INTRODUCTION

Pseudomonas syringae is a model plant pathogen for studying bacterial pathogenicity, in particular the molecular mechanisms of disease establishment. The P. syringae species complex is currently represented by 64 pathovars, with each pathovar identified as infecting a select host plant genus, but often possessing the capacity to infect more, being restricted by plant immunity and environmental parameters (Berge et al., 2014; Xin et al., 2016; Morris et al., 2019; Laflamme et al., 2020). Collectively, the pathovars of P. syringae cause disease in the vast majority of agriculturally important crop plants.
demonstrating the ubiquity and flexibility of this species globally. Regular disease outbreaks, caused by emergent P. syringae strains, frequently threaten global agricultural productivity (Xin et al., 2018).

Comparative and population genomics studies of isolates collected from agricultural and environmental sources have transformed our understanding of diseases caused by P. syringae (McCann et al., 2017; Dillon et al., 2019a, 2019b). The P. syringae species complex is divided into 13 phylogroups on the basis of multilocus sequence analysis, with the three major pathogenic phylogroups (numbered 1–3) with their core genomes forming distinct lineages, but with accessory genetic components shared between them through horizontal gene transfer (Berge et al., 2014; Dillon et al., 2019b). A near-universally common genetic component of these plant pathogens is a canonical tripartite pathogenicity island composed of an hrp and hrc gene cluster encoding a type III secretion system (T3S), flanked by the conserved effector locus (CEL) and the exchangeable effector locus (EEL) (Xin et al., 2018). The T3S delivers bacterial effector proteins (T3Es) into host cells and is necessary for pathogenesis (Büttner and He, 2009; Clarke et al., 2010). While effectors encoded by the EEL vary between pathohar strains, with several expected to be functionally redundant, the CEL encodes between two and four highly conserved effector genes: hopAA1-1, hopM1, avrE1, and occasionally hopN1 (Deng et al., 2003).

Evolutionarily conserved CEL-encoded T3Es are required for (and predictive of) pathogenicity (Alfano et al., 2000; Baltrus et al., 2011; Dillon et al., 2019b). There is evidence of redundancy in this T3E group, but studies of this phenomenon as well as associated contributions of each CEL effector are limited to the type strain P. syringae pv. tomato (Pto) DC3000. These studies have revealed functional redundancy between CEL T3Es hopM1 and avrE1, and an avrE-related non-CEL T3E, hopR1, in a host species-dependent manner (Badel et al., 2006; Kvitko et al., 2009). Whether these results are representative of other P. syringae strains on their respective hosts is currently unknown.

In this study, the CEL locus T3Es and their non-CEL homologs/orthologs were characterized from the kiwifruit bacterial canker pathogen, P. syringae pv. actinidiae (Psa), and compared and contrasted with orthologs from Pto DC3000. Psa and Pto strains are closely related in P. syringae phylogroup 1 (PG1), albeit from different clades, PG1a and PG1b, respectively (Berge et al., 2014). Psa HopM1 was found to be nonfunctional due to truncation of its chaperone ShcM, while AvrE1 and HopR1 were found to be functional and nonredundantly required for full virulence in Psa infection of kiwifruit.

2 | RESULTS

2.1 | The CEL is required for pathogenicity of Psa3 in Actinidia chinensis var. chinensis 'Hort16A'

The CEL of Psa biovar 3 ICMP 18884 (hereafter Psa3 V13) was identified previously during genome annotation via the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Templeton et al., 2015) and comprises T3E genes hopN1 (with its chaperone shcN), hopAA1-1, the T3S helper gene hrpW1, hopM1 (with its chaperone shcM), and avrE1 (with its chaperone shcE) (Figure 1a). It is highly similar to and syntenic with the CEL of type strain Pto DC3000 (Table 1). In Psa3, however, there is a 14-bp indel in the genomic sequence of hopAA1-1, resulting in an early truncation of its peptide sequence and thus poor similarity in this effector (Badel et al., 2006). To investigate whether the CEL in Psa3 V13 has an essential role in pathogenicity, a knockout of the entire 14.2 kb region of the CEL was generated (∆CEL). We assessed "pathogen fitness" by measuring in planta bacterial growth and "virulence" by examining disease symptom development. Pathogenicity testing of the ∆CEL mutant was carried out on Actinidia chinensis var. chinensis 'Hort16A' plantlets along with wild-type Psa3 V13 and the T3S-deficient ∆hrcC strain. Loss of the CEL resulted in significant reduction in Psa3 V13 fitness, as assessed by in planta growth at both 6 and 12 days postinoculation (dpi) (Figure 1b), but not to the extent of that observed for the type III secretion system ∆hrcC mutant. Reflecting the reduction of in planta growth was a significant reduction in both leaf lesion development and plant death in Hort16A plants at 50 dpi, compared to the wild-type strain, indicating an associated reduction in virulence (Figure 1c). Taken together, these results suggest that the CEL is required for full virulence of Psa3 on Hort16A kiwifruit plants.

2.2 | AvrE1 is responsible for CEL-conferring virulence of Psa3

Having established a significant role for the CEL in the pathogenicity of Psa3 V13, the contribution of individual CEL-encoded effectors to Psa3 pathogenicity was investigated. Broad host-range plasmid-borne copies of each functional effector gene from the CEL were transformed into the Psa3 V13 ∆CEL mutant (Figure 2a). In planta bacterial growth assays in Hort16A plantlets revealed that only plasmid-borne avrE1 (p.avrE1) significantly restored in planta growth to the ∆CEL mutant (Figure 2b), but Psa3 V13 ∆CEL + p.avrE1 was reduced compared to wild type at 12 dpi. This difference was not due to plasmid loss during in planta growth (Figure S1). None of the plasmid-borne CEL effectors were able to fully restore virulence as demonstrated by the lack of lesion development and plant death at 50 dpi, however (Figure 2c).

The lack of full complementation by p.avrE1 may in part be explained by either the absence of its chaperone shcE (Lorang and Keen, 1995) or an unknown effect of genomic localization for avrE1 (and/or hopM1) required for appropriate levels of expression and/or secretion. Alternatively, T3S helper hrpW1, which was not included in the original screen, may be involved in conferring full virulence. This gene is expressed at high levels during in planta infection of Psa3 compared to other functionally redundant helpers (Figure S2) (Kvitko et al., 2007; McAtee et al., 2018). To assess this, genomic knockin constructs of hrpW1, hopM1 (with chaperone shcM), or avrE1 (with chaperone shcE) were generated with
their native HrpL box promoters and reintegrated into Psa3 V13 genome at the CEL knockout site (Figure 3a). Assessment of in planta bacterial growth confirmed our previous result that avrE1 restored in planta growth to the ΔCEL mutant, and, further, that inclusion of shcE restored higher growth at both 6 and 12 dpi (Figure 3b). In addition, the genomic shcE:avrE1 knockin fully restored symptomology with leaf lesions and plant death observed at 50 dpi, suggesting virulence at this locus is largely conferred by AvrE1 (Figure 3c). In contrast, genomic knockin of hopM1 with chaperone shcM (shcM:hopM1) did not restore in planta growth or virulence to the Psa3 ΔCEL mutant.

### 2.3 HopM1 function in Psa3 is affected by truncation of its chaperone ShcM

The shcM:hopM1 genomic knockin was unable to complement the Psa3 ΔCEL mutant, despite Psa3 having a full-length hopM1 allele (Figure 4a) (Templeton et al., 2015). Closer examination of the Psa hopM1 genomic region including shcM (and its related alleles in P. syringae phylogroup 1 compared to alleles from the P. syringae phylogroup type strains Pto DC3000, P. syringae pv. syringae [Psy] B728A, and P. syringae pv. phaseolicola [Pph] 1448A; Figure 4b,c) suggested that the Psa lineage lacks the ability to deliver a fully functional HopM1 due to an early truncation in either shcM (P. syringae pv. morsprunorum [Pmp], c.70G > T; Psal–1, c.283delA) or hopM1 itself (P. syringae pv. actinidifoliorum [Pfm; previously Psa4], c.1297_1316 delAACCAGAAAAAAGTTCAGG) (Figure 4a). All Psa biovars shared a single nucleotide insertion in the associated chaperone shcM that is unique to Psa. Pmp possesses a single nucleotide deletion in its shcM sequence, and Pfm is truncated by a short nucleotide deletion in hopM1. Psa3 strains possess an additional single nucleotide deletion downstream of this aforementioned deletion (c.365delT), but
Plasmid-borne avrE1 is able to largely restore virulence to Pseudomonas syringae pv. actinidiae ICMP 18884 biovar 3 (Psa3) ΔCEL. (a) Schematic of pBBR1MCS-5 vector constructs (Jayaraman et al., 2017) for under the AvrRps4 HrpL box for hopM1 with chaperone shcN, hopM1 with chaperone shcM, or avrE1 without its chaperone. The effectors were tagged with 6× HA, and plasmids were selected for by a gentamicin resistance cassette under a strong Pc promoter. The 5′ (AATG; including the first codon of the chaperone/effector) and 3′ (TTGC; non-native sequence) overhangs used for assembly of the final construct are indicated. (b) Psa3 V13 (wild type, WT), conserved effector locus mutant (ΔCEL), ΔCEL carrying empty vector (p.EV), or ΔCEL plasmid complementation mutants, were flood-inoculated at c.10⁶ cfu/ml into Actinidia chinensis var. chinensis ‘Hort16A’ leaves, and bacterial growth was determined 0, 6, and 12 days postinoculation (dpi). Error bars represent standard error of the mean from four pseudobiological replicates. Asterisks indicate results of a two-tailed Student’s t test between the selected sample and wild type (Psa3 V13); *p < .05, **p < .001. The experiment was conducted three times with similar results. (c) Symptom development in Hort16A leaves at 50 dpi for strains inoculated in (b). Photographs taken of a single representative pot and a representative leaf displaying typical disease lesion symptoms, if present.

This is a silent mutation because the upstream deletion inserts a stop codon into the coding sequence.

To assess whether HopM1 delivery was affected by the truncation in the ShcM protein, plasmid-borne versions of each of the CEL effectors (with their chaperones, if available) were transformed into wild-type Pseudomonas fluorescens (Pfo) Pfo-1, or an artificially generated T3S-carrying P. fluorescens strain, Pfo Pfo-1(T3S) (Thomas et al., 2009), and infiltrated into Nicotiana benthamiana leaves for protein expression (Figure 5) or cell death analysis (Figure S4). To avoid the effects of differences in promoter sequences affecting expression, shcM from Pto DC3000 (shcMₚₚₒ) was cloned under the same avrRps4 promoter to control expression of the plasmid-borne hopM1 operon used to assess shcMₚₚₒ described earlier (Jayaraman et al., 2017). shcMₚₚₒ was cloned as a cis-complementation construct ahead of hopM1 in Pto Pfo-1(T3S) strains for comparison.

Interestingly, at higher bacterial loads (OD₆₃₀ = 1), AvrE1 (without chaperone ShcE) and HopM1 (with native chaperone ShcMₚₚₒ) were both able to trigger a cell death response in N. benthamiana. However, the cell death triggered by HopM1 was weak/sporadic (Figure S3a), suggesting poor expression or delivery. This was confirmed by in planta effector secretion assays (Figure 5). In contrast, cis-complementation with full-length shcMₚₚₒ along with hopM1 in Pfo Pfo-1(T3S) was able to confer robust HopM1-triggered cell death even at reduced bacterial loads (Figure S3b,c) and improved secretion of HopM1 (Figure 5).

### 2.4 HopM1 triggers immunity in A. chinensis ‘Hort16A’

To determine whether shcM complementation restored HopM1-mediated virulence, the Psa3 V13 (wild type), Psa3 ΔCEL mutant, Psa3 ΔCEL + hopM1 (genomic knockin of shcMₚₚₒ:opm1), and Psa3 ΔCEL + avrE1 (genomic knockin of shcEavrE1) strains were transformed with plasmid-borne shcMₚₚₒ (trans-complemented), and assessed by in planta bacterial growth assays in Hort16A plants. Intriguingly, while shcMₚₚₒ complementation did not restore virulence, it consistently reduced the in planta growth of Psa3 V13 (wild type) and Psa3 ΔCEL + hopM1, but not of Psa3 ΔCEL and Psa3 ΔCEL + avrE1, suggesting that HopM1 triggers an immune response (Figure S4a). This difference was not due to plasmid loss during in planta growth (Figure S4b).

To further confirm the contribution of the shcM allele in triggering immunity in Hort16A plants, cis-complementation by the
in planta growth was accompanied by an associated increase in disease symptoms (Figure 6b). This decrease in plant growth by a lack of reduction in disease symptoms (Figure 6b). This decrease was made at the native M1u site indicated in the 3′ region of the ΔCEL knockout construct. Coloured ovals indicate inclusion of the relevant HrpL binding site promoters included in the knockin constructs. (b) Psa3 V13 (wild type, WT), ΔCEL mutant, ΔCEL with avrE1 plasmid-complemented mutant, or ΔCEL with genomic knockin hrpW1, shcM:hopM1, or shcE:avrE1 were inoculated by flooding at c.10^6 cfu/ml into Actinidia chinensis var. chinensis ‘Hort16A’ leaves, and bacterial growth was determined 0, 6, and 12 days postinoculation (dpi). Error bars represent standard error of the mean from four pseudobiological replicates. Asterisks indicate results of a two-tailed Student’s t-test between the selected sample and wild type (Psa3 V13): *p < .05, **p < .01, ***p < .001. The experiment was conducted three times with similar results. (c) Symptom development in Hort16A leaves at 50 dpi for strains inoculated in (b). Photographs taken of a single representative pottle and a representative leaf displaying typical disease lesion symptoms, if present.

avrE1 genomic knockin with shcE fully restores virulence to Pseudomonas syringae pv. actinidiae ICMP 18884 biovar 3 (Psa3) ΔCEL. (a) Schematic of the conserved effector locus (CEL) locus (wild type), knockout with introduced XbaI site (ΔCEL), knockin for hrpW1 (ΔCEL + hrpW1), knockin for hopM1 with chaperone shcM (ΔCEL + shcM:hopM1), or knockin for avrE1 with chaperone shcE (ΔCEL + shcE:avrE1). The knockin was made at the native M1u site indicated in the 3′ region of the ΔCEL knockout construct. Coloured ovals indicate inclusion of the relevant HrpL binding site promoters included in the knockin constructs. (b) Psa3 V13 (wild type, WT), ΔCEL mutant, ΔCEL with avrE1 plasmid-complemented mutant, or ΔCEL with genomic knockin hrpW1, shcM:hopM1, or shcE:avrE1 were inoculated by flooding at c.10^6 cfu/ml into Actinidia chinensis var. chinensis ‘Hort16A’ leaves, and bacterial growth was determined 0, 6, and 12 days postinoculation (dpi). Error bars represent standard error of the mean from four pseudobiological replicates. Asterisks indicate results of a two-tailed Student’s t-test between the selected sample and wild type (Psa3 V13): *p < .05, **p < .01, ***p < .001. The experiment was conducted three times with similar results. (c) Symptom development in Hort16A leaves at 50 dpi for strains inoculated in (b). Photographs taken of a single representative pottle and a representative leaf displaying typical disease lesion symptoms, if present.

shcM_Psa construct alongside the original shcM_Psa construct in Psa3 V13 wild type, Psa3 ΔCEL, and Psa3 ΔCEL + avrE1 (genomic knockin of shcE:avrE1) was tested. When transformed with plasmid-borne shcM_Psa, a consistent reduction of in planta growth confirmed that the restoration of HopM1 delivery by Psa3 reduces in planta growth in a HopM1-dependent manner (Figure 6a). Despite this finding, no changes in shcM-dependent virulence were detected as determined by a lack of reduction in disease symptoms (Figure 6b). This decrease in plant growth was accompanied by an associated increase in AvrRKK70a defence gene expression in Hort16A plants at 24 hr postinoculation (Figure 6c). Notably, the cis-complementation of shcM_Psa allowed for increased in vitro secretion of HopM1 in a T3S-dependent manner, suggesting that the ShcM_Psa-mediated increase in secretion of HopM1 facilitated this change in response (Figure S5). Therefore, the HopM1-dependent reduction of in planta growth and associated defence gene induction indicates that HopM1 may trigger immunity in Hort16A plants, and suggests that the loss of ShcM function through truncation is a mechanism of reducing HopM1-triggered immunity in its host kiwifruit plants.

Comparison of the hopM1 alleles for type strains from phylogroups 1, 2, and 3 suggests that the Psa lineage (Psa, Pfm, Pmp, P. syringae pv. theae [Pth], and P. syringae pv. avellanea [Pav]), a subset of clade 1b in phylogroup 1, possesses a hopM1 allele that has a distinct evolutionary path incongruent with strain phylogeny (Figures 4b,c and S6a). In contrast, the adjacent genes hrpW1 and avrE1 have an evolutionary history congruent with their respective phylogroups. The Psa1–6 hopM1 alleles are all significantly different from the previously characterized hopM1 in Pto DC3000, and this variation may be associated with the functional deletions in the shcM operon. A broader assessment of the shcM and hopM1 operon across phylogroup 1 strains suggests multiple independent mutation events leading to either loss of ShcM (Pmp or Psa strains) or HopM1 itself (Pfm and Pto T1-like strains), possibly affected by host-dependent selection (Figure S6b).

2.5 | HopR1 and AvrE1 are additively required for Psa3 virulence

Effectors outside the CEL locus have been reported to be functionally redundant with those in the CEL of Pto DC3000 (Kvitko et al., 2009; Cunnac et al., 2011). Psa alleles of hopAA1-2 from the CEL and plasmid-borne hopAA1-2, though highly homologous to their counterparts in Pto DC3000, have both been pseudogenized by a
14 nucleotide or miniature inverted-repeat transposable element (MITE) insertion, respectively (Table 1 and Figure S7a) (Templeton et al., 2015; Poulter et al., 2018). Psa3 hopR1, however, appears to be fully functional and highly homologous (94.2% and 95.9% nucleotide and amino acid identities, respectively) to hopR1 from Pto DC3000 (Figure S7b). Interestingly, hopR1 sequences within the Psa lineage appear to group into two clades, with the Psa3 V13 allele group - ing with those from Pto DC3000, biovars 2 and 6, as well as Pfm (Figure S8). The more distantly related hopR1 sequences from Psa1 and Psa5, and closely related Pmp, appear to be significantly different in sequence, but are not characterized further here.

Because HopR1 was previously found to be redundantly required with CEL effectors in Pto, HopR1 function in Psa3 V13 virulence was investigated. A hopR1 knockout in Psa3 V13 ($\Delta$hopR1), assessed by in planta growth, showed that hopR1 is required for full Psa3 virulence in Hort16A leaf tissues at both 6 and 12 dpi (Figure 7a). This was mirrored in disease symptomology with the $\Delta$hopR1 mutant displaying reduced lesions and plant death at 50 dpi (Figure 7b). Intriguingly, a double CEL and hopR1 knockout strain ($\Delta$CEL/$\Delta$hopR1) was more severely impacted than $\Delta$CEL alone, as assessed by both in planta growth as well as disease symptom development (Figure 7c,d). This demonstrates additive contributions to Psa3 virulence from both AvrE1 and HopR1, in a nonredundant manner.

3 | DISCUSSION

Herein, Psa3 in planta growth (pathogen fitness) and symptom production (virulence) encoded by the evolutionary conserved CEL locus have been linked largely to effector AvrE1. The contribution to in planta growth and virulence is particularly dependent on the AvrE1 chaperone ShcE combination because supplying plasmid-encoded AvrE1 without its chaperone only partially restored in planta growth and still lacked obvious symptom production long term. Due
hopR1 in *P. syringae* strains, indicating that CEL and associated effectors appear to be critical for this group of plant-pathogenic bacteria. In fact, Xin et al. (2018) suggest one of the earliest acquisitions of *P. syringae* on its evolutionary journey to pathogenicity would have been an AvrE1 and/or HopM1-like effector for water acquisition in the apoplast. Large-scale studies in *Arabidopsis* have identified that, of all environmental strains present on this model plant, the lineage of most successful bacterial colonizers (and potential pathogens) all carried a functional T3S and largely possessed *avrE1* (Karavos et al., 2018). Environmental isolates of *P. syringae* have been found to possess all three CEL effectors (*avrE1*, *hopM1*, and *hopAA1*) as well as *hopR1* in bulk-sequenced assemblies, and both *avrE1* and *hopAA1* are conserved in over 95% of *P. syringae* species with *hopM1*, as well being present in a majority (Monteil et al., 2013; Dillon et al., 2019b).

In their large-scale environmental and agricultural sampling study, Dillon et al. (2019b) proposed that conserved ecologically and evolutionarily significant loci (like the CEL) are sites of increased interphylogroup recombination, amplifying genetic cohesion in the *P. syringae* species complex. The Psa lineage (a subset of phylogroup 1b defined here as strains including Psa, Pfm, Pth, and Pmp) alleles of *hopM1* appear to bear the hallmarks of such a recombination event (Figures S6a and 4b, c) and may be driven by selection for evasion of host immunity in their native plant hosts. The variation in the *hopM1* sequence compared to flanking *avrE1* and *hrpW1* sequences either suggests a recombination event substituting the *hopM1* region specifically in the Psa lineage strains or indicates evolutionary diversifying selection on the *hopM1* gene that has been noted previously for this gene across a number of *P. syringae* pathovars (Baltrus et al., 2011). Despite possessing an apparently full-length amino acid sequence, Psa3 *hopM1* appeared to be nonfunctional due to a truncation in its chaperone *shcM*, unlike previously published nonfunctional *hopM1* alleles in the Pto lineage (T1-like strains) with mutations in the effector sequence (Cai et al., 2011). In a similar vein, a broader examination of the *shcM-hopM1* region from phylogroup 1 strains suggests at least three independent mutation events in either *shcM* (Pmp or Psa mutations) or *hopM1* (Pfm mutation) that lead to potential loss of HopM1 functionality in the Psa clade, mimicking the diversifying selection in Pto T1-like strains (Figure S6b). The loss of function suggests independent selection on Psa clade strains during growth on kiwifruit (or possibly another shared host) for loss of HopM1 function. Nevertheless, when chaperone function was restored in Psa3 V13, in both cis and trans, the HopM1-dependency of reduced in planta growth was reminiscent of proposed HopM1-triggered recognition by tomato plants (Cai et al., 2011). This highlights that effector variation through evolutionary pressure encompasses both its native gene sequence as well as associated accessory genes required for efficient delivery into plant cells, a parameter underexplored in current research. Future evolutionary studies examining the presence/absence of effectors should consider chaperone presence, in both cis (present in operon) and trans (distal genomic presence), and sequence variation in the chaperone that could further impact on delivery efficiency, in the process of assessing an effector’s role in pathogenicity.

![FIGURE 5 Pseudomonas fluorescens (Pfo) Pfo-1 carrying shcM from *P. syringae* pv. *tomato* (Pto) DC3000 shows increased in planta secretion of HopM1 in a type III secretion (T3S)-dependent manner. In planta secretion assay for empty vector (EV), HopN1 (vector with shcN), HopM1 (vector with shcM<sub>PSA</sub> or shcM<sub>Pto</sub>), or AvrE1 (no chaperone) proteins tagged with 6 × HA from Pfo Pfo-1 (T3S+) or Pto Pto-1 (wild type, T3S–). Pfo strains were infiltrated at OD<sub>600</sub> = 1.5 into *Nicotiana benthamiana* plants and leaf samples harvested at 6 hr postinfiltration, protein extracted and western blots conducted using α-HA antibody. Red asterisks indicate expected sizes for each tagged protein band. The Coomassie brilliant blue (CBB)-stained band for RuBisCO is presented as a parameter underexplored in current research. Future evolutionary pressure encompasses both its native gene sequence as well as associated loci conserved in over 95% of *P. syringae* species with *hopM1*, as well being present in a majority (Monteil et al., 2013; Dillon et al., 2019b).](image-url)
P. syringae pathogens that possess the canonical tripartite pathogenicity island have at least one of the conserved effectors hopM1, avrE1, or hopAA1-1, indicating a critical function for these effectors in pathogenicity (Alfano et al., 2000; DebRoy et al., 2004; Dillon et al., 2019a). Similar critical roles for CEL effector orthologs in multiple bacterial plant pathogens including Erwinia spp., Pantoea spp., Pectobacterium spp., and Dickeya spp. have been described (Alfano et al., 2000; Degrave et al., 2015). Interestingly, unlike Pto DC3000, AvrE1 homologues WtsE from Pantoea stewartii, and DspE from Erwinia amylovora, Pantoea agglomerans pv. glycines, and Pectobacterium carotovorum play a major, nonredundant role in pathogenicity (reviewed in Degrave et al., 2015). An avrE1-like effector was also recently found in the oomycete pathogen Hyaloperonospora arabidopsis, suggesting a conservation and functional role beyond bacterial pathogens (Deb et al., 2018). Effectors from the Pto DC3000 CEL show redundancy in their ability to promote disease, including hopM1 and avrE1 (Kvitko et al., 2009). Recently, further evidence of redundancy in the CEL was discovered through the fact that the same resistance protein that recognizes AvrE1 in Arabidopsis also recognizes HopAA1, suggesting a shared mechanism of action (e.g., a shared target monitored by this resistance gene) for these two effectors (Kvitko et al., 2009).

Intriguingly, Psa3 AvrE1 and HopR1 appear to be functionally independent (Figure 7). In fact, the additive nature of AvrE1 and HopR1 contributions to Psa3 pathogenicity indicates that, unlike their orthologs in Pto DC3000, the Psa3 versions do not appear to share a REG, at least for the susceptible kiwifruit host Hort16A plants tested. This may suggest they have target(s) in kiwifruit that do not overlap. The remainder of CEL and associated effectors appear to be largely pseudogenized and nonfunctional in Psa3; HopM1 is nonfunctional due to truncation of its chaperone, while HopAA1-1 and HopAA1-2 are truncated through independent mutations. This suggests that AvrE1 alone contributes to virulence in the CEL-associated functions, and HopR1 independently contributes to an
Associated role for AvrE1 and partial redundancy between HopR1 was recently found to be able to suppress HopQ1-triggered plant disease. However, Arabidopsis and Pseudomonas syringae pv. actinidiae have been described to date. Nevertheless, Arabidopsis and Pseudomonas syringae pv. actinidiae have been found to suppress salicylic acid-mediated immunity by target-ting signalling-related protein phosphatase 2A (DebRoy et al., 2004; Jin et al., 2016), hamper immunity-related secretion (Nomura et al., 2011), and induce water-soaking (Xin et al., 2016). HopAA1-1 is associated with formation of necrotic lesions and is a virulence factor, making both attractive targets for durable resistance breeding.

While CEL effectors are required for pathogenicity, their precise role in disease establishment is still currently being elucidated. Pto DC3000 CEL (and associated) effectors HopM1 and AvrE1 have been found to suppress salicylic acid-mediated immunity by target-ting signalling-related protein phosphatase 2A (DebRoy et al., 2004; Jin et al., 2016), hamper immunity-related secretion (Nomura et al., 2011), and induce water-soaking (Xin et al., 2016). HopAA1-1 is associated with formation of necrotic lesions and is a virulence factor, sharing partial redundancy for disease symptom production with the phytoalexin coronatine biosynthetic gene cmol (Munkvold et al., 2009; Worley et al., 2013). This is reminiscent of the symptom-associated role for AvrE1 and partial redundancy between avrE1 and hopAA1-1 seen earlier (Badel et al., 2006; Cunnac et al., 2011), and mirrored in shared recognition by the CAR1 resistance gene in Arabidopsis (Laflamme et al., 2020). No conclusive function or mechanism of action for HopR1 has been described to date. However, HopR1 was recently found to be able to suppress HopQ1-triggered cell death in a strain-specific manner, suggesting an anti-immunity role (Zembek et al., 2018).

As yet, despite all key Pto DC3000 CEL and associated REG (CEL-REG) effectors triggering cell death in N. benthamiana, the resistance gene(s) recognizing HopM1, HopR1, HopAA1, and/or AvrE1 is unknown (Wei et al., 2007, 2018). Nevertheless, the CEL effectors’ ability to render Pto DC3000 avirulent on N. benthamiana is hampered by the presence of suppressor effectors Hop1 and AvrPtoB (Wei et al., 2018). This suggests that while CEL-REG effectors may be conserved for function in a number of different plant pathogens, a required suppressor-REG also exists in these bacterial pathogens to counter the plant hosts’ ability to recognize and trigger immunity against the CEL-REG effectors. This may also be true for Psa and is a significant focus of current research. The interest in this proposed suppressor-REG is largely because functionally redundant suppressor effectors make attractive targets for future programmes for plant resistance breeding because resistance to these suppressor effectors, in turn, is likely to be durable due to their requirement in masking immunity triggered by multiple virulence-critical CEL and associated effectors.

**FIGURE 7** hopR1 is nonredundantly required for virulence of *Pseudomonas syringae* pv. *Actinidia* ICMP 18884 biovar 3 (Psa3). (a) Psa3 V13 (wild type), ΔhrcC, or ΔhopR1 mutants were inoculated by flooding at c.10^6 cfu/ml on *Actinidia chinensis* var. *chinensis* ‘Hort16A’, and bacterial growth was determined 0, 6, and 12 days postinoculation (dpi). Error bars represent standard error of the mean from four pseudobiological replicates. Letters above the bars indicate results of a one-way analysis of variance (ANOVA) followed by Tukey’s HSD post hoc test with samples per time point assigned different letters if significantly different (p < .05). The experiment was conducted three times with similar results. (b) Symptom development in Hort16A at 50 dpi for strains inoculated with samples per time point assigned different letters if significantly different (p < .05). The experiment was conducted three times with similar results. (c) Psa3 V13 (wild type), ΔhrcC, ΔCEL, ΔhopR1 mutants, or ΔCEL/ΔhopR1 double mutant were inoculated by flooding at c.10^6 cfu/ml on Hort16A, and bacterial growth was determined 0, 6, and 12 dpi. Error bars represent standard error of the mean from four pseudobiological replicates. Letters above the bars indicate results of a one-way ANOVA followed by Tukey’s HSD post hoc test with samples per time point assigned different letters if significantly different (p < .05). The experiment was conducted three times with similar results. (d) Symptom development in Hort16A at 50 dpi for strains inoculated in (c). Photographs taken of a single representative pottle and a representative leaf displaying typical disease lesion symptoms, if present.
Understanding pathogen emergence in new crops such as kiwifruit requires the discrimination of nonredundant disease-critical elements from those that may be redundant. Most *P. syringae* strains appear to have a narrow spectrum of hosts they may infect (Morris et al., 2019). Interestingly, kiwifruit appears highly resistant to most characterized *P. syringae* pathovars, with the obvious exception of Psa strains that appear to be pathogenic on a number of plants, suggesting that they possess an intrinsic ability to cause more virulent disease (Morris et al., 2019). Thus, understanding the core effectors in Psa that are required for pathogenicity and are associated with increased virulence as well as the redundancy in this system is likely to impact on our understanding of other *P. syringae* pathogens that infect and trigger virulent disease in a large number of plant species.

4 | EXPERIMENTAL PROCEDURES

4.1 | Bioinformatics and sequences

Genome sequences for Psa3 ICMP 18884 (Psa3 V13) and other strains in this study were obtained from NCBI GenBank (CP011972.2 and CP011973.1). The Psa3 V13 genome was annotated previously (Templeton et al., 2015). Sequences for T3Es from Psa3 V13, other Psa lineage strains, and type strains from phylogroups 1–3 were analysed on Geneious R11 software (https://www.geneious.com; Biomatters) with built-in Geneious DNA and amino acid sequence alignments, tree building (RAxML v. 8 with 100 bootstrapping replicates), and annotation tools.

4.2 | Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table S1. Psa3 V13 strains were grown in lysogeny broth (LB) at 20°C with shaking at 200 rpm. Escherichia coli strains were grown in LB with appropriate antibiotics at 37°C. The concentrations of antibiotics used in selective media were kanamycin 50 μg/ml, gentamicin 25 μg/ml, nitrofurantoin 12.5 μg/ml, cephalaxin 40 μg/ml, chloramphenicol 10 μg/ml, and tetracycline 5 μg/ml (all from Sigma-Aldrich). Plasmids were transformed into electrocompetent Psa3 (Choi et al., 2006; Mesarich et al., 2017) or *E. coli* by electroporation using a BioRad Gene Pulser Xcell and recovered for 1 hr in LB before plating on selective media.

4.3 | Effector gene knockout

To make the CEL or hopR1 deletions in Psa3 V13, an approach derived from that described for Pto DC3000 was used (Kvitko et al., 2009). Briefly, DNA fragments containing the upstream (c.1 kb) and downstream (c.1 kb) regions of the effector (T3E) of interest were PCR-amplified using primer pairs Psa_(T3E)-KO_UP-F/ Psa_(T3E)-KO_UP-R and Psa_(T3E)-KO_DOWN_F/Psa_(T3E)-KO_DOWN_R, respectively (Table S2), with the up-R and down-F primers carrying XbaI restriction enzyme sites. Each PCR fragment was then gel-purified using an EZNA gel extraction kit (Custom Science), digested with XbaI, repurified with an EZNA PCR product purification kit, and ligated to form the c.2 kb KO fragment. The c.2 kb fragment containing both the upstream and downstream fragments for each T3E was then reamplified by PCR using primers Psa_(T3E)-KO_UP-F and Psa_(T3E)-KO_DOWN-R. This PCR product was cloned into the Eco53Kl blunt-end restriction enzyme site of pk18mobsacB (Schäfer et al., 1994) that had first been mutagenized to remove the non-MCS Eco53Kl site to generate pK18B-E. The pK18B-E vector carrying the c.2 kb KO fragment, called pΔ(T3E), was transformed into *E. coli* DH5α, plated on X-gal/ IPTG/kanamycin LB agar plates for blue/white selection and positive transfectants confirmed by Sanger sequencing (Macrogen). For each T3E knockout, Psa3 V13 was transformed with the relevant pΔ(T3E) construct and transconjugants were selected on LB plates with nitrofurantoin, cephalaxin, and kanamycin. Selected colonies were subsequently streaked onto LB plates containing 10% (wt/vol) sucrose to counterselect plasmid integration. ΔCEL or ΔhopR1 mutants were screened using colony PCR with primers Psa_(T3E)-KO_Check-F and Psa_(T3E)-KO_Check-R, and sent for Sanger sequencing with the cloning Psa_(T3E)-KO_UP-F and Psa_(T3E)-KO_DOWN-R primers, as well as further confirmed using internal T3E-specific primers and plating on kanamycin-containing medium to confirm loss of the sacB gene.

4.4 | Effector gene knockin

The sequences for *hrpW1, hopM1* (with chaperone *shcM*), and *avrE1* (with chaperone *shcE*) were synthesized with their native HrpL box promoter sequences, synonymous mutations to remove internal MluI sites, and flanking Ascl restriction enzyme sites added in the pUC57 vector (GenScript). These pUC57 vectors carrying the knockin gene of interest were then single-pot cloned (with Ascl, MluI, and T4 DNA ligase enzymes in T4 buffer; NEB) into the MluI site in the pΔCEL vector. The pΔCEL vector carrying the knockin gene, called pΔCEL + hrpW1, pΔCEL + shcM:hopM1, or pΔCEL + shcE:avrE1, was transformed into *E. coli* DH5α, plated on kanamycin LB agar plates and screened by colony PCR for the inserted gene. Positive transfectants were confirmed by Sanger sequencing (Macrogen). For each gene knockin, Psa3 V13 ΔCEL was transformed with the relevant pΔCEL + (gene-of-interest) construct and transconjugants were selected on LB agar plates with nitrofurantoin, cephalaxin, and kanamycin. Selected colonies were subsequently streaked onto LB agar containing 10% (wt/vol) sucrose to counterselect plasmid integration. Insertion mutants, ΔCEL + hrpW1, ΔCEL + hopM1, or ΔCEL + avrE1, were screened using colony PCR with primers Psa_ (T3E)-KI_Check-F and Psa_(T3E)-KI_Check-R, and sent for Sanger sequencing with internal gene-specific primers, as well as further confirmed by plating on kanamycin-containing medium to confirm loss of the sacB gene.
4.5 | shcM<sub>Pto</sub> complementation

shcM<sub>Pto</sub>, including its HrpL box promoter from Pto DC3000, was PCR-amplified using shcM<sub>Pto</sub>-F and shcM<sub>Pto</sub>-R primers (for trans-complementation) or shcM<sub>Pto</sub>-GG-F and shcM<sub>Pto</sub>-GG-R Golden Gate primers (for cis-complementation by assembly into the modified broad host-range vector pBBR1MCS-5-GG:avrRsp4<sub>pro</sub> (Jayaraman et al., 2017) (Table S2). The resulting PCR fragment was gel-purified as above. For the trans-complementation construct, the extracted PCR product was blunt-end-ligated into the Eco53Kl site of broad host-range vector pBBR1MCS-5 (Kovach et al., 1995). For the cis-complementation construct, the extracted PCR product was blunt-end-ligated into the Eco53Kl site of shuttle vector pICH41021 (Engler et al., 1995). Both constructs were transformed into E. coli DH5α, plated on X-gal/IPTG-containing (for blue/white selection) LB agar plates with gentamicin (trans) or ampicillin (cis) selection, and positive transformants confirmed by Sanger sequencing (Macrogen). The pICH41021-shcM<sub>Pto</sub> construct was then used to Golden Gate-assemble the pBBR1MCS-5:avrRsp4<sub>pro</sub>:shcM<sub>Pto</sub>:hopM1 construct with a 6xHA tag added to C-terminus of HopM1, as done previously for other effector constructs (Choi et al., 2017; Jayaraman et al., 2017). Both trans- and cis-complementation constructs were transformed into relevant Psa3 strains by electroporation, as described previously, and transformants screened for presence of shcM<sub>Pto</sub> by gene-specific colony PCR, followed by digestion by Ndel (specific for Psa3 allele of shcM).

4.6 | Infection assays

Psa infection assays were based on previous conditions (McAtee et al., 2018). A. chinensis var. chinensis ‘Hort16A’ plantlets, grown from axillary buds on Murashige and Skoog rooting medium without antibiotics in sterile 400-ml plastic tubs (“pottles”), were purchased from Multiflora. Plantlets were grown at 20°C under Gro-Lux fluorescent lights under long-day conditions (16 hr:8 hr, light:dark) and used when the plantlets were at 8–12 weeks old. Overnight cultures of wild-type or mutant strains of Psa3 were pelleted at 6,000 × g, resuspended in 10 mM MgSO<sub>4</sub>, cell density determined by measuring the optical density at 600 nm, and reconstituted at OD<sub>600</sub> = 0.05 (c.10<sup>7</sup> cfu/ml, determined by plating) in 500 ml of 10 mM MgSO<sub>4</sub>. Surfactant Silwet L-77 (Lehle Seeds) was added to the inoculum at 0.0025% (vol/vol) to facilitate leaf wetting. Pottles of Hort16A plantlets were flooded with the inoculum, submerging the plantlets for 3 min, drained, sealed, and then incubated under previously described plant growth conditions.

4.7 | Pathogen growth and symptom development

Psa3 V13 in planta growth assays were based on previous conditions (McAtee et al., 2018). Briefly, leaf samples of four leaf discs per pseudobiological replicate, taken randomly with a 1-cm diameter cork-borer from three plants, were harvested at 2 hr (day 0), day 6, and day 12 postinoculation. All four replicates per treatment, per time point were taken from the same pottle. To estimate Psa3 growth inside the plant, the leaf discs were surface-sterilized, placed in Eppendorf tubes containing three sterile stainless-steel ball bearings and 350 μl 10 mM MgSO<sub>4</sub>, and macerated in a Storm 24 Bullet Blender (Next Advance) for two bursts of 1 min each at maximum speed. A 10-fold dilution series of the leaf homogenates was made in sterile 10 mM MgSO<sub>4</sub> until a dilution of 10<sup>−8</sup> and plated as 10 μl droplets on LB medium supplemented with nitrofurantoin and cephalaxin. After 2 days of incubation at 20°C, the cfu per cm<sup>2</sup> of leaf area was ascertained from dilutions. Plasmid loss was investigated by plating the pBBR1MCS-carrying strains on LB agar medium with and without gentamicin (Gm or non-Gm). To observe pathogenic symptoms on the plants, infected pottles were kept up to 50 days postinoculation and photographs taken of pottles and a representative infected leaf. Infection severity was qualitatively assessed based on typical symptoms: necrotic leaf spots, chlorotic haloes, leaf death, and plant death. Each of these growth assay experiments were conducted at least three times.

4.8 | In planta effector secretion assay

Broad host-range plasmid constructs (pBBR1MCS-5-GG:avrRsp4<sub>pro</sub>) of each Psa3 T3E were transformed by electroporation into Pf0 Pf0-1(wild type) or Pf0-1(T3S) strains (Thomas et al., 2009) and plated on selective medium with chloramphenicol, gentamicin, and tetracycline (only + T3S strains). Positive transformants were confirmed by gene-specific colony PCR. Pf0-1(T3S) carrying empty vector or Psa3-T3E constructs were streaked from glycerol stocks onto LB agar plates with antibiotic selection and grown for 2 days at 28°C. Bacteria were then harvested from plates, resuspended in 10 mM MgSO<sub>4</sub>, and diluted to required OD<sub>600</sub> = 1.5 (c.10<sup>7</sup> cfu/ml). Infiltrations were carried out on fully expanded leaves of 4- to 5-week-old N. benthamiana with a blunt-end syringe, on two half-leaf sections of N. benthamiana leaves (replicates). Leaf samples were harvested at 6 hr postinfiltration and snap frozen in liquid nitrogen, ground with mortar and pestle, boiled in 1 × Laemmli buffer with dithiothreitol and run on SDS-PAGE for immunoblot for presence of the 6 × HA-tagged T3E using α-HA antibody. Membranes were subsequently stained with Coomassie brilliant blue to visualize total protein for gel loading control.

4.9 | Defence gene expression by quantitative PCR

Total RNA was extracted from four leaf discs per replicate (three replicates, each sampled from two independent flood-inoculated Hort16A plantlets) via a Spectrum Plant Total RNA kit (Sigma Aldrich). RNA was treated with DNase I (Sigma Aldrich)
and cDNA was synthesized from RNA using the High-Capacity cDNA Reverse Transcription kit following manufacturer’s instructions (Thermo Fisher). Quantitative PCR (qPCR) was carried out on an Illumina Eco Real-Time PCR machine using the EvaGreen SsoFast qPCR mix (Bio-Rad). The primers used for qPCR are listed in Table S2.

4.10 | N. benthamiana hypersensitive response assays

Broad host-range plasmid constructs (pBBR1MCS-5) of each P. syringae pv. actinidiae ICMP 18884 biovar 3 (Psa3) V13 T3E were transformed by electroporation into Pfo Pf0-1 (wild type) or Pf0-1 (T3S) strains (Thomas et al., 2009) and plated on selective media with chloramphenicol, gentamicin, and tetracycline (only + T3S strains). Positive transformants were confirmed by gene-specific colony PCR. Pf0-1(T3S) carrying empty vector or Psa3-T3E constructs were streaked from glycerol stocks onto LB agar plates with antibiotic selection and grown for 2 days at 28°C. Bacteria were then harvested from plates, resuspended in 10 mM MgSO4, and diluted to the required OD600 = 0.6 or 1 (c.10^8–9 cfu/ml). Infiltrations were carried out on fully expanded leaves of 4- to 5-week-old N. benthamiana using a blunt-end syringe on two or three leaves (replicates), with the full experiment repeated three times. Hypersensitive cell death response was assayed visually and photographs taken at 3 dpi.

4.11 | In vitro effector secretion assay

For detection of T3E secretion in vitro, the protocols used were based on those described previously (Huynh et al., 1989; Roiné et al., 1997). Briefly, Psa3 V13 conserved effector locus mutant (ΔCEL) or Psa3 V13 type III secretion system mutant (ΔhrCC) (Colombi et al., 2017) carrying the relevant broad-host range plasmid constructs (pBBR1MCS-5 or pBBR1MCS-2) were grown in LB medium with antibiotic selection overnight, pelleted at 6,000 × g, washed with hrp-inducing minimal medium supplemented with 10 mM fructose (Huynh et al., 1989) and then resuspended in hrp-inducing minimal medium and incubated for 6 hr with shaking for hrp induction. Following hrp induction, cells were pelleted, the supernatant carefully separated and passed through a 0.2 μm filter, and proteins from both pellet and supernatant were resolved by SDS-PAGE and immunoblotted for the presence of the 6 × HA-tagged T3E using α-HA antibody.

ACKNOWLEDGMENTS

This work was funded (including a post-doctoral fellowship to J.J.) by the Bio-protection Research Centre (Tertiary Education Commission). We would like to thank Jo Bowen (PFR), Erik Rikerink (PFR), and Carl Mesarich (Massey University) for critically reading the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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**How to cite this article:** Jayaraman J, Yoon M, Applegate ER, Stroud EA, Templeton MD. AvrE1 and HopR1 from *Pseudomonas syringae* pv. *actinidiae* are additively required for full virulence on kiwifruit. *Molecular Plant Pathology*. 2020;21:1467–1480. [https://doi.org/10.1111/mpp.12989](https://doi.org/10.1111/mpp.12989)