**Ralstonia solanacearum** Requires PopS, an Ancient AvrE-Family Effector, for Virulence and To Overcome Salicylic Acid-Mediated Defenses during Tomato Pathogenesis

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**ABSTRACT** During bacterial wilt of tomato, the plant pathogen *Ralstonia solanacearum* upregulates expression of *popS*, which encodes a type III-secreted effector in the AvrE family. PopS is a core effector present in all sequenced strains in the *R. solanacearum* species complex. The phylogeny of *popS* mirrors that of the species complex as a whole, suggesting that this is an ancient, vertically inherited effector needed for association with plants. A *popS* mutant of *R. solanacearum* UW551 had reduced virulence on agriculturally important *Solanum* spp., including potato and tomato plants. However, the *popS* mutant had wild-type virulence on a weed host, *Solanum dulcamara*, suggesting that some species can avoid the effects of PopS. The *popS* mutant was also significantly delayed in colonization of tomato stems compared to the wild type. Some AvrE-type effectors from gammaproteobacteria suppress salicylic acid (SA)-mediated plant defenses, suggesting that PopS, a betaproteobacterial ortholog, has a similar function. Indeed, the *popS* mutant induced significantly higher expression of tomato SA-triggered pathogenesis-related (PR) genes than the wild type. Further, pretreatment of roots with SA exacerbated the *popS* mutant virulence defect. Finally, the *popS* mutant had no colonization defect on SA-deficient NahG transgenic tomato plants. Together, these results indicate that this conserved effector suppresses SA-mediated defenses in tomato roots and stems, which are *R. solanacearum*’s natural infection sites. Interestingly, PopS did not trigger necrosis when heterologously expressed in *Nicotiana* leaf tissue, unlike the AvrE homolog DspE, from the necrotroph *Pectobacterium carotovorum* subspp. *carotovorum*. This is consistent with the differing pathogenesis modes of necrosis-causing gammaproteobacteria and biotrophic *R. solanacearum*.

**IMPORTANCE** The type III-secreted AvrE effector family is widely distributed in high-impact plant-pathogenic bacteria and is known to suppress plant defenses for virulence. We characterized the biology of PopS, the only AvrE homolog made by the bacterial wilt pathogen *Ralstonia solanacearum*. To our knowledge, this is the first study of *R. solanacearum* effector function in roots and stems, the natural infection sites of this pathogen. Unlike the functionally redundant *R. solanacearum* effectors studied to date, PopS is required for full virulence and wild-type colonization of two natural crop hosts. *R. solanacearum* is a biotrophic pathogen that causes a nonnecrotic wilt. Consistent with this, PopS suppressed plant defenses but did not elicit cell death, unlike AvrE homologs from necrosis-causing plant pathogens. We propose that AvrE family effectors have functionally diverged to adapt to the necrotic or nonnecrotic lifestyle of their respective pathogens.

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Plant-pathogenic bacteria cause destructive diseases that limit crop production worldwide. Many Gram-negative phytopathogenic bacteria use a type III secretion system (T3SS) to inject effector proteins into host cells. These generally modulate host immunity and physiology for pathogenesis (1–4). Individual effectors rarely contribute measurably to virulence but rather function as a consortium (5). Because of their redundancy and subtle biological activities, the functions of individual type III (T3) effectors remain largely unknown.

Plant immune systems have evolved complex signaling responses to defend against microbial invasion. Plants use specific protein receptors to detect conserved features of pathogen products such as flagellin, chitin, lipopolysaccharide, and elongation factor Tu (EF-Tu); perception of these microbe-associated molecular patterns (MAMPs) triggers basal immunity (1). In addition, plant R genes specifically recognize pathogen effectors. The plant hormone salicylic acid (SA) is a major defense signal molecule (6), and upon recognition of a pathogen, SA production induces basal immune responses such as callose deposition (7, 8). Accumulation of SA in plants also induces expression of pathogenesis-related (PR) defense genes to resist microbial infection and sometimes triggers rapid host cell death (9, 10). Studies of a few genera in the gammaproteobacteria have revealed how pathogen T3 effectors disrupt immune signaling and suppress SA-mediated defenses (5, 11, 12).

The AvrE family of effectors is well conserved across agricul-
were based on comparative analysis of whole genomes of 11 representative sequenced strains. (A) PopS, HopR (XopAM) from *Xanthomonas* spp. and *Pseudomonas* spp.; (B) PopS from sequenced plant-pathogenic *Ralstonia*; (C) *R. solanacearum* species complex. For panels A and B, amino acid sequences were aligned using CLUSTAL-W, from which a maximum-likelihood phylogenetic tree was created with MEGAS. The percentage of replicate trees in which the individual orthologs clustered together in the bootstrap test (200 replicates) is noted at each branch. For panel C, a phylogenetic tree of the *R. solanacearum* species complex was derived from a MUM index (MUMi) distance matrix of whole-genome sequences using the neighbor-joining clustering method.

FIG 1  PopS, an ancient core T3 effector in plant-pathogenic *Ralstonia* species, forms a distinct clade of the AvrE/DspE/HopR effector family. Phylogenetic trees were based on comparative analysis of whole genomes of 11 representative sequenced strains. (A) PopS, HopR (XopAM) from *Xanthomonas* spp. and *Pseudomonas* spp.; (B) PopS from sequenced plant-pathogenic *Ralstonia*; (C) *R. solanacearum* species complex. For panels A and B, amino acid sequences were aligned using CLUSTAL-W, from which a maximum-likelihood phylogenetic tree was created with MEGAS. The percentage of replicate trees in which the individual orthologs clustered together in the bootstrap test (200 replicates) is noted at each branch. For panel C, a phylogenetic tree of the *R. solanacearum* species complex was derived from a MUM index (MUMi) distance matrix of whole-genome sequences using the neighbor-joining clustering method.

Naturally important phytopathogens, including enterobacteria, xanthomonads, and pseudomonads (gammaproteobacteria) and *Ralstonia* spp. (betaproteobacteria) (13). Effectors in this family, which includes AvrE, DspE, and WtsE, induce host cell death and suppress defense signaling (12–14). AvrE from *Pseudomonas syringae* pv. *tomato* DC3000 and its ortholog DspE<sub>DC3000</sub> from *Erwinia amylovora* promote pathogen growth and overcome plant immunity by inhibiting SA-mediated defense responses (12). Despite their broad relevance to the interactions of plant-pathogenic bacteria with their hosts (13), little is known about AvrE-like effectors outside plant-pathogenic gammaproteobacteria.

The bacterial wilt pathogen *R. solanacearum* is responsible for diseases of many crops in tropical and subtropical climates worldwide. This bacterium enters plant roots from the soil and colonizes the host vasculature, which eventually leads to wilt and plant death (15). *R. solanacearum* requires a T3SS for root and stem invasion and colonization (16), and T3SS-deficient strains are essentially unable to wilt host plants (16). The *R. solanacearum* genome encodes an extensive effector repertoire (2, 17). Mutants lacking individual effectors generally do not have virulence defects (18), likely because the effectors have redundant functions (3, 5). The defense-suppressing functions of the individual effectors during the infection cycle remain unknown.

We previously used gene expression analysis to define the *R. solanacearum* in planta transcriptome, the set of bacterial genes expressed during growth in wilting tomato plant stems (19). An orthologous gene encoding an AvrE-family effector was expressed in planta in two ecologically and phylogenetically distinct strains. This locus ([RSRL]03375 in strain UW551 and [RSrl]128J in strain GMI1000) encodes a secreted T3 effector in the AvrE/DspE/HopR protein family (20), herein named PopS. Relative to expression in rich culture medium, UW551 and GMI1000 upregulate *popS* in planta 14- and 8-fold, respectively (19). Expression of *popS* is dependent on HrpB, the transcriptional activator of the T3SS and its effectors (21–23). Most effector genes are upregulated in planta via HrpB (19, 21, 24).

This study characterizes the role of PopS throughout the tomato infection process. We determined that this effector, which has ancient roots in the *R. solanacearum* species complex, is required for normal host colonization and virulence on multiple *Solanum* spp. crops. One host, *Solanum dulcamara*, suggested that it has species-specific virulence activity within the genus *Solanum*. PopS forms a unique clade in the AvrE family of effectors. Although it is highly divergent from its closest orthologs, we found that PopS retains the function of suppressing SA-mediated plant defenses. In contrast, PopS did not cause plant cell death or necrosis as do the AvrE-family proteins of necrosis-causing pathogens, such as DspE<sub>DC3000</sub> and WtsE from *Pectobacterium carotovorum* subsp. *carotovorum* and *Pantoea stewartii* subsp. *stewartii*. Together, our phylogenetic, virulence, and gene expression data suggest that PopS suppresses SA-mediated host defenses but lacks the ability to cause cell death, which may have helped this pathogen adapt to its nonnecrotic lifestyle.

RESULTS

PopS is a conserved, vertically inherited T3 effector in the *R. solanacearum* species complex. The AvrE effector family is widely present among plant-pathogenic bacteria (13). PopS most closely resembles HopR in *Pseudomonas* spp. and *Xanthomonas* spp. (also known as XopAM; [http://www.xanthomonas.org/t3e.html](http://www.xanthomonas.org/t3e.html)) (13), which were, respectively, 25 to 26% and 27 to 28% identical to PopS at the amino acid level. To understand the phylogenetic relationships among these proteins, we developed a maximum-likelihood phylogenetic tree in MEGA5 based on protein sequences of available PopS, AvrE, DspE, and HopR effector or-
Virulence Factor PopS Suppresses SA Defenses

R. solanacearum T3 effector PopS is required for full virulence on several hosts. To study the virulence function of this effector, we disrupted popS in strain UW551 (phylogroup II, sequevar 1) via allelic exchange to create strain UW551 popS:Kmr' (referred to here as the popS mutant). This mutant grew indistinguishably from the wild type (WT) in culture medium (data not shown), indicating that popS is not required for in vitro growth. The popS mutant retained wild-type ability to grow on sucrose as the sole carbon source, indicating that insertion of the Km' cassette did not disrupt expression of the scrK sucrose kinase gene (RRSL_03374) immediately downstream of popS. Quantitative reverse transcriptase PCR (qRT-PCR) analysis revealed that UW551 WT, but not the popS mutant, accumulates popS transcript when grown in minimal medium, confirming that popS is not expressed in the mutant strain (data not shown). To test the hypothesis that PopS contributes to bacterial wilt virulence, we used a naturalistic soil soak virulence assay to compare wilt disease progress of UW551 WT and the popS mutant on susceptible and moderately resistant tomato (Solanum lycopersicum cv. Bonny Best and H7996, respectively), susceptible potato (S. tuberosum cv. Russet Norkotah), and a natural weed host, S. dulcamara (bittersweet nightshade) (19). Briefly, pots containing unwounded plants were soaked with bacterial suspensions, and disease progress was rated daily. The popS mutant was significantly less virulent than the wild type on susceptible (P < 0.005; repeated-measures ANOVA) and resistant tomato and potato (P < 0.05) but not on bittersweet nightshade (P = 0.2).

To test the hypothesis that popS has been vertically inherited over the evolution of the R. solanacearum species complex, we developed a neighbor-joining tree based on 58 amino acid sequences, demonstrated that PopS forms a unique clade in the AvrE/DspE/HopR effector family and that this large family of effectors forms three distinct clusters: AvrE/DspE, HopR, and PopS (Fig. 1A). The R. solanacearum species complex is divided into four subgroups called phylotypes (26). A single copy of popS is found in the genomes of all 12 sequenced strains in the R. solanacearum species complex, which includes the blood disease bacterium (BDB) and the clove pathogen Ralstonia syzygii, both fastidious pathogens with limited host ranges (17, 27–33). A maximum-likelihood phylogenetic tree of genes encoding the T3 effector PopS is found in the whole-genome phylogenetic analysis (Fig. 1C) (17, 34). The MUMi distance matrix and phylogenetic tree of the species complex (Fig. 1B and C) (26). There is no indication of interstrain movement of popS via horizontal transfer, suggesting that PopS is an ancient core effector in the species complex. This conserved, vertically inherited gene is potentially useful for typing strains and identifying their phylogenetic positions in the R. solanacearum species complex.

R. solanacearum strain UW551 (black) and the popS mutant (red) were inoculated by pouring a bacterial suspension onto unwounded roots of wilt-susceptible tomato (A) and wilt-resistant tomato (B) (Bonny Best and H7996, respectively), potato (cv. Russet Norkotah) (C), and Solanum dulcamara (bittersweet nightshade) (D). Symptoms were rated daily using a disease index scale of 0 to 4 (0, healthy; 1, 1 to 25% of leaves wilted; 2, 26 to 50% of leaves wilted; 3, 51 to 75% of leaves wilted; 4, 76 to 100% of leaves wilted). Each point represents the mean disease index from three independent experiments, each containing 16 plants per treatment (A, B, and D), or one experiment (C). The popS mutant was significantly less virulent than the wild type on susceptible (P < 0.005; repeated-measures ANOVA) and resistant tomato and potato (P < 0.05) but not on bittersweet nightshade (P = 0.2).
petiole with WT UW551, the popS mutant, or the hrcC mutant, and bacterial colonization was quantified over time.

After direct petiole inoculation, the popS mutant was slightly delayed in virulence compared to WT UW551 (Fig. 3A) ($P < 0.001$; repeated-measures ANOVA). The popS mutant also colonized resistant H7996 tomato stems significantly more slowly than its wild-type parent ($P < 0.03$; Mann-Whitney test), although its population size reached wild-type levels by 96 h postinoculation (Fig. 3C). Complementing the popS mutant by adding a single copy of popS under the control of its native promoter restored the ability of the popS mutant to both wilt and colonize tomato stems ($P < 0.05$; Mann-Whitney test) (Fig. 3C). These results suggest that PopS is required for bacterial success in planta after the early stages of root infection. This result is congruent with a previous observation that a popS mutant of phytophthora I strain GM11000 had reduced fitness in eggplant leaves (36), although there are significant biological differences between the apoplast and xylem tissue.

As expected (16), the T3SS-deficient hrcC mutant was avirulent and did not effectively colonize either susceptible or resistant tomato stems (Fig. 3). The hrcC mutant never reached population sizes greater than $1.5 \times 10^7$ CFU/g stem on either host. Population sizes of the hrcC mutant declined gradually over the 4 days of the assay to $1.6 \times 10^6$ CFU/g stem.

**The popS mutant induced higher SA defenses in plant roots.** Following infection by pathogens, plant tissues accumulate SA, which induces expression of several PR defense genes (6, 9). Specifically, tomato plants upregulate the SA-mediated PR genes PR-1a and PR-1b in response to infection by R. solanacearum (37). Effectors AvrE in P. syringae pv. tomato DC3000 and DspEsp in E. amylovora both suppress plant defenses mediated by SA (12). Because UW551 PopS shares 23% amino acid identity with DspEsp and AvrE, we tested the hypothesis that it similarly suppresses SA-mediated host defense gene expression.

We measured expression of PR-1a and PR-1b in roots of moderately resistant H7996 tomato inoculated with GM11000, UW551, the UW551 popS mutant, or a water control. Twenty-fours hours postinoculation, plants inoculated with wild-type strains GM11000 or UW551, respectively, increased expression of PR-1a by 2.4- and 4.9-fold and PR-1b by 2.5- and 3.2-fold (Fig. 4A and B). This is consistent with our previous finding that UW551 triggers a faster response in H7996 than GM11000 (37). Plants inoculated with the UW551 popS mutant had much higher levels of PR-1a (15.8-fold increase) and PR-1b (13.3-fold increase) than those inoculated with WT (Fig. 4A and B). This result indicates that PopS functions to suppress expression of host plant SA-mediated defense genes. Complementation of the popS mutant with the wild-type popS locus restored the ability of the mutant to suppress tomato SA-mediated defenses (see Fig. S1 in the supplemental material).

**SA-treated tomato plants have increased resistance to a popS mutant.** Because roots upregulated PR-1a and PR-1b in response to the UW551 popS mutant and SA induces tomato PR defense gene expression (6, 38, 39), we predicted that pretreating tomato plants with SA would specifically increase their resistance to the UW551 popS mutant. We primed the SA defenses by soaking the soil of unwounded susceptible tomato plants (cv. Bonny Best) with 7.5 mM sodium salicylate (for an estimated soil concentration of 0.75 mM SA/g soil) 6 h before inoculating the plants with either UW551 WT or the popS mutant. As predicted, plants pretreated with 7.5 mM sodium salicylate upregulated the SA-triggered defense genes PR-1a and PR-1b relative to water-treated control plants (Fig. 4C). SA treatment did not trigger expression of ethylene- or jasmonic acid-dependent defense genes ACO5 or pin2, respectively (Fig. 4C), suggesting that PR-1a and PR-1b induction is specific to SA.

SA treatment delayed wilt symptom development in plants inoculated with UW551 WT by 2 days compared to water-treated controls (Fig. 4D and E). By the end of the assay, SA-treated tomato plants inoculated with WT UW551 wilted all tomato plants (Fig. 4E). This demonstrates that SA triggers defenses that increase plant resistance to R. solanacearum.
PopS is required to overcome SA-mediated defense induction. (A and B) Expression of tomato SA-induced PR defense genes increases in response to a popS mutant of R. solanacearum. Quantitative reverse transcriptase PCR was used to measure expression of plant defense genes in roots of the resistant tomato strain H7996 24 h after inoculation with R. solanacearum phytophthora I strain GMI1000, phytophthora II strain UW551 (WT), or the UW551 popS mutant. Expression of PR-1a (A) and PR-1b (B) was normalized to that of the tomato GAPDH gene, and the change in expression was determined using the ΔΔCt method comparing pathogen-treated plants to water-inoculated control plants. Results reflect two replicates, each including 10 to 13 pooled roots per treatment; error bars indicate standard errors. (C) Expression of tomato defense genes PR-1a, PR-1b, ACO5, and pin2 was measured by qRT-PCR 6 h after soil soak treatment of Money-maker tomato plants with 0.75 mM SA; RNA was extracted from midstem tissue, and expression levels are shown relative to those of water-treated controls. Results shown are the averages of two replicates, each including 5 pooled stem samples per treatment; bars indicate standard errors. (D and E) Treating plants with SA exacerbates the virulence delay of the popS mutant. Average symptom development of susceptible tomato plants (cv. Bonny Best) that were soil soak inoculated with approximately 1 × 10⁸ CFU/g soil of R. solanacearum strain UW551 (WT) (open bars) or the UW551 popS mutant (filled bars). Six hours preinoculation, roots of the plants were drenched with either water (D) or 0.75 mM sodium salicylate (E) (10 plants per strain per treatment). A representative of two replicates is shown. By the end of the assay, all plants treated with water and WT UW551 or the popS mutant or with SA and WT UW551 were completely wilted, and 40% of plants treated with SA and the UW551 popS mutant were asymptomatic (P = 0.036; Student’s t test). Of these, 75% were colonized with >10⁹ CFU/g and 25% contained no detectable bacteria. (F and G) Stems of susceptible cv. Money maker tomato plants (F) or a SA-deficient NahG transgenic derivative of Money-maker (G) were inoculated through cut petioles with 40,000 cells of wild-type R. solanacearum strain UW551 (white), a popS effector mutant (gray), or a T3SS-deficient hrcC mutant (black). Columns represent the average bacterial population sizes (CFU/g stem) of 5 plants per treatment at each time point, determined by dilution plating ground stem tissue 24 and 48 hpi; error bars indicate standard errors. Asterisks represent statistically significant differences between wild-type UW551 and the popS mutant or wild-type UW551 and the hrcC mutant (Mann-Whitney test).
Interestingly, pretreatment with SA significantly exacerbated the popS mutant virulence defect. SA-treated plants that were inoculated with the popS mutant never reached WT levels of disease (P = 0.0336; Student’s t test) (Fig. 4E). In fact, 40% of SA-treated tomato plants inoculated with the UW551 popS mutant remained asymptomatic. Quantification of bacterial populations in these plants showed that three of the four asymptomatic plants harbored large R. solanacearum populations (average, 10^{10} CFU/g stem) but the remaining plant contained no detectable R. solanacearum cells. This observation was consistent across replicates (data not shown). In response to the popS mutant, SA-primed roots had decreased rates of initial stem infection and also delayed symptom development. These SA treatment experiments offer further evidence that R. solanacearum uses PopS to overcome SA-induced defenses.

SA-deficient NahG tomato plants restore the colonization defect of the popS mutant. If the function of PopS is to repress SA-mediated defenses, then reduced levels of SA in planta should allow a popS mutant to be more successful. We tested this hypothesis using transgenic Money-maker tomato plants expressing nahG, which encodes a bacterial salicylate hydroxylase that degrades salicylic acid and reduces SA-mediated defenses (40–42). We measured growth of WT UW551, the popS mutant, and the T3 secretion-deficient hrcC mutant in petiole-inoculated stems of wilt-susceptible cv. Money-maker and an isogenic SA-deficient NahG transgenic line. Both the popS and hrcC mutants were significantly delayed in colonization of nontransgenic Money-maker (P < 0.05 and P = 0.004, respectively; Mann-Whitney test) (Fig. 4F); after 48 h, the popS mutant grew to 5.6 × 10^{8} CFU/g stem, compared to 1.5 × 10^{8} CFU/g stem for the wild-type strain (Fig. 4F). These results demonstrated that Money-maker and the susceptible cultivar Bonny Best respond similarly to these R. solanacearum strains (Fig. 3C). The hrcC mutant grew equally poorly in both tomato lines, indicating that absence of SA alone is not enough to restore the stem growth defect of a completely T3SS-deficient strain (Fig. 4F and G). However, the UW551 popS mutant grew as well as its wild-type parent in the NahG tomato lines, indicating that absence of SA alone is not enough to restore the stem growth defect of a completely popS mutant.

PopS does not elicit cell death in Nicotiana benthamiana. Some AvrE orthologs possess not only the ability to suppress SA-mediated defenses but also to cause cell death when they are expressed transiently in leaf tissue of Nicotiana benthamiana (14, 43). AvrE-like proteins contain conserved WXXXE motifs; at least two of these motifs are required to trigger cell death or for virulence (14, 43). A multiple alignment of PopS and other AvrE orthologs revealed that PopS contains all conserved tryptophans shown to be important for function in other AvrE family members (Fig. 5A) (14, 43). To determine if R. solanacearum PopS elicits plant cell death, we transiently expressed a C-terminally hemagglutinin (HA)-tagged PopS (PopS-HA) via Agrobacterium tumefaciens (14, 43). AvrE ortholog sequences from strains (NCBI sequence reference) analyzed include R. solanacearum UW551 (ZP_00944047.1), P. syringae pv. tomato DC3000 (NP_791204.1), P. stewartii subsp. stewartii (AAG1467.2), P. carotovorum subsp. carotovorum (CW2790:NP_058733.1, WPP14:NP_058733.1, WPP38:NP_058733.1, and WPP15:NP_058733.1), and P. carotovorum subsp. carotovorum (JN3125:NP_058733.1, WPP14:NP_058733.1, WPP38:NP_058733.1, and WPP15:NP_058733.1). Conserved tryptophans known to be important for virulence or cell death activity (14, 43) are highlighted in red. (B to D) Agrobacterium tumefaciens-mediated transient expression in N. tabacum (B and C) and N. benthamiana (D). Leaves were infiltrated with A. tumefaciens pGW14::popS (PopS-HA), A. tumefaciens pGW14::DspE (DspE from P. carotovorum [Pcc] WPP14) as a positive control, or buffer as a negative control (neg.). Plant symptoms were imaged 48 h postinoculation. In panel C, trypan blue staining shows cell death caused by DspE. Each infiltration was repeated for at least three biological replicates. (E) Western blot analysis of PopS-HA (pGW14::popS) or negative control (pGW14::empty) from N. benthamiana leaf tissue.

**DISCUSSION**

It is well established that T3-secreted effectors are essential for R. solanacearum virulence (16), but the biological roles of specific effectors remain unexplored. In this study, we characterized the function of PopS, an AvrE family effector that is present...
throughout the *R. solanacearum* species complex. This effector family is widely conserved among plant-pathogenic bacteria, but its members make various contributions to pathogenesis (12–14, 45). In enteric plant pathogens such as *E. amylovora* and *P. carotovorum*, disrupting *dspE* renders the pathogen completely avirulent (44–46). In contrast, *avrE* mutants of *P. syringae* pv. *tomato* have no detectable colonization or virulence defects, although *AvrE* apparently works with other effectors, such as HopM1, to suppress host immunity and facilitate pathogenesis (12). *PopS* falls in the middle of this functional spectrum, because *popS* mutants are significantly delayed in virulence and plant colonization but can still cause bacterial wilt disease. The virulence and colonization defects of the *popS* mutant suggest that none of *R. solanacearum*’s more than 70 putative effectors is fully redundant with *PopS* activity (2, 22, 47). Nonetheless, a completely *T3SS*-deficient *hrcC* mutant was much less able to colonize plants than the *popS* mutant, confirming that additional T3-secreted effectors contribute to this process. As shown for *P. syringae* pv. *tomato*, multiple effector polymutants may identify those effectors that promote colonization and wilt in the absence of *PopS* (5, 13). Overall, the *AvrE* family’s wide conservatism and consistent role in virulence suggest that this effector has ancient origins in the evolutionary history of bacterial plant pathogens.

We determined that *PopS* contributes measurably to *R. solanacearum* virulence on several different hosts in the genus *Solanum*. Most strains of this broad-host-range pathogen have multiple effector families (e.g., GALA and AWR), whose homologs together potentiate virulence on solanaceous crop hosts such as tomato and eggplant (48–50). For example, individual GALA-family effectors are not required for full virulence on solanaceous hosts, but deleting three or more GALA effector genes delays wilt on tomato and eggplant (48, 49). *PopS* is a single-copy effector present in all members of the species complex, and our data indicate that it is needed for success on two agriculturally important *Solanum* hosts. Notably, *PopS* was dispensable for virulence on *S. dulcamara*, a common weed that can shelter and disseminate *R. solanacearum* (51). This difference suggests that *PopS* can have plant species-specific activity. As a result of selection pressures in natural ecosystems, wild hosts like *S. dulcamara* may have evolved to avoid *PopS* activity by modifying or eliminating the *PopS* target. Further studies are needed to define the specific mechanisms that permit *S. dulcamara* to resist *PopS*.

The *popS* mutant had the largest virulence defect on moderately wilt-resistant H7996 tomato, which upregulated its SA-induced *PR* defense genes to a greater degree in response to the *popS* mutant than in response to wild-type UW551. No such difference was observed in the response of roots of susceptible cv. Bonny Best (data not shown). We previously found that after infection by UW551, H7996 upregulates SA-mediated defense gene expression faster than Bonny Best (37). Consistent with this previous observation, we detected no differences in expression of *PR-1a* and *PR-1b* in Bonny Best roots inoculated with wild-type UW551 or the *popS* mutant (data not shown). We suspect that the larger virulence defect of the *popS* mutant on H7996 is directly correlated to the magnitude and timing of the defense signaling in H7996. This hypothesis was supported by our finding that susceptible tomato plants were more resistant to infection by the *popS* mutant when roots were pretreated with SA, which induces *PR* gene expression. It seems likely that *PopS*, like other *AvrE*-like effectors (12–14), also suppresses SA-induced immune responses, such as callose deposition, that are triggered by recognition of MAMPs. The specific *R. solanacearum* MAMPs are undetermined, but purified *R. solanacearum* exopolysaccharide (EPS), a conserved and essential virulence factor, triggers increased *PR* gene expression in quantitatively resistant H7996 but not in susceptible Bonny Best (37). Further studies are needed to determine how *R. solanacearum*’s T3 effectors suppress MAMP- and EPS-triggered plant defenses.

The tomato pathogenesis-related protein *PR-1a* is triggered by SA (52). *PR-1b* has been described in the literature as ethylene responsive (53–55). Based on this, we previously used *PR-1b* as a marker of ethylene pathway activation (37). However, *PR-1b* has also been described as SA responsive (56, 57) and there is some evidence that it is upregulated under both conditions (52, 58, 59). We therefore directly tested the effect of SA exposure on expression of this gene in H7996 tomato. This experiment revealed that under our conditions, both *PR-1a* and *PR-1b* are upregulated around 35-fold in response to SA treatment (Fig. 4C).

The importance of *PopS* for tomato plant stem colonization and wilt is consistent with our previous observation that many *R. solanacearum* T3SS genes are highly expressed at midstage disease in planta (19). Further, SA-induced defenses are not expressed in tomato stems until *R. solanacearum* reaches 10⁶ CFU/g stem (37). Together these results affirm that T3 effectors are active not only at low pathogen cell densities early in colonization, as previously suggested (60, 61), but also at a later stage in the disease cycle. Between initial root infection and the end-stage collapse and death of the plant, *R. solanacearum* primarily inhabits the xylem elements, which are composed of nonliving tracheids; this raises the question of where T3SS effectors might act during midstage wilt disease. It has been suggested that bacteria in xylem elements inject effectors into the living xylem parenchyma cells that are adjacent to tracheids and accessible through the pits in xylem cell walls (19).

As expected (16), the T3SS-deficient UW551 *hrcC* mutant could not reach the 10⁶-CFU/g cell densities in stems required for bacterial wilt symptom development (62) and *hrcC* populations declined in tomato stems over time. This suggests that the T3SS is important not only for growth but also for persistence in planta. Both animal- and plant-pathogenic *Pseudomonas* spp. use the T3SS to persist in host tissue (63–66). Unlike the *popS* mutant, growth of the *hrcC* mutant was not restored in SA-deficient plants. We suspect that the constraints that limit success of the *hrcC* mutant include an inability to overcome basal immunity (1) or manipulate host physiology (4).

To the best of our knowledge, this is the first study to explore the defense-suppressing functions of an *R. solanacearum* effector in roots and stems, which are the important niches for *R. solanacearum* during natural pathogenesis. Using *PopS* as an example, we propose a model for effector functions during the bacterial wilt disease cycle where *R. solanacearum* uses T3 effectors for (i) root invasion and colonization, (ii) suppression of root defenses, (iii) stem colonization and growth, and (iv) induction of wilt symptoms. Further studies using adjustable promoters or inducible deletion mutations could reveal when these virulence traits are required during the disease process.

Three independent lines of evidence supported our conclusion that *PopS* suppresses SA-mediated defenses. First, a *popS* mutant strain elicited 3– to 4-fold-higher expression of SA-triggered *PR* genes in tomato than WT UW551. Second, pretreating tomato
plants with SA increased the magnitude of the popS mutant’s virulence defect, as would be expected if that defect resulted from an inability to modulate SA-triggered plant defenses. Third, PopS was dispensable for colonization of SA-defective NahG transgenic tomato, as would be predicted if the popS mutant’s colonization defect was caused by SA-mediated plant defenses. These results suggest that AvrE-family effectors generally function to suppress SA-mediated defenses, in beta- as well as gammaproteobacteria. Although PopS shares 23% amino acid sequence identity with its closest AvrE and DspE orthologs, protein sequence alignments revealed many scattered, moderately conserved regions, including the three conserved tryptophans important for the virulence activity in other gammaproteobacterial AvrE homologs (14, 43). Given the diversity of hosts that can be manipulated by AvrE-family proteins, it seems likely that this effector family interacts with a broadly conserved element of the plant defense system that indirectly or directly impacts SA-mediated responses.

Most surprisingly, we determined that PopS does not elicit cell death when transiently expressed in leaf tissue, unlike other AvrE homologs from hemibiotrophic and necrotrophic pathogens. Of the many AvrE-containing plant pathogens studied to date, R. solanacearum is the only one that causes a nonnecrotic wilt. As a biotroph, it multiplies to high cell densities in the xylem without causing necrosis. We speculate that as AvrE, WtsE, DspE-popS, and PopS diverged from a common ancestor, they adapted to the pathogenic lifestyles (necrotrophy, hemibiotrophy, and biotrophy) of each bacterium (Fig. 6). T3 effectors from hemibiotrophic Pseudomonas induce cell death in host tissue more often than effectors from biotrophic R. solanacearum (67), which suggests that in general, T3 effectors may function differently based on a pathogen’s lifestyle. Necrotrophs such as P. carotovorum subsp. carotovorum kill host tissue upon contact and during multiplication. P. carotovorum subsp. carotovorum DspE-popS elicits cell death but does not suppress SA-mediated defenses (43, 44) (Fig. 6), which is consistent with the observation that SA is not a major signal associated with necrotrophic infection. AvrE possessing hemibiotrophs (e.g., P. syringae pv. tomato and P. s. subsp. stewartii) multiply in living host tissue but elicit necrosis during pathogenesis. Their AvrE homologs (AvrE and WtsE, respectively) not only elicit plant cell death when heterologously introduced into plant tissue but also indirectly or directly suppress SA-mediated defenses, which are known to defend plants against biotrophs and hemibiotrophs (Fig. 6) (12, 14; S. Y. He, personal communication). It remains to be determined if P. syringae HopR, the closest ortholog of PopS, causes cell death like AvrE, although it also suppresses SA-mediated defenses (13). R. solanacearum does not cause necrosis during infection and wilt, placing it on the biotrophic end of the spectrum. PopS does not elicit plant cell death but suppresses SA-mediated plant defenses, consistent with the biology of R. solanacearum. Functional studies of chimeric AvrE homologs and heterologous complementation across pathogens could reveal the specific domains that contribute to the distinct and common biological activities of this conserved effector.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The bacterial strains used in this study are listed in Table 1. *Escherichia coli* was grown at 37°C in Luria-Bertani medium (68). *R. solanacearum* was cultivated at 28°C on rich Casamino Acids-peptone-glucose (CPG) medium (pH 7.0) (69). When required, media were supplemented with kanamycin (Km) (25 μg/ml), gentamicin (Gm) (15 μg/ml), tetracycline (Tc) (15 μg/ml), or rifampin (Rif) (25 μg/ml).

**Phylogenetic analysis.** Phylogenetic trees of PopS and AvrE/DspE/HopR orthologs were created with MEGA5 (25). The amino acid or gene sequences of PopS orthologs from NCBI or MAGE databases were aligned with CLUSTAL-W, from which a maximum-likelihood phylogenetic tree was adapted from Fig. 1. DspE from the necrotroph *P. carotovorum* subsp. *carotovorum* (Pcc) causes cell death when transiently expressed in plant tissue but does not suppress SA-mediated defenses (43, 44). WtsE and AvrE from the hemibiotrophs *P. stewartii* subsp. *stewartii* (Pss) and *P. syringae* pv. tomato (Pst), respectively, cause cell death when heterologously introduced into plant tissue and suppress SA-mediated defenses against virulence (12, 14). *R. solanacearum* (Rs) is a nonnecrotic wilt pathogen. We found that PopS does not elicit cell death when expressed in plant tissue but does suppress SA-mediated defenses during tomato wilt. This model suggests that these effectors have undergone adaptation to the pathogenic lifestyles of their respective microbes.
Aqu unwounded plants were soaked with a water suspension of UW551 or
joining cluster analysis. The complex tree was created from a MUMi distance matrix using neighbor-
as well as a gain/loss of DNA segments (34). The R. solanacearum distances by considering divergence of the
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grinding and dilution plating stem segments on appropriate antibiotic
resistant H7996 tomato. Bacterial colonization was quantified daily by
7.5 mM SA or sterile water as described above.
10 to 14 plants per treatment. The effect of salicylic acid on defense gene
of leaves wilted). Each assay from two independent experiments included
scale from 0 to 4 (0, no wilt symptoms; 1, 1 to 25% of leaves wilted; 2, 26
to 50% of leaves wilted; 3, 51 to 75% leaves of wilted; and 4, 76% to 100% of
leaves wilted). Each assay from two independent experiments included 10
plasms for whole-genome analysis were computed for each pair of se-
sequenced genomes of R. solanacearum using the web server (http://genome.
jouy.inra.fr/mumi/index.cgi). Briefly, the MUMI estimated the genomic
distances by considering divergence of the R. solanacearum core genome as well as a gain/loss of DNA segments (34). The R. solanacearum species
cplex tree was created from a MUMI distance matrix using neighbor-
joining cluster analysis.
Plant assays. To evaluate pathogen virulence, pots containing individ-
ual unwounded plants were soaked with a water suspension of UW551 or
or the popS mutant to create a final inoculum density of 1 × 10^6 CFU/g soil.
Hosts included 17-day-old susceptible tomato plants (cv. Bonny Best),
14- to 16-day-old moderately resistant Hawaii 7996 tomato plants, 21-
day-old potato plants (cv. Russet Norkotah) grown from minitubers, and
18- to 19-day-old bittersweet nightshade plants (Solanum dulcamara). Each
treatment contained 14 plants and was repeated three times, except
potato, which represents one biological replicate. Inoculated plants were
incubated at 28°C with a 12-h light cycle. To evaluate the impact of sali-
cylic acid on disease development, susceptible tomato (cv. Bonny Best)
plants were pretreated with either 50 ml water or 50 ml 7.5 mM sodium
salcylic acid in water, followed 6 h later by soil soak inoculation with either
UW551 WT or the popS mutant. Disease was rated daily on a disease index
scale from 0 to 4 (0, no wilt symptoms; 1, 1 to 25% of leaves wilted; 2, 26
up to 50% of leaves wilted; 3, 51 to 75% leaves of wilted; and 4, 76% to 100% of
leaves wilted). Each essay from two independent experiments included 10
plants per treatment. The effect of salicylic acid on defense gene
expression was measured by extracting RNA as described below from
Tomato RNA was extracted and purified from pooled tissue samples
in liquid nitrogen using an RNeasy minikit (Qiagen, Valencia,
were performed in 50-ml conical tubes, and seeds were incubated on a
shaker at 200 rpm at room temperature. Seeds were then rinsed 5 to 7
times with sterile water to remove residual ethanol. For uniform germi-
nation, seeds were stored at 4°C overnight in the dark in water and then
germinated on 1% water agar for 48 h at room temperature in the dark.
Germinated seedlings were transferred to plates containing 1% agar and
0.5% Murashige and Skoog basal salts medium plus Gambourg’s vitamins
(MS medium) (MP Biomedical, Santa Ana, CA) and incubated for 2 days
at 28°C with a 12-h light cycle. Root tips were inoculated with 2 µl con-
taining 2 × 10^6 CFU/ml of either GMI10000, WT UW551, or the popS
mutant. Plant root tissue was harvested 2.54 cm from the inoculation
point 24 hpi, immediately frozen in liquid nitrogen, and stored at −80°C.
Results are averages of data from 7 to 12 plants per treatment.
Tomato RNA was extracted and purified from pooled tissue samples
ground in liquid nitrogen using an RNeasy minikit (Qiagen, Valencia,
followed the manufacturer’s instructions, except that initial flow-
through was applied to column twice. RNA was eluted in 30
CA) following the manufacturer’s instructions, except that initial flow-
(defined by RNA integrity numbers greater than 8.0) and high purity
(defined as A_{260/230} and A_{260/280} of >1.9) were used for analysis.
Plant defense gene expression from tomato roots and stems was mea-
using quantitative reverse transcription PCR (qRT-PCR). One mi-

| Strain or plasmid | Genotype and characteristics | Source or reference |
|-------------------|-----------------------------|---------------------|
| Escherichia coli Top10 | F' mcrA Δ(mrr-heatRMS-mcrBC) 430lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL endA1 supG4 ΔSm' | Life Technologies |
| Agrobacterium tumefaciens GV3101 | Gm' Rif' | 75 |
| Ralstonia solanacearum | | |
| UW551 | Wild-type geranium isolate; phylootype II, sequevar 1 | 76 |
| RSW19 | UW551 popS::Km' | This study |
| RSW35 | UW551 popS::Km'; pUC18-miniTn7T-Gm-GW::popS Km' Gm' | This study |
| RSW36 | UW551 hrcC::Gm', type III secretion deficient, Gm' | This study |
| Plasmids | | |
| pSTBlue-1 | Cloning vector, Ap' Km' | EMD Bioscience |
| pSUP202 | Cloning vector, Ap' Km' Te' Cm' | 71 |
| pUCGM | Ap' Gm' | 77 |
| pENTR/D-TOPO | Cloning vector, Km'; Gateway (Life Technologies) | Life Technologies |
| pGW2B | Expression vector, 355 promoter, Km' | 78 |
| pGW14 | Expression vector, 355 promoter, C-terminal HA, Km' | 78 |
| pUC18-miniTn7T-Gm-GW | Complementation vector, Gm'; Gateway (Life Technologies) | 79 |
| pTNS1 | Helper plasmid for transposition; Ap' | 79 |
| ECW34 | pSUP202::popS::Km' Ap' Km' Cm' | This study |
| ECW35 | pSUP202::hrcC::Gm'; Ap' Gm' Cm' | This study |
| pENTR/D-TOPO::popS | –6.5-kb fragment containing UW551 popS with its native | This study |
| pUC18-miniTn7T-Gm-GW::popS | promoter cloned into pENTR/D-TOPO; Km' | |
| pUC18-miniTn7T-Gm-GW::popS | promoter cloned into pUC18-miniTn7T-Gm-GW; Gm' | |
| pGW14::popS | –5.2-kb gene encoding UW551 PopS cloned into pGW14; Km' | This study |
| pGW2B::dspE | –4.9-kb gene encoding Pectobacterium carotovorum | 43 |

Virulence Factor PopS Suppresses SA Defenses
crogram of total RNA per sample was reverse transcribed using SuperScript III reverse transcriptase first-strand synthesis Supermix (Life Technologies, Carlsbad, CA) with oligo(dT) and random hexamer primers, following the manufacturer’s protocol. qRT-PCR was performed in duplicate with 1× PowerSYBR green master mix (Life Technologies, Carlsbad, CA), 400 nM forward and reverse primers, and 50 ng cDNA template for a final volume of 25 µL. The reaction conditions were as follows: 10 min polymerase activation and 40 cycles of 95°C for 15 s and 57°C for 1 min. Relative gene expression was quantified for the tomato defense genes PR-1a and PR-1b using previously described primers (52) and normalized to that of a reference gene (GAPDH). Relative expression of treatment compared to control was defined using the ∆∆CT method (73).

4. Transient expression of Posp in leaf tissue. The popS gene was amplified as described above and inserted into Gateway vector pENTR/D-TOPO following the manufacturer’s instructions (Life Technologies, Carlsbad, CA). The popS gene was inserted into expression vector pGWB14 for C-terminal HA fusion protein expression using LR cloning (Life Technologies, Carlsbad, CA). The resulting plasmid, pGWB14::popS, was confirmed with sequencing and transformed into Agrobacterium tumefaciens, followed by selection with the appropriate antibiotics. Leaves from ~30-day-old N. benthamiana and N. tabacum plants were infiltrated with either buffer control, A. tumefaciens pGWB2::dspP::popS (positive control) (43), A. tumefaciens pGWB14 (empty vector control), or A. tumefaciens pGWB14::popS. Leaf symptoms were observed and captured by scanning leaves at 48 h postinoculation. To verify the visible cell death elicited by DspE, plant leaves were stained with trypan blue as previously described (74). Each treatment was carried out in triplicate over three independent experiments.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00875-13/-/DCSupplemental.

Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.
Figure S1, DOCX file, 0.1 MB.

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