Ralstonia solanacearum Type III Effector RipJ Triggers Bacterial Wilt Resistance in *Solanum pimpinellifolium*

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Ralstonia solanacearum causes bacterial wilt disease in solanaceous crops. Identification of avirulence type III-secreted effectors recognized by specific disease resistance proteins in host plant species is an important step toward developing durable resistance in crops. In the present study, we show that *R. solanacearum* effector RipJ functions as an avirulence determinant in *Solanum pimpinellifolium* LA2093. In all, 10 candidate avirulence effectors were shortlisted based on the effector repertoire comparison between avirulent Pe_9 and virulent Pe_1 strains. Infection assays with transgenic strain Pe_1 individually carrying a candidate avirulence effector from Pe_9 revealed that only RipJ elicits strong bacterial wilt resistance in *S. pimpinellifolium* LA2093. Furthermore, we identified that several RipJ natural variants do not induce bacterial wilt resistance in *S. pimpinellifolium* LA2093. RipJ

belongs to the YopJ family of acetyltransferases. Our sequence analysis indicated the presence of partially conserved putative catalytic residues. Interestingly, the conserved amino acid residues in the acetyltransferase catalytic triad are not required for effector-triggered immunity. In addition, we show that RipJ does not autoacetylate its lysine residues. Our study reports the identification of the first *R. solanacearum* avirulence protein that triggers bacterial wilt resistance in tomato. We expect that our discovery of RipJ as an avirulence protein will accelerate the development of bacterial wilt-resistant tomato varieties in the future.

Keywords: avirulence, bacterial wilt resistance, plant immunity, plant-pathogenic bacteria, *Ralstonia solanacearum*, *Solanum pimpinellifolium*

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Owing to their sessile nature, plants are ubiquitous hosts for a diverse range of phytopathogens. In order to protect themselves, plants have evolved two main layers of immunity comprising receptors localized at either the cell surface or various intracellular compartments (Dodds and Rathjen 2010; Dou and Zhou 2012). The first layer of this surveillance system is pattern-triggered immunity (PTI), which is activated by the recognition of microbe-associated molecular patterns by pattern-recognition receptors (Boller and Felix 2009; Jones and Dangl 2006). In order to circumvent PTI and promote virulence, many bacterial pathogens deploy effector molecules into host cells via a type III secretion system (Alfano and Collmer 2004). On the other hand, plants have evolved a second layer of immunity consisting of disease resistance (R) proteins, including nucleotide-binding and leucine-rich repeat (LRR) receptor (NLR) proteins and their associated guarders or decoys (Jones and Dangl 2006). NLR proteins directly or indirectly recognize type III secreted effectors (T3SEs) and activate effector-triggered immunity (ETI)

(Huang et al. 2019). The T3SEs that are perceived by R proteins and trigger immunity are referred to as avirulence effectors.

Ralstonia solanacearum is a bacterial phytopathogen that causes bacterial wilt disease in many plant species, especially Solanaceae crops such as tomato, potato, pepper, and eggplant, mainly in tropical and subtropical regions (Hayward 1991; Mansfield et al. 2012; Yabuuchi et al. 1995). Due to exceptionally broad host range and high genetic diversity, the *R. solanacearum* species complex (RSSC) has been divided into four phylotypes that reflect geographical origin: phylotype I (Asia), phylotypes IIa and IIb (Americas), phylotype III (Africa), and phylotype IV (Australia and Indonesia) (Fegan and Prior 2005; Wicker et al. 2012).

R. solanacearum generally possesses over 70 T3SEs, or “*Ralstonia*-injected proteins” (Rips). Several *R. solanacearum* T3SEs have been studied in the past for their roles in bacterial virulence during infection of host plants. For example, RipG, belonging to the multigenic GALA effector family, has been shown to interfere with ubiquitin signaling to suppress PTI (Angot et al. 2006; Remigi et al. 2011; Wang et al. 2016). RipAY is known to inhibit PTI by interfering with glutathione production (Fujiwara et al. 2016; Mukaihara et al. 2016; Wei et al. 2017). Several Rips have also been studied for their propensity to confer avirulence. RipP2 (formerly known as PopP2), belonging to the *Yersinia* outer protein J (YopJ) family of acetyltransferases (Tasset et al. 2010), acetylates the WRKY domain of the NLR protein resistance to *R. solanacearum* 1 (RRS1) and activates immunity in *Arabidopsis* (Deslandes et al. 2002; Le Roux et al. 2015; Sarris et al. 2015; Williams et al. 2014). RipP1 (formerly PopP1) is another acetyltransferase effector from the YopJ family that induces immunity in species of *Petunia* (Lavie et al. 2002; Poueymiro et al. 2009). RipB was shown to act as an avirulence effector in *Nicotiana benthamiana* (Nakano and Mukaihara 2019). Recently, the *N. benthamiana* NLR recognition of XopQ 1 (Roq1) was shown to recognize RipB and confers bacterial wilt resistance when expressed in tomato (Schultink et al. 2017; Thomas et al. 2020). RipAX2, a zinc-dependent protease, is another avirulence effector potentially recognized by a protein encoded in the *EBWR9* (Eggplant bacterial wilt resistance 9) quantitative trait locus (QTL) in eggplant AG91-25 (Nahar et al. 2014; Morel et al. 2018a). Another *Ralstonia* effector, RipAZ1, has been recently identified to trigger bacterial wilt resistance in *Solanum americanum* accession SP2273 (Moon et al. 2021). These examples demonstrate the wide variety of contributions to virulence and avirulence of the large effector repertoire of *R. solanacearum* and hints at the future first step at resistance breeding through understanding complexities in effector–host dynamics.

Wild plant species are considered excellent sources of *R* genes with novel recognition specificities (Henry 2019; Maxted and Kell 2009; Pimentel et al. 1997). Some sources of bacterial wilt resistance have been identified in various tomato species such as *S. lycopersicum* var. *cerasiforme*, *S. pimpinellifolium*, and cultivated tomato *S. lycopersicum* (Aslam et al. 2017; Yang and Francis 2006). *S. pimpinellifolium*, considered to be the closest wild relative of cultivated tomato (The 100 Tomato Genome Sequencing Consortium et al. 2014; The Tomato Genome Consortium et al. 2012), harbors economically important traits, including fruit quality (Chen and Foolad 1999; Foolad 2007), abiotic stress tolerance, and disease resistance (Lin et al. 2010; Razali et al. 2018; Villalta et al. 2008). The well-described R protein Pto, which confers resistance to bacterial speck disease caused by *Pseudomonas syringae* in tomato, has been isolated from *S. pimpinellifolium* (Pedley and Martin 2003; Thapa et al. 2015). Owing to these characteristics, *S. pimpinellifolium* has historically been a part of various breeding programs in tomato. One such effort to exploit the genetic variation between

S. pimpinellifolium and *S. lycopersicum* was made by developing a recombinant inbred line (RIL) population from a cross between *S. lycopersicum* breeding line NCEBR-1 (LA4504) and *S. pimpinellifolium* accession LA2093 (Ashrafi et al. 2009). However, despite the amenability of genetic studies in *S. pimpinellifolium*, no comprehensive investigation of bacterial wilt resistance has been carried out with this species thus far.

In this work, we show that *S. pimpinellifolium* accession LA2093 is resistant to several *R. solanacearum* phylotype I strains. We discovered that a type III effector RipJ from avirulent strains induces bacterial wilt resistance in LA2093. In addition, based on sequence and functional analyses, we demonstrate that some RipJ natural variants have lost their avirulence. Finally, we show that the predicted catalytic residues conserved among the YopJ family of acetyltransferases are not essential for RipJ avirulence in *S. pimpinellifolium* LA2093.

RESULTS

S. pimpinellifolium accession LA2093 shows bacterial wilt resistance.

To identify avirulent strains, we tested virulence of the previously reported phylotype I strains Pe_1, Pe_9, Pe_18, and Pe_39 in *S. pimpinellifolium* LA2093 and *S. lycopersicum* LA4504 (Prokhorchik et al. 2020; Segonzac et al. 2017). *R. solanacearum* strains Pe_1 and Pe_39 caused severe wilt symptom in both *Solanum* spp. (Fig. 1A). Interestingly, strains Pe_9 and Pe_18 showed significantly reduced virulence in *S. pimpinellifolium* LA2093 as compared with *S. lycopersicum* LA4504 (Fig. 1A). Pe_1 and Pe_9 were chosen for further analysis based on the reproducibility of disease assay results. Next, in order to quantify *S. pimpinellifolium* LA2093-specific bacterial wilt resistance to the Pe_9 strain, we monitored the survival rate of infected plants using the Kaplan-Meier survival curves (Fig. 1B). Consistent with disease assay results (Fig. 1A), only the Pe_9 strain-infected *S. pimpinellifolium* LA2093 showed a significantly high survival rate as compared with the other compatible interactions (Fig. 1B). These results suggest that *R. solanacearum* Pe_9 may harbor an avirulence effector that can trigger bacterial wilt resistance in *S. pimpinellifolium* LA2093. However, strain Pe_9 did not induce a clear hypersensitive response when infiltrated in *S. pimpinellifolium* LA2093 leaves (data not shown).

Recently, the effector repertoires of multiple *R. solanacearum* phylotype I strains, including Pe_1 and Pe_9, were reported (Prokhorchik et al. 2020). In order to shortlist candidate avirulence T3SEs, the effector repertoire of avirulent strain Pe_9 was compared with that of virulent strain Pe_1. The T3SEs RipA1, RipAH, RipAX2, and RipP1 were present in the avirulent strain Pe_9 and absent in the virulent strain Pe_1 (Table 1). In addition, RipJ, RipAF1, RipD, RipB, RipAX1, and RipBA, which showed less than 95% amino acid sequence identity between the two strains, were also selected for further investigation (Table 1).

RipJ confers *R. solanacearum* avirulence in *S. pimpinellifolium* LA2093.

To identify the effector that confers Pe_9 avirulence in LA2093, we introduced each of the avirulence candidate effectors, expressed under the *hrpB* promoter, into the genome of the virulent strain Pe_1 (Monteiro et al. 2012) (Table 1; Supplementary Fig. S1A; Supplementary Table S3). The genomic integration of effectors in the resulting *R. solanacearum* Pe_1 strain was confirmed using gene-specific PCR (Supplementary Fig. S1B). In disease assays, *S. lycopersicum* LA4504 was susceptible to all tested transgenic Pe_1 strains, indicating that the genomic integration of effectors did not alter the virulence of Pe_1 (Supplementary Fig. S2A and B). Conversely, *S. pimpinellifolium*

LA2093 developed significantly reduced wilt symptom after infection with Pe_1 carrying *ripJ*_{Pe_9}, whereas severe disease development was observed following infection with the other Pe_1 strain (Supplementary Fig. S2A). Consistently, Pe_1 carrying *ripJ*_{Pe_9} showed significantly higher survival rates than nine other effector candidates or the empty vector (EV) when infected in *S. pimpinellifolium* LA2093 (Supplementary Fig. S2B). To test whether recognition of RipJ is affected when expressed under its native promoter, *S. pimpinellifolium* LA2093 and *S. lycopersicum* LA4504 plants were infected with the virulent *R. solanacearum*

Fig. 1. *Ralstonia solanacearum* phylotype I strain Pe_9 is avirulent in *Solanum pimpinellifolium*. **A**, *S. pimpinellifolium* accession LA2093 shows bacterial wilt resistance. Four-week-old *S. pimpinellifolium* (Sp) LA2093 and *S. lycopersicum* (Sl) LA4504 plants were infected with *R. solanacearum* phylotype I strain Pe_1, Pe_9, Pe_18, and Pe_39 using a soil-drenching method. Representative photographs of infected plants were taken at 10 days postinfection (dpi). S and R represent susceptibility and resistance, respectively. White scale bars indicate 5 cm. **B**, *S. pimpinellifolium* LA2093 plants infected with *R. solanacearum* Pe_9 have a significantly higher survival rate compared with strain Pe_1. Surviving plants were counted for 10 dpi consecutively. The *P* value indicated in each graph (significant if *P* < 0.05) was calculated using Mantel-Cox log-rank test comparing curves.

strain Pe_1 carrying either *ripJ*_{Pe_9} whose expression was driven by its native promoter or pRCKgg EV as a control (Supplementary Table S1). As expected, the virulent strain Pe_1 carrying EV or *ripJ*_{Pe_9} caused strong bacterial wilt disease in *S. lycopersicum* LA4504 (Fig. 2A). Importantly, infection with Pe_1 expressing *ripJ*_{Pe_9} under its native promoter resulted in a strong resistance response in *S. pimpinellifolium* LA2093 (Fig. 2A; Supplementary Fig. S2A and B). Consistent with disease assays, Pe_1 carrying *ripJ*_{Pe_9} showed a significantly higher survival rate than Pe_1 carrying EV in *S. pimpinellifolium* LA2093 but not in *S. lycopersicum* LA4504 (Fig. 2B). To better characterize the resistance mediated by *ripJ*_{Pe_9}, in planta bacterial growth of Pe_1 carrying EV or *ripJ*_{Pe_9} was quantified from the stem of root-infected *S. pimpinellifolium* LA2093 and *S. lycopersicum* LA4504 plants at 5 and 10 days postinfection (dpi) (for detailed experimental procedure, see Materials and Methods). Consistent with our survival assay result (Fig. 2B), in planta bacterial growth was significantly restricted only when LA2093 was infected with Pe_1 carrying *ripJ*_{Pe_9} (Fig. 2C). Taken together, these results demonstrate that RipJ_{Pe_9} functions as an avirulence effector in *R. solanacearum* interactions with *S. pimpinellifolium* LA2093.

Some naturally occurring RipJ variants do not confer avirulence.

Often, natural sequence variation found in avirulence effectors causes loss of recognition (Armstrong et al. 2005; Bos et al. 2006; van Poppel et al. 2008). To investigate the natural variation of RipJ avirulence activity, we first compared the RipJ amino acid sequences from *R. solanacearum* GMI1000 and 30 phylotype I strains that were recently reported (Prokhorchik et al. 2020). Based on amino acid sequence analysis, six representative groups and their representative variants (RipJ_{Pe_9}, RipJ_{Pe_3}, RipJ_{Pe_1}, RipJ_{Pe_57}, RipJ_{To_28}, and RipJ_{GMI1000}) were selected for further analysis (Fig. 3A; Supplementary Tables S2 and S3). In comparison with RipJ_{Pe_9} (avirulent variant), RipJ_{GMI1000} and RipJ_{Pe_3} carried only four and one nonsynonymous single-nucleotide polymorphisms, respectively (Fig. 3A; Supplementary Table S2). On the other hand, RipJ_{Pe_1} (virulent variant) and RipJ_{Pe_57} showed greater polymorphism. In addition, RipJ_{To_28} carried 15 unique amino acids at the C terminus, leading to a stop codon that caused a 51-amino-acid C-terminal truncation compared with RipJ_{Pe_9} (Fig. 3A; Supplementary Table S2).

To test whether these naturally occurring RipJ variations affected avirulence, we introduced representative alleles of these RipJ natural variants expressing under RipJ_{Pe_9} native promoter

Table 1. List of candidate avirulence effectors that induce bacterial wilt resistance in *Solanum pimpinellifolium* LA2093^a

Pe_9 (avirulent)	Pe_1 (virulent) (%)
<i>RipA1</i>	—
<i>RipAH</i>	—
<i>RipAX2</i>	—
<i>RipP1</i>	—
<i>RipJ</i>	94.0
<i>RipAF1</i>	94.4
<i>RipD</i>	91.8
<i>RipB</i>	85.5
<i>RipAX1</i>	70.8
<i>RipBA</i>	64.1

^a Candidate avirulence effectors were selected by comparing the effector repertoires of *Ralstonia solanacearum* Pe_9 and Pe_1 strains. Effectors that are present in Pe_9 and absent or showing significant polymorphism (<95% amino acid sequence identity) in Pe_1 were selected and indicated in the table; — represents the absence of effectors. Indicated percentages represent amino acid sequence identity between the two effector variants from Pe_9 and Pe_1.

A

R. solanacearum Pe_1

B

C

Fig. 2. Introduction of RipJ_{Pe_9} in a virulent *Ralstonia solanacearum* Pe_1 strain confers avirulence in *Solanum pimpinellifolium* (Sp) LA2093. **A**, *Sp* LA2093 but not *S. lycopersicum* (Sl) LA4504 shows bacterial wilt resistance to *R. solanacearum* strain Pe_1 carrying *ripJ*_{Pe_9}. Four-week-old plants were infected with *R. solanacearum* Pe_1 carrying pRCKgg empty vector (EV) or *ripJ*_{Pe_9}. Representative photographs of infected plants were taken at 10 days postinfection (dpi). S and R represent susceptibility and resistance, respectively. White scale bars indicate 5 cm. **B**, *S. pimpinellifolium* LA2093 plants infected with strain Pe_1 carrying *ripJ*_{Pe_9} display enhanced survival rate. Surviving plants were counted for 10 dpi consecutively. The *P* value indicated in each graph (significant if *P* < 0.05) was calculated using a Mantel-Cox log-rank test comparing curves. **C**, In planta growth of *R. solanacearum* strain Pe_1 carrying EV or *ripJ*_{Pe_9} inoculated on *S. pimpinellifolium* LA2093 and *S. lycopersicum* LA4504 plants at 5 and 10 dpi. Plants were inoculated using a root drenching method (1×10^8 CFU/ml). Each dot corresponds to the data obtained from a single infected plant. Statistical analyses was performed by one-way analysis of variance with *P* value < 0.0001 using Graphpad Prism.

Fig. 3. Some naturally occurring RipJ variants do not confer avirulence. **A**, Graphical representation of RipJ natural variants from *Ralstonia solanacearum* phylotype 1 strain. Experimentally confirmed avirulent RipJ_{Pe_9} variant was used as a reference. Nonsynonymous mutations are indicated using dashed lines. Amino acid residues shared by the virulent variants Pe_1 and Pe_57 are indicated with asterisks. Amino acid sequence changes caused by frameshift mutations are indicated as a gray box. Avr = avirulent, Vir = virulent, and aa = amino acids. **B**, Several RipJ natural variants have lost avirulence in *Solanum pimpinellifolium* (Sp) LA2093. Plants were infected with *R. solanacearum* GMI1000 wild-type (WT) and Pe_1 carrying pRCKgg empty vector (EV) or *ripJ* natural variant. Representative photographs of infected plants were taken at 10 days postinfection (dpi). Sl = *S. lycopersicum* LA4504. S and R represent susceptibility and resistance, respectively. White scale bars indicate 5 cm. **C**, *S. pimpinellifolium* LA2093 plants infected with *R. solanacearum* Pe_1 carrying an avirulent *ripJ* natural variant display an enhanced survival rate. Surviving plants were counted for 10 dpi. The *P* value indicated in each graph (significant if *P* < 0.05) was calculated using a Mantel-Cox log-rank test comparing curves.

in the virulent Pe₁ strain (Fig. 3A; Supplementary Fig. S1A and C). Subsequently, we observed bacterial wilt symptom development in *S. lycopersicum* LA4504 and *S. pimpinellifolium* LA2093 infected with the transgenic Pe₁ strains. As expected, strong bacterial wilt disease symptoms were observed in *S. lycopersicum* LA4504 infected with Pe₁ carrying EV or RipJ variants (Fig. 3B). Importantly, the introduction of *ripJ*_{GMI1000} or *ripJ*_{Pe₃} into Pe₁, as seen earlier for *ripJ*_{Pe₉}, resulted in bacterial wilt resistance in *S. pimpinellifolium* LA2093 (Fig. 3B). In contrast, Pe₁ carrying *ripJ*_{Pe₅₇} or *ripJ*_{To₂₈} caused strong bacterial wilt symptoms in *S. pimpinellifolium* LA2093 (Fig. 3B). Consistently, only *S. pimpinellifolium* LA2093 plants infected with Pe₁ carrying *ripJ*_{GMI1000}, *ripJ*_{Pe₃}, or *ripJ*_{Pe₉} showed significantly higher survival rates compared with the EV (Fig. 3C). To confirm that the *ripJ*_{Pe₁} allele does not play a role in avirulence of strain Pe₁ in *S. pimpinellifolium* LA2093, we generated a virulent Pe₁ carrying the *ripJ*_{Pe₁} deletion mutant (Δ ripJ) (Supplementary Table S3; Supplementary Fig. S3A and B). Furthermore, the transgenic Pe₁ Δ ripJ strains carrying the *ripJ* allele from Pe₃, Pe₉, Pe₅₇, To₂₈, or GMI1000 were developed. Pe₁ Δ ripJ showed virulence levels comparable with the Pe₁ wild type infected in *S. pimpinellifolium* LA2093 (Supplementary Fig. S4A and B). Moreover, the Pe₁ Δ ripJ strain carrying Pe₃, Pe₉, or GMI1000 allele of *ripJ* showed an avirulence phenotype, whereas introduction of the Pe₅₇ or To₂₈ allele of *ripJ* did not alter Pe₁ Δ ripJ virulence in *S. pimpinellifolium* LA2093 (Supplementary Fig. S4A and B).

Taken together, these results suggest that Pe₁, Pe₅₇, and To₂₈ variants of RipJ have lost their avirulence activity. Interestingly, the sequence polymorphisms V9A, Q274H, M317T, and F363S were commonly found in RipJ_{Pe₁} and RipJ_{Pe₅₇}, suggesting that these mutations might have an important role in evading recognition by the LA2093 immune system (Fig. 3A; Supplementary Table S2).

Mutations in putative acetyltransferase catalytic residues do not alter RipJ avirulence in LA2093.

Previous studies suggest that RipJ belongs to the YopJ acetyltransferase family (Mukaihara et al. 2010; Peeters et al. 2013). YopJ acetyltransferase family effectors harbor a catalytic triad consisting of histidine, glutamate/aspartate, and cysteine (Ma and Ma 2016). Protein alignment of RipJ_{Pe₉} with the characterized acetyltransferase effectors RipP2, HopZ1, HopZ3, HopZ4, HopZ5, AvrBsT, YopJ, and XopJ revealed that RipJ_{Pe₉} harbors only two of the three conserved catalytic residues, histidine 214 and aspartate 235, but not cysteine 287 (Fig. 4A; Supplementary Fig. S6A). The cysteine residue typical of a catalytic triad is substituted by a serine at position 287 in RipJ_{Pe₉} (Supplementary Fig. S6B). These residues (H214, D235, and S287) are conserved among all RipJ variants of the six *R. solanacearum* phylotype I strains GMI1000, Pe₉, Pe₃, Pe₁, Pe₅₇, and To₂₈ (data not shown).

We hypothesized that RipJ_{Pe₉} is an acetyltransferase; mutations in the putative catalytic residues should abolish avirulence in *S. pimpinellifolium* LA2093. To determine the role of the conserved key residues H214 and D235 in avirulence, we generated two RipJ_{Pe₉} catalytic mutants (H214A or D235A) and introduced these mutants into the Pe₁ strain (Supplementary Table S3; Supplementary Fig. S1A and D). Subsequently, *S. pimpinellifolium* LA2093 and *S. lycopersicum* LA4504 were infected with Pe₁ carrying EV, *ripJ*_{Pe₉}, or the two *ripJ*_{Pe₉} catalytic mutants. As expected, *S. lycopersicum* LA4504 developed strong bacterial wilt symptoms against all of the strains tested (Fig. 4A). Notably, strain Pe₁ carrying either of the RipJ catalytic mutants H214A or D235A caused symptoms and survival rates comparable with wild-type RipJ_{Pe₉} in *S. pimpinellifolium* LA2093 (Fig. 4B and C). To rule out the possibility of a native

*ripJ*_{Pe₁} allele complementing the catalytic variants H214A and D235A in trans, we generated the *ripJ*_{Pe₁} deletion mutant in the Pe₁ virulent strain and complemented it with catalytic variants H214A or D235A. Infection assays were performed and survival

Fig. 4. Mutations in predicted acetyltransferase catalytic residues do not alter RipJ avirulence. **A**, Schematic representation of key residues of RipJ_{Pe₉}. Indicated key residues were predicted by comparing the amino acid (aa) sequences of RipJ_{Pe₉} and other YopJ family effectors shown in Supplementary Figure S3. Indicated amino acid residues are conserved in *Ralstonia solanacearum* GMI1000 and all phylotype I strains available in Prokhorchik et al. (2020). Among the predicted acetyltransferase catalytic triad residues, H214 and D235 but not S287 are conserved among YopJ family effectors. K342 indicates the predicted autoacetylation residue. **B**, Mutations in the conserved catalytic residues do not affect RipJ avirulence. Plants were infected with virulent strain *R. solanacearum* Pe₁ carrying pRCKg empty vector (EV), *ripJ*_{Pe₉} wild-type (WT), or *ripJ*_{Pe₉} catalytic mutants H214A and D235A. *Sp* = *Solanum pimpinellifolium* LA2093 and *Sl* = *S. lycopersicum* LA4504. Representative photographs of infected plants were taken at 10 days postinfection (dpi). S and R represent susceptibility and resistance, respectively. White scale bars indicate 5 cm. **C**, Mutations in conserved catalytic residues do not affect RipJ-triggered immunity. Plant infection was performed as described in B. Surviving plants were counted for 10 dpi. The P value indicated in each graph (significant if $P < 0.05$) was calculated using Mantel-Cox log-rank test comparing curves.

rates were quantified. Results indicate that Pe_1 Δ ripJ strains complemented with catalytic variants H214A or D235A maintain their avirulence phenotype in *S. pimpinellifolium* LA2093 whereas they continue to cause strong bacterial wilting in *S. lycopersicum* LA4504 (Supplementary Fig. S5A and B). Together, these results indicate that mutations in the putative acetyltransferase catalytic residues do not abolish the avirulence of RipJ.

Autoacetylation of the conserved lysine residue in HopZ5, RipP2, or HopZ1a was shown to be required for avirulence (Jayaraman et al. 2017; Lee et al. 2012; Mukaiharu et al. 2010). Our alignment result indicated that this lysine residue (K342) is present in RipJ_{Pe_9} (Fig. 4A; Supplementary Fig. S6C). We hypothesized that RipJ might also be autoacetylated if it carries acetyltransferase activity. To test this, we expressed two natural variants of RipJ (RipJ_{Pe_9} and RipJ_{Pe_1}) in *N. benthamiana* leaf cells by *Agrobacterium*-mediated transient transformation. The wild type and C218A mutant of HopZ5 were used as positive and negative controls, respectively (Jayaraman et al. 2017). Immunoblot analysis with anti-AcK antibody suggested that neither RipJ_{Pe_9} nor RipJ_{Pe_1} is acetylated on lysine residues (Fig. 5). However, we cannot exclude the possibility that a serine or threonine residue of RipJ is acetylated.

DISCUSSION

RipJ has previously been predicted to belong to the YopJ family of acetyltransferases (Ma and Ma 2016; Mukaiharu et al. 2010; Peeters et al. 2013). Here, we report that RipJ acts as an avirulence effector in *S. pimpinellifolium* accession LA2093. We further show that some naturally occurring RipJ variants do not induce bacterial wilt resistance in LA2093. Interestingly, the conserved acetyltransferase catalytic residues of RipJ are not required for avirulence.

Fig. 5. RipJ does not autoacetylate lysine residues. HopZ5 wild-type (WT) and acetyltransferase catalytic mutant C218A were used as positive and negative controls, respectively. The C-terminally FLAG-tagged RipJ or HopZ5 variants were expressed in *Nicotiana benthamiana* leaf cells using *Agrobacterium*-mediated transient transformation. Total proteins were extracted from the infiltrated leaves at 2 days postinfection (dpi) and detected with antiacetyl lysine antibody (α -AcK) after immunoprecipitation with anti-FLAG antibody (α -FLAG). Ponceau staining shows the Rubisco band as a measure of similar loading of total protein. Protein bands of expected sizes are indicated with yellow asterisks.

S. pimpinellifolium LA2093 is a new source of bacterial wilt resistant tomato breeding.

Two major QTLs, *Bacterial wilt resistance locus-12* (*Bwr-12*) and *Bwr-6*, were shown to be associated with bacterial wilt resistance in *S. lycopersicum* ‘Hawaii 7996’ (Carneille et al. 2006; Wang et al. 2000). Recently, it was shown that *Bwr-12* harbors the genes encoding LRR receptor-like proteins (Kim et al. 2018). Several *R. solanacearum* T3SEs such as RipAX2, RipP1, RipP2, RipN, and RipS5 were predicted to be associated with avirulence in Hawaii 7996 (Pensec et al. 2015). However, the avirulence effector that is recognized in Hawaii 7996 is unknown. Several wild tomato species have been extensively used to introduce beneficial traits; in particular, disease resistance to crops. Large-scale screens in multiple *Solanum* spp. showed that three *S. lycopersicum* and one *S. peruvianum* accessions are resistant to *R. solanacearum* (Kim et al. 2016). Interestingly, this study did not identify *S. pimpinellifolium* as a resistance source. As demonstrated in our study, some natural variants of RipJ do not confer avirulence. Therefore, it is plausible that *R. solanacearum* strain WR-1 used by Kim et al. (2016) did not carry an avirulent allele of RipJ.

S. pimpinellifolium is the closest relative of cultivated tomato varieties and a source of important traits such as disease resistance (Miller and Tanksley 1990). Owing to several desirable traits, a high-quality genome sequence of *S. pimpinellifolium* accession LA2093 was recently assembled (Wang et al. 2020). Moreover, a genotyping-by-sequencing-based high-resolution genetic map of the RIL population constructed from a cross between *S. pimpinellifolium* LA2093 and *S. lycopersicum* LA4504 has been developed (Gonda et al. 2019). Analysis of this RIL population for RipJ-triggered bacterial wilt resistance may help accelerate the identification of corresponding disease resistance genes. Based on the sequence analysis of currently available *R. solanacearum* genomes, the corresponding *R* gene for RipJ may provide disease resistance to approximately 50% of the RSSC. According to the RalstoT3E database, RipJ is mostly present in *R. solanacearum* phylotype I, IIa, and IIb strains but absent in phylotype III and IV strains. Thus, if the corresponding *R* gene is identified, it may be a valuable source to develop bacterial wilt-resistant tomato varieties against phylotype I and II strains. However, we cannot exclude the possibility that RipJ is present in phylotype III and IV strains, whose genome sequences are not available.

Identification of RipJ as a unique member of the YopJ type III effector family.

The YopJ family is a unique T3SE family found in both animal and plant pathogens (Ma and Ma 2016). Previous phylogenetic analysis have predicted RipJ as a group V effector of the AvrRxv/YopJ superfamily, along with RipP2 and RipAE (Ma and Ma 2016). YopJ family effectors share an evolutionarily conserved His/Glu/Cys or His/Asp/Cys catalytic triad (Zhang et al. 2016). The catalytic triad is essential for enzymatic activity and virulence of YopJ effectors (Üstün and Bornke 2015; Üstün et al. 2013). Studies on avirulence of the YopJ effector family members revealed that the catalytic triad and its corresponding function are important for perception by host plant R proteins (Orth et al. 2000; Roden et al. 2004; Whalen et al. 2008). In this study, we identified RipJ as a unique member of the YopJ family because it only has two of the conserved catalytic triad residues (i.e., H214 and D235). Serine (S287) is conserved in RipJ in the same position where the cysteine of the triad was expected. Moreover, unlike other YopJ family effectors identified to date, mutations in the RipJ catalytic triad do not alter avirulence. However, the contribution of the S287 residue to avirulence requires further investigation (Ma et al. 2015).

Often, autoacetylation of YopJ family effectors is required for in planta function. For instance, HopZ5, a T3SE of *P. syringae* pv. *actinidiae*, autoacetylates a lysine residue (K278) to trigger defense in *Arabidopsis* and *Nicotiana* spp. (Jayaraman et al. 2017). In addition, *R. solanacearum* effector RipP2 also autoacetylates a lysine residue (K383) for activation of RRS1-mediated immunity (Tasset et al. 2010). Therefore, it is surprising that the RipJ autoacetylation at the conserved lysine residue (K342) was undetected in our study. However, we cannot exclude the possibility that RipJ autoacetylates serine or threonine residues. Alternatively, the presence of an unknown cofactor that is absent in *N. benthamiana* might be required for RipJ acetyltransferase activity. Further biochemical experiments seem to be necessary to better understand RipJ function in the future. In addition, identification of an in planta target protein would be required to reveal the biochemical activity of RipJ in the future.

MATERIALS AND METHODS

Plant material and growth conditions.

Tomato germplasms of two accessions, *S. pimpinellifolium* LA2093 and *S. lycopersicum* LA4504 (NCEBR-1), were obtained from the C. M. Rick Tomato Genetics Resource Center. Seed were soaked in water and incubated at 28°C for 5 days in darkness to induce germination. Seedlings were transplanted in soil 2 to 3 days after germination. Plants were grown in a growth chamber at 24°C with a photoperiod of 16 h of light and 8 h of darkness.

Bacterial strains and culture condition.

R. solanacearum strains Pe_1 and Pe_9, described by Prokhorchik et al. (2020) and Segonzac et al. (2017), were isolated from pepper or tomato hosts in various geographical regions of South Korea. Wild-type and modified strains were cultured in liquid BG medium (1% Bacto peptone, 0.1% yeast extract, and 0.1% casamino acid) containing appropriate antibiotics when required and were grown at 28°C with shaking at 150 rpm overnight. For pathogenicity assays, cells were incubated at 28°C for 48 h on BG complete medium supplemented with 1.5% Bacto agar, 0.5% glucose, 0.005% triphenyl tetrazolium chloride (TTC), and relevant antibiotics.

Pathogenicity assays and statistical analysis.

S. pimpinellifolium and *S. lycopersicum* plants were grown for 4 weeks in a controlled room at 24°C with a photoperiod of 16 h of light and 8 h of darkness, before infection. *R. solanacearum* cells were cultured and resuspended in 10 mM MgCl₂. The bacterial suspension was adjusted to an optical density at 600 nm absorbance (OD₆₀₀) of 0.1 (approximately 10⁸ CFU/ml) with 10 mM MgCl₂. Plant roots were cut by passing a blade through the soil in a circle with a 1-cm distance from the stem and 15 ml of the bacterial suspension was poured onto the soil. Bacterial wilt symptoms were scored 10 to 12 dpi. Eight plants per accession were tested for each *R. solanacearum* strain. The severity of the wilting symptoms was quantified daily and its rate was plotted with Kaplan-Meier survival curves by using GraphPad Prism version 8.4.3 (GraphPad Software, San Diego, CA, U.S.A.). The log rank Mantel-Cox method was used to compute the *P* value of the survival distribution (Morel et al. 2018a). For bacterial load quantification in the stem, 1 cm of the stem just above the cotyledon was sampled, weighed, and surface sterilized with 70% ethanol solution. The sample was sectioned into six parts and immersed in 1 ml of sterile water for a minimum of 30 min for bacteria to ooze out of the stem. Appropriate dilutions were plated and the bacterial load was quantified as described by Morel et al. (2018b). Experiments were conducted three times with similar results.

Type III effector cloning.

Candidate avirulence effectors (Table 1) were first cloned in pICH41021 (a modified pUC19 shuttle vector, wherein the *BsaI* site has been removed via mutagenesis). *ripA1*, *ripAH*, *ripAX2*, *ripP1*, and *ripAF1* were cloned from *R. solanacearum* genomic DNA. *R. solanacearum* genomic DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega Corp., Madison, WI, U.S.A.) as per the manufacturer's protocol. Internal *BsaI* sites in the cloned effector sequences were eliminated through site-directed mutagenesis. *ripJ*, *ripD*, *ripB*, *ripAX1*, and *ripBA* were codon optimized and synthesized by Integrated DNA Technologies (IDT, Coralville, IA, U.S.A.) (sequences available upon request). *BsaI* and *SfiI* enzyme sites were eliminated before synthesis to aid in Golden Gate assembly and plasmid linearization, respectively. DNA sequences of the constructs were confirmed by sequencing using the M13F and M13R primer pair, performed by Solgent (Daejeon, South Korea). The pRC vector toolkit is a specific delivery system that allows the incorporation of genes or constructs in a permissible site in the *R. solanacearum* genome through double crossover (Monteiro et al. 2012). The pRCK (Monteiro et al. 2012) was modified to generate pRCKgg, which is a Golden-Gate-compatible version, as described by Moon et al. (2021). Effector constructs in pICH41021 were assembled into the destination pRCKgg vector through Golden Gate assembly (Engler et al. 2008), with a *hrpB* (230 bp) or *ripJ*_{Pe_9} native promoter (1,000 bp). A residual Shine-Dalgarno sequence AGGAG or GAGG is present at -891 to -867 or -14 to -10 bp from the start codon in *hrpB* or *ripJ*, respectively (Supplementary Fig. S7). The final constructs in pRCKgg were confirmed through gene-specific restriction enzyme digestion. For *Agrobacterium*-mediated delivery, effectors were assembled in pICH86988 binary vector with an appropriate C-terminal epitope tag through Golden Gate assembly. Assembled constructs were selected with appropriate antibiotics of the concerned destination vector. Integration of the constructs was screened with a blue-white screen using 15 µl of 100 mM of isopropyl-β-D-thiogalactoside and 800 µg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside. Correct integration was ultimately confirmed with restriction enzyme digestion. Constructs were transformed into *Agrobacterium tumefaciens* strain AGL1 through electroporation.

RipJ knockout was constructed as described by Wu et al. (2018). *ripJ* knockout plasmid was constructed by amplifying 989 bp of the 5' sequence of *ripJ* open reading frame (ORF) and 494 bp consisting of 117 bp of the *ripJ* ORF sequence and 377 bp of the 3' sequence of *ripJ* ORF from genomic DNA of *R. solanacearum* Pe_1 (virulent strain) (Supplementary Fig. S3A). *BsaI* sites were added to 5' and 3' ends for cloning in pICH41021. A Spec^R cassette was amplified from pCR8 (Invitrogen) consisting of a *BsaI* site on both 5' and 3' ends and was cloned in pICH41021. Modules were assembled in pRCgg plasmid using *BsaI*. Ampicillin and spectinomycin were used for selecting the colonies. Assemblies were further verified using restriction enzyme digestion and visualized through agarose gel electrophoresis (Supplementary Fig. S3A and B).

Natural transformation of *R. solanacearum*.

All candidate avirulence T3SEs cloned in pRCKgg were transformed into *R. solanacearum* strain Pe_1 (Table 1) using a modified protocol (Perrier et al. 2018). A single colony of *R. solanacearum* Pe_1 was cultured in 15 ml of one-fourth strength M63 minimal medium (15 mM ammonium sulfate, 1.8 µM iron(II) sulfate, 100 mM monopotassium phosphate, and 1 mM magnesium sulfate heptahydrate adjusted to pH 7 with KOH) supplemented with 2% glycerol and incubated at 28°C for approximately 36 h, to reach an OD₆₀₀ of 0.4 to 0.6. Each T3SE construct (500) was linearized using the *SfiI* restriction

enzyme. Linearized plasmid was purified using Sepharose 4B column. Bacterial culture (100 µl) was mixed with the linearized DNA. The mixture was spotted on BG complete medium supplemented with TTC and agar and incubated at 28°C for 48 h. The grown cells were then harvested in 1 ml of liquid BG medium and plated onto a new BG agar medium supplied with TTC and kanamycin at 50 µg/ml. Plates were incubated at 28°C for 48 to 56 h to allow transformants to appear. Transformed colonies were selected by PCR as described in Supplementary Figure S1.

Agrobacterium-mediated transient transformation.

Cultured *A. tumefaciens* AGL1 cells were harvested by centrifugation and resuspended in *Agrobacterium* infiltration buffer (10 mM MgCl₂ and 10 mM 2-(N-morphino) ethanesulfonic acid adjusted to pH 5.6 with KOH). OD₆₀₀ of the suspension was adjusted to 0.4. The bacterial suspension was infiltrated into the abaxial side of 4-week-old plant leaves with a blunt-end needle-less syringe. Leaf discs were harvested 2 days postinfiltration for immunoblot analysis.

Protein extraction, sodium dodecyl sulfate polyacrylamide gel electrophoresis, and immunoprecipitation.

For immunoprecipitation, agroinfiltrated leaves of *N. benthamiana* expressing the T3SE of interest were ground to a fine powder using liquid nitrogen. The sample was resuspended in extraction buffer (150 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.2% Nonidet P40, 2% polyvinylpyrrolidone, 10 mM dithiothreitol, and one tablet of protease inhibitor cocktail) and centrifuged at 5,000 × g for 20 min at 4°C. The supernatant was filtered and clear lysate was incubated using anti-FLAG beads (Sigma-Aldrich, St. Louis, MO, U.S.A.) for 2 h at 4°C. Finally, magnetic beads were washed thrice in extraction buffer and the sample was eluted by boiling the beads in 80 µl of 1× sodium dodecyl sulfate (SDS) sample buffer at 95°C for 5 min. Proteins were separated with SDS polyacrylamide gel electrophoresis and were transferred onto a polyvinylidene difluoride membrane using standard protocols of Western blot. Membranes were probed with α-FLAG (Sigma-Aldrich) or with antiacetyl-lysine antibody (α-AcK) used at a dilution 1:10,000. Hrp-conjugated antimouse was used at a dilution 1:5,000 as a secondary antibody. Proteins were visualized with SuperSignal West Dura Extended Substrate and SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL, U.S.A.) in an ImageQuant LAS 500 Chemiluminescence CCD Camera (GE Healthcare, Chicago, IL, U.S.A.). Visualization of the loaded total protein was achieved through Ponceau S staining.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

C. M. Rick Tomato Genetics Resource Center: <https://tgrc.ucdavis.edu/RalstoT3E> database:
<https://iant.toulouse.inra.fr/bacteria/annotation/site/prj/T3Ev3/>

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