RALSTONIA SOLANACEARUM REQUIRES POPS, AN ANCIENT AVR-E-FAMILY EFFCTOR, 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 DspEVC from the necrotroph Pectobacterium carotovorum subsp. 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.
were based on comparative analysis of whole genomes of 11 representative sequenced strains. (A) PopS, HopR (XopAM) from *Xanthomonas*
2 *Pseudomonas* sp.- (Pseudomonas sp.- (HopR)) from *R. solanacearum* pv. *suppress defense signaling* (12–14). *AvrE* from *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* 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 *P. syringae* in two ecologically and phylogenetically distinct strains. This locus (*RRSL_03375* in strain UW551 and *RSp1281* in strain GM11000) 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 GM11000 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. crop hosts. PopS was dispensable for virulence on a weed, *Solanum dulcamara*, suggesting 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* 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

The conserved, vertically inherited gene is potentially useful to distinguish the stem colonization process from root colonization. To study the virulence function of this effector, we disrupted popS in strain UW551 (phylotype 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 Kmr cassette did not disrupt expression of the scrK sucrose kinase gene (RRSL_03374) immediately downstream of popS. Quantitative reverse transcription 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. Lear), 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 distinguish the stem colonization process from root colonization, we compared the rates at which strain UW551 WT and the popS mutant wilted and colonized the stems of wilt-resistant tomato plants. Virulence and colonization rates for a completely T3SS-deficient hrcC mutant were also measured. To distinguish the stem colonization process from root invasion, tomato stems were directly inoculated through a cut

**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 (phylotype 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 Kmr cassette did not disrupt expression of the scrK sucrose kinase gene (RRSL_03374) immediately downstream of popS. Quantitative reverse transcription 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. Lear), 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).

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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. 3). 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. 3). 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 phylotype I strain GMII1000 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 populations greater than 1.5 × 10⁷ CFU/g stem on either host. Population sizes of the hrcC mutant declined gradually over the 4 days of the assay to 1.6 × 10⁶ 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 DspE₉ in *E. amylovora* both suppress plant defenses mediated by SA (12). Because UW551 PopS shares 23% amino acid identity with DspE₉ 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 GMII1000, UW551, the UW551 popS mutant, or a water control. Twenty-four hours postinoculation, plants inoculated with wild-type strains GMII1000 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 GMII1000 (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 *ACOS* 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*. 

![Image](mbio.asm.org on May 18, 2015 - Published by mbio.asm.org Downloaded from)
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* phylotype I strain GMI1000, phylotype 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 × 10^8 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* = 0.0336; Student’s t test). Of these, 75% were colonized with >10^10 CFU/g and 25% contained no detectable bacteria. (F and G) Stems of susceptible cv. Money maker (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 per 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-primated 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 × 10^9 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 plant stems (Fig. 4G). Thus, an SA-deficient plant host could restore the popS mutant’s colonization rate to wild-type levels, offering independent evidence that a direct or indirect function of PopS is to suppress SA-mediated plant defenses.

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 WXXXXE 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 known 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). Our AvrE homologs (PopS, AvrE_{pop}, WtsE_{pop}, and DspE_{pop}) are 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

**FIG 5** Transient expression of PopS in N. benthamiana leaves did not induce cell death. (A) Sequence alignment based on T-Coffee analysis of AvrE orthologs (PopS_{pop}, AvrE_{pop}, WtsE_{pop}, and DspE_{pop}). 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 (AAAG1467.2), P. carotovorum subsp. carotovorum WPP14 (ZP_03833468.1). 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 pgWB14::popS (PopS-HA), A. tumefaciens pgWB2::dspE (DspE from P. carotovorum subsp. carotovorum [Pcc] WPP14) as a positive control, A. tumefaciens pgWB14 (empty vector 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 (pgWB14::popS) or negative control (pgWB14; empty) from N. benthamiana leaf tissue.
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 conservation 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 calllose 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 inhales 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 tryptophan 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
 subsp. 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. E. coli hrcC mutant (hrcC::Kmr) was created. The ORF popS from UW551 genomic DNA and pSTBlue-1, respectively, using Phusion high-fidelity DNA polymerase (Finnzymes, Vantaa, Finland). The Kmr cassette underwent adaptation to the pathogenic lifestyles of their respective microbes.

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).

Recombinant DNA techniques and mutagenesis. Genomic and plasmid DNA was isolated by standard protocols (68). E. coli and R. solanacearum were transformed as previously described (68). PCR primer sequences are listed in Table S2 in the supplemental material. To disrupt the R. solanacearum UW551 locus RRSI_03375 (popS), flanking regions from popS and a kanamycin resistance (Kmr) cassette were amplified from UW551 genomic DNA and pSTBlue-1, respectively, using Phusion high-fidelity DNA polymerase (Finnzymes, Vantaa, Finland). The Kmr cassette was inserted between the popS-flanking fragments by splicing by overlap extension (SOE)-PCR (70). Similar methods were employed to create a hrcC mutant (hrcC::Kmr), except that a Gm cassette amplified from vector pUCGM was inserted in the regions flanking hrcC. The resulting SOE-PCR product was gel purified, phosphorylated with T4 polynucleotide kinase (Promega, Madison, WI), and ligated into the EcoRV site of cloning vector pSUP202 (71) to create pSUP202-popS::Kmr. Wild-type UW551 was transformed with pSUP202-popS::Kmr, and double recombinant mutants were selected for Km resistance but screened for Tc to ensure proper allelic exchange. To complement the R. solanacearum popS::Kmr mutant, popS and its upstream native promoter were amplified from the UW551 genome and directly inserted into pENTR/D-TOPO (Life Technologies, Carlsbad, CA) following the manufacturer’s instructions. UW551 popS was transferred to complementation vector pUC18-miniTn7-Gm-GW via LR Gateway cloning as described by the manufacturer’s protocol (Life Technologies, Carlsbad, CA). R. solanacearum popS::Kmr mutant competent cells were transformed with pUC18-miniTn7-Gm-GW and Tn7 helper plasmid pTNS1 as previously described (72). Mutations were confirmed with PCR.

Phylogenetic analysis. Phylogenetic trees of PopS and AvrE/DspE/HopR orthologs were created with MEGAS (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 created. The R. solanacearum strains used in this analysis are listed in Table S2 in the supplemental material. The percentage of replicate trees in which individual orthologs clustered together in the bootstrap test (200 replicates) was calculated and noted at each branch. MUM index (MUMI)
differences for whole-genome analysis were computed for each pair of 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 complex tree was created from a MUMi distance matrix using neighbor-joining cluster analysis.

**Plant assays.** To evaluate pathogen virulence, pots containing individual unwounded plants were soaked with a water suspension of UW551 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), 18- to 19-day-old bittersweet nightshade plants (*Solanum dulcamara*), 14- to 16-day-old moderately resistant Hawaii 7996 tomato plants, 21-day-old potato plants (cv. Russet Norkotah) grown from minitubers, and 21- to 22-day-old moderately resistant Hawaii 7996 tomato plants, 21- to 22-day-old potato plants (cv. Russet Norkotah) grown from minitubers, and 21- to 22-day-old potato plants (cv. Russet Norkotah) grown from minitubers.

*E. coli Top10* F′ mcrA Δ(mrr-hsdRMS-mcrBC) q80lacZΔM15 ΔlacX74 recA1 araD139 Δ ara-leu)7697 galU galK rpsL endA1 nupG A− Smr

*Agrobacterium tumefaciens GV3101* Gmr Rifr

*Ralstonia solanacearum* UW551 Wild-type geranium isolate; phytootype II, sequevar 1

RSW19 UW551 popS::Kmr Kmr

RSW35 UW551 popS::Kmr pUC18-miniTn7T-Gm-GW::popS Km r Gmr

RSW36 UW551 hrcC::Gmr, type III secretion deficient, Gmr

Plasmids pSTBlue-1 Cloning vector, Ap r Km r EMD Bioscience

pSUP202 Cloning vector, Ap r Km r Tc r Cmr

pUCGM Cloning vector, Km r; Gateway (Life Technologies)

pENTR/D-TOPO Expression vector, 35S promoter, Km r Life Technologies

pGW2 Cloning vector, Km r; Gateway (Life Technologies)

pGW14 Expression vector, 35S promoter, C-terminal HA, Km r

pUC18-miniTn7-Gm-GW Complementation vector, Gmr; Gateway (Life Technologies)

pTNS1 Helper plasmid for transposition; Ap r

ECW34 pSUP202::popS Km r Ap r Km r Cmr

ECW35 pSUP202::hrcC::Gmr Ap r Km r Cmr

pENTR/D-TOPO::popS −6.5-kb fragment containing UW551 popS with its native promoter cloned into pENTR/D-TOPO; Km r

pUC18-miniTn7-Gm-GW::popS −6.5-kb fragment containing UW551 popS with its native promoter cloned into pUC18-miniTn7-Gm-GW; Gmr

pGW14::popS −5.2-kb gene encoding UW551 PopS cloned into pGW14; Km r

pGW2B:: dspE −4.9-kb gene encoding *Pectobacterium carotovorum* subsp. *carotovorum* DspE

*Virulence Factor PopS Suppresses SA Defenses*

Plants were pretreated with either 50 ml water or 50 ml 7.5 mM sodium salicylate 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 to 50% of leaves wilted; 3, 51 to 75% of leaves wilted; and 4, 76% to 100% of leaves wilted). Each assay from two independent experiments included 10 to 14 plants per treatment. The effect of salicylic acid on defense gene expression in tomato was measured by extracting RNA as described below from Money-maker tomato stems 6 h after soil treatment with either 7.5 mM SA or sterile water as described above.

To measure bacterial colonization, plant stems were inoculated by applying a droplet of bacterial suspension to the cut petiole of the first true leaf. Plant stems were inoculated by applying a droplet of bacterial suspension to the cut petiole of the first true leaf. Host plants used for colonization were susceptible Bonny Best and resistant H7996 tomato. Bacterial colonization was quantified daily by grinding and dilution plating stem segments on appropriate antibiotic plates. Four or five plants were sampled for bacterial colonization each day per treatment in two biological replicates. To evaluate the impact of plant salicylic acid levels on stem colonization, we measured bacterial colonization of wilt-susceptible tomato cultivar Money-maker and transgenic Money-maker expressing NahG (42). The experiment included five plants per treatment in two biological replicates.

**Plant defense gene expression.** Seeds of Bonny Best or H7996 tomato were surface sterilized with 50 ml 10% bleach for 10 min, followed by an ethanol wash in 50 ml 70% ethanol for 5 min. Ethanol and bleach washes 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 germination, 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 Biomedicals, Santa Ana, CA) and incubated for 2 days at 28°C with a 12-h light cycle. Root tips were inoculated with 2 μl containing 2 × 10^6 CFU/ml of either GMI1000, 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, CA). RNA integrity numbers greater than 8.0) and high purity (as defined by A 260/280 of >1.9) were used for analysis.

**Plant defense gene expression.** Seeds of Bonny Best or H7996 tomato were surface sterilized with 50 ml 10% bleach for 10 min, followed by an ethanol wash in 50 ml 70% ethanol for 5 min. Ethanol and bleach washes 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 germination, 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 Biomedicals, Santa Ana, CA) and incubated for 2 days at 28°C with a 12-h light cycle. Root tips were inoculated with 2 μl containing 2 × 10^6 CFU/ml of either GMI1000, 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.

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RNA was eluted in 30 μl lysis buffer. RNA purity and quality were evaluated on a NanoDrop (Thermo Genomics, Santa Clara, CA), respectively. Samples with high quality A 260/280 (0.9) were used for analysis.
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 ΔΔCt method (73).

**Transient expression of PopS 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 pGW14 for C-terminal HA fusion protein expression using LR cloning (Life Technologies, Carlsbad, CA). The resulting plasmid, pGW14::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 pGW2::dsPEpop (positive control) (43), A. tumefaciens pGW14 (empty vector control), or A. tumefaciens pGW14::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.

**REFERENCES**

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