strains with their known hosts along with their respective phylotype and RipB allele accession information. Table 2 illustrates that strains lacking putative functional RipB alleles correlate with strains that are virulent on tobacco, which contains a native Roq1 gene. All strains in Table 2 except for tomato pathogenic strains K60, Y45, BK1002, and OE1-1 contain putative full-length and functional RipB alleles. Relative to other RipB alleles, the K60 RipB allele is truncated after residue 437 and missing approximately 65 C-terminal residues and the OE1-1 allele is truncated after residue 417, missing approximately 77 residues based on a published genome sequence (Hayes et al., 2017) (Supplementary Figure S5). Y45 does not have a predicted RipB allele based on a draft genome sequence (Li et al., 2011). Published gene models for RipB disagree on which start codon is the correct one, leading some RipB alleles to look like they are missing part of the N terminus or have N-terminal extensions. Analysis of the DNA sequence of diverse RipB alleles showed that out of three possible in-frame start codons, only a single putative start codon is conserved among *Ralstonia* strains from all four phylotypes.
DISCUSSION

Roq1 expression in tomato confers strong resistance to X. perforans, X. euvesicatoria, and P. syringae pv. tomato. Its effectiveness is dependent on the presence of the recognized effector protein XopQ/HopQ1 (Figures 1, 2). Field trials revealed that tomatoes expressing Roq1 were less susceptible to X. perforans than wild-type tomatoes in conditions approximating commercial production (Table 1). Roq1 conferred a similar level of resistance as the Bs2-containing resistant check variety in one season and was slightly weaker in the other two. Bacterial spot caused by X. perforans can cause lesions on fruits, making them unsuitable for commercial sale, and also reduce plant productivity by damaging leaf tissue. The onset of fruit lesions requires high disease pressure during a particular phase of fruit development. Environmental conditions did not favor the development of fruit lesions and we did not observe significant fruit lesion formation on any of the genotypes in any of the three seasons. Despite showing a strong reduction in foliar disease symptoms, the Roq1 line did not have a significantly greater yield than the susceptible parental variety. A possible explanation for this finding is that bacterial spot did not appear to be a major constraint on yield in any of the three seasons. In spring 2018, weather conditions promoted the development of disease only late in the season after much of the yield was already set. Fall 2018 was unseasonably hot and dry for most of the season resulting in low disease pressure and very poor yield for all genotypes. Of the three seasons, spring 2019 had weather conditions expected to be most conducive for observing an impact of bacterial spot on marketable yield with mid-season rain promoting the development of disease symptoms. The average marketable yield for the Roq1 tomatoes was 27% higher than wild-type in this season, although a relatively small sample size (four replicate plots of 10 plants each) and a large variability of yield between plots resulted in a p-value of 0.08 by Student’s t-test. Notably the yield of the resistant check variety expressing Bs2 was not significantly higher than the susceptible control in this season, though it was previously reported to give a yield increase of 1.5–11x relative to susceptible varieties under high disease pressure (Horvath et al., 2012). This suggests that bacterial spot was not severe enough to have a strong impact on yield in this season and that Roq1 may result in an increase in marketable yield under stronger disease pressure.

It was unclear if Roq1 could confer resistance to Ralstonia because it colonizes different tissues than Xanthomonas and P. syringae. While Xanthomonas and P. syringae colonize tomato leaf tissue, Ralstonia enters through the roots and colonizes xylem vessels. Although the type III secretion system is essential for virulence in Ralstonia, it is not clear when and where the pathogen delivers effectors into host cells. It was therefore not clear if Roq1 would be able to confer resistance to this pathogen in tomato. Here we demonstrated that tomato plants expressing Roq1 had strong resistance to Ralstonia as measured by both soil soak and cut-petiole inoculation assays (Figure 5). In addition to conferring resistance to the phytophyle type I strain GMI1000, Roq1 also confers resistance to Ralstonia race 3 biovar 3 strain UW551, a phytophyle type II strain that can overcome other known sources of bacterial wilt resistance in tomato (Milling et al., 2011). Some but not all of the Roq1 tomatoes inoculated with GMI1000 by soil soak were colonized by a moderate or low population of Ralstonia (Supplementary Figure S10). This observation suggests that Roq1-mediated immune responses may act to both restrict the establishment of vascular colonization and separately reduce bacterial titers if colonization does occur. Activation of immune receptors, including Roq1, is known to induce many defense-associated genes with different putative activities (Sohn et al., 2014; Qi...
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et al., 2018), presumably acting to inhibit pathogen virulence by distinct mechanisms. The observation that Roq1 inhibits both colonization establishment and population growth suggests that at least two independent downstream defense responses mediate the observed resistance phenotype.

The Roq1 tomatoes were susceptible to a Ralstonia mutant lacking RipB, indicating that the resistance depends on the interaction between RipB and Roq1. This is consistent with the observation that several naturally occurring Ralstonia strains that can infect tobacco have a truncated or are missing the RipB effector (Table 2) (Nakano and Mukaihara, 2019), suggesting that losing RipB can allow the pathogen to overcome the native Roq1 gene present in N. tabacum. Tobacco-infecting strains K60 and OE1-1 contain independently truncated RipB alleles (Figure 3 and Supplementary Figure S5) and there have likely been multiple independent gene loss events which enable strains to evade Roq1-mediated resistance. Similarly, HopQ1 has been lost in strains of P. syringae that can infect tobacco (Denny, 2006; Ferrante and Scortichini, 2009; Li et al., 2011). This suggests that this effector is not essential for virulence in all circumstances and it would therefore be prudent to deploy Roq1 in combination with other disease resistance traits to avoid resistance breakdown due to pathogens losing XopQ/HopQ1/RipB. Although minor foliar symptoms were observed on the Roq1 tomatoes, particularly in spring 2019 (Table 1), we do not believe this was due to a naturally occurring XopQ mutant arising during the trial. Instead, we think that these low disease scores may have been the result of fungal diseases, which can cause foliar symptoms that look similar to bacterial spot, or by the X. campestris races that cause disease in crop plants including kiwi (P. syringae pv. actinidiae), banana (Ralstonia and X. campestris pv. musacearum), stone fruit (P. syringae), pepper (X. eurivestitoria), citrus (X. citri), strawberry (X. fragariae), brassica (X. campestris), rice (X. oryzae), potato (Ralstonia), and others. Ralstonia race 3 biovar 2 strains are of particular concern because they are cold tolerant and potentially threaten potato cultivation in temperate climates. As a result, Ralstonia race 3 biovar 2 strains are strictly regulated quarantine pathogens in Europe and North America and is on the United States Select Agent list. The ability of Roq1 to protect tomato from the race 3 biovar 2 strain UW551 (Supplementary Figure S8) suggests that Roq1 can also protect potato from this high-concern pathogen. This work demonstrates the widespread potential of using naturally occurring plant immune receptors to safely, sustainably, and economically manage diverse and difficult to control pathogen species.

### DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/Supplementary Material.

### AUTHOR CONTRIBUTIONS

NT and AS wrote the manuscript and performed Pseudomonas and Ralstonia petiole infection assays. AS carried out...
Xanthomonas infection and Agrobacterium transient expression experiments. UG and SH performed Xanthomonas field experiments. CH constructed the Ralstonia knockout and performed Ralstonia soil soak assays, supervised by CA. All authors analyzed the results and edited and approved the manuscript.

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SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00463/full#supplementary-material

REFERENCES


Conflict of Interest: AS and NT are employees of and have a financial stake in Fortiphyte Inc., which has intellectual property rights related to the Roq1 resistance gene.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Figure S1. Growth of *Pseudomonas syringae pv. tomato* Race 1 in wild-type and Roq1 tomatoes. *Pseudomonas syringae pv. tomato* Race 1 was infiltrated into wild-type and Roq1 tomatoes. At four days post infiltration disease symptoms were imaged (top) and colony forming units were determined by dilution plating of homogenized tissue. Error bars indicate standard deviation. ** = p < 0.01 by Student’s t-test.
**Supplementary Figure S2. Disease assay of complemented HopQ1/XopQ mutants.** The indicated genotypes of *Pseudomonas syringae* strain DC3000 (*Ps*) and *Xanthomonas euvesicatoria* strain 85-10 (*Xe*) were infiltrated into leaf tissue of wild-type tomato and tomato expressing *Roq1* at a low density (OD$_{600} = 0.00005$ and 0.0001 respectively). Punches of leaf tissue were homogenized and plated to determine colony forming units (CFU) at four days post infiltration for *Pseudomonas syringae* and six days post infiltration for *Xanthomonas euvesicatoria*. The error bars show the standard deviation from three biological replicates per condition. The visual disease symptoms for *Pseudomonas syringae* were imaged at four days post infiltration.
Supplementary Figure S3. Resistance phenotype of independent tomato lines expressing Roq1. Wild-type and XopQ deletion mutants of Xanthomonas euvesicatoria 85-10 and Xanthomonas perforans 4B were infiltrated at a low inoculum (OD$_{600}$ = 0.0001) into leaf tissue of wild-type tomatoes and two independent tomato lines expressing Roq1. Six days post infiltration, the leaf tissue was homogenized and plated to quantify bacterial abundance by colony forming units (CFU). Error bars indicate standard deviation from six replicates for each condition. ** indicates $p$-value < 0.01 in comparison to the wild-type by Student’s t-test. Roq1 line #2 was selected for further characterization and use in field trials.
Supplementary Figure S4. Growth phenotype of Roq1 tomato plants under no and low disease pressure. Images of 19-day old and 35-day old growth chamber grown, and field grown wild-type Fla. 8000 and Roq1 tomatoes show no obvious stunting, necrosis, or other growth defects in the Roq1 tomatoes. Although some foliar disease symptoms are visible for the field grown wild-type tomatoes here, the plants were under low disease pressure at this stage due to the lack of warm and rainy conditions favoring disease.
### Supplementary Figure S5. Protein alignment of XopQ, HopQ1 and RipB

Protein sequences from *Xanthomonas euvesicatoria* 85-10 (XopQ), *Pseudomonas syringae* DC3000 (HopQ1), and the *Ralstonia* strains GMI1000, MolK2, K60, and BK1002 (RipB) were used to generate the alignment using ClustalO. The putative full length BK1002 RipB sequence based on analysis shown in Supplementary Figure S9 was used for this alignment and differs from the NCBI accession BBI29704.1. The boxed region indicates a motif that is conserved in XopQ, HopQ1 and the GMI1000 and MolK2 alleles of RipB but absent in the putatively truncated K60, BK1002 and OE1-1 alleles.
Supplementary Figure S6. Sequence of roq1 N. tabacum mutants. A CRISPR / CAS9 construct was transformed into N. tabacum with a guide targeting the first exon of the Roq1 gene (A). Transformed plants were selected by resistance to kanamycin and then genotyped by PCR and Sanger sequencing to look for the presence of mutations at the target site. A mutant containing a single base pair A insertion at the predicted cut site was identified and named Nt roq1-1 (B). The target sequence of this guide is conserved between N. tabacum and N. benthamiana and was also used for the generation of N. benthamiana roq1 mutants published in Qi et al. 2018.
Supplementary Figure S7. *Ralstonia* disease symptoms from soil soak inoculation. This image shows representative plants from the *Ralstonia* disease assay depicted in Figure S 5A. The plants were infected with wild-type *Ralstonia* strain GMI1000 by soil soak with a 50 mL solution containing 1x10^8 colony forming unit / mL. The plants were imaged 8 days post inoculation at an age of 25 days. The wild-type tomato plants exhibited severe wilting (left) whereas the *Roq1* tomatoes showed no or minor disease symptoms (right).
Supplementary Figure S8. Disease assay with *Ralstonia solanacearum race 3 biovar 2*. Wild-type tomato plants (cv. Fla. 8000) and tomato plants expressing *Roq1* were soil-soak inoculated with *R. solanacearum* race 3 biovar 2 strain UW551. Disease symptoms were scored over the course of two weeks, with a Disease Index of 0 corresponding to no symptoms and a Disease Index of 4 corresponding to complete wilting. Error bars indicate standard error from three biological replicates.
Supplementary Figure S9. Putative start codon annotation of RipB. The N-terminal amino acids and codons of GMI1000 RipB were aligned to the DNA sequence of other RipB alleles. In-frame start codons are boxed, with the green box indicating the start codon that is conserved across RipB accessions from all four *Ralstonia* phylotypes. The start codons boxed in orange have been annotated for some RipB accessions but are not conserved across all the phylotypes and may therefore be incorrect. Nucleotide polymorphisms relative to GMI1000 are shown in bold, with synonymous changes in green and non-synonymous changes in red. Notably the phylotype IV strains R24, PSI07 and BDB A2-HR MARDI (for which only six codons are shown) are all lacking the downstream start codon.
Supplementary Figure S10. Colonization of Roq1 tomato plants following inoculation with *Ralstonia*. Tomato plants expressing *Roq1* were infected with *Ralstonia* strain GMI1000 using the soil soak method. After two weeks all wild-type tomato plants had wilted but nearly all the *Roq1* tomato plants appeared healthy with no or minimal disease symptoms (Figure S 5A). Bacterial colonization in the *Roq1* plants were measured by homogenizing and dilution plating mid-stem sections to determine colony forming units per gram stem tissue. Out of 19 plants tested from three independent biological replicates, twelve had no detectable colonization (limit of detection = 100 colony forming units / gram stem tissue), five had low colonization (<1x10⁵) and two had moderate colonization (~2x10⁸). The wild-type tomatoes were dead and unable to be assayed at this timepoint, but susceptible tomato plants typically reach colonization densities of 10⁹ or 10¹⁰ CFU / g stem tissue (Lowe-Power et al., 2018; Zhang et al., 2018; Zhang et al., 2019). All the *Roq1* tomato plants in this assay had a Disease Index of 0 except for one plant which had a Disease Index of 3 and a colonization of 2.2 x 10⁸.
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**Supplementary Table S1.** Sequence of oligonucleotide primers used in this research, listed in 5’ to 3’ orientation. Note that AS-941 was designed for genotyping the *Nicotiana benthamiana* allele and has a 1 bp mismatch relative to *Nicotiana tabacum* but is still functional.