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Four candidate avirulence effector loci contribute to *Pseudomonas syringae* pv. *actinidiae* biovar 3 recognition in *Actinidia arguta*

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Introduction

A pandemic isolate of *Pseudomonas syringae* pv. *actinidiae* biovar 3 (Psa3) has devastated kiwifruit orchards growing cultivars of *Actinidia chinensis* since 2008. In contrast, *A. arguta* (kiwiberry) is resistant to Psa3. Host range in the *P. syringae* species complex is largely driven by the composition of the effector complement, which consists of at least 66 effector families[1]. Effectors act to promote bacterial virulence by interacting with host targets to suppress host immunity, allowing the pathogen to invade host tissue and cause disease[2]. However, plants have, in turn, evolved a secondary means of pathogen resistance, known as effector-triggered immunity[2]. Under effector-triggered immunity, plant resistance proteins monitor the integrity of the defence signalling cascade and can detect subversion by bacterial effectors, thus restoring plant resistance[2]. While previous research has identified Psa3 conserved effector locus (CEL) effectors required for virulence[3], no specific Psa3 avirulence effectors recognised by the host have been identified in *Actinidia*.

Methods

- The isolate Psa3 ICMP 18884 (hereafter Psa3) was used as the wild-type (WT) for a Psa effector knockout library using the pK18mobsacB-based system.
- A complete library of 21 Psa3 effector knockout strains was developed with effectors knocked out either individually, in pairs if homologs were present (*hopAM1-1*/*hopAM1-2*), or as a functional group (CEL; *hopZ5*/*hopH1*; and three iterations of the exchangeable effector locus (EEL) – xEEL, fEEL, and sEEL: Figure 1).
- Effector knockout plasmids were developed for Psa3 using the methodology established by Kvitko & Collmer[4] and described in Jayaraman et al.[3].
- Psa pathogenicity was assessed using an *in planta* flooding assay, as established in McAtee et al.[5].
- Real-time quantitative PCR (qPCR) was carried out on an Illumina Eco Real-Time PCR platform, following the protocol outlined in Barrett-Manako et al.[6].
- The hypersensitive response was assessed using young leaves of *A. arguta* AA07_03, which were co-bombarded with DNA-coated gold particles carrying pRT99-GUS and pICH86988 with the effector of interest, as described in Jayaraman et al.[7].

Results

To determine whether Psa3 effectors triggered resistance on *A. arguta* AA07_03, 21 knockout strains covering all 30 Psa3 effectors were screened on *A. arguta* AA07_03 plantlets by flood-inoculation and sampled at 12 dpi (Figure 2). Four candidate avirulence effector loci contributed to Psa3 recognition in *A. arguta*. Psa3 Δ*fEEL*, Psa3 Δ*sEEL*, Psa3 Δ*hopZ5*/Δ*hopH1*, Psa3 Δ*avrRpm1* and Psa3 Δ*hopF2* all achieved significantly higher growth than WT Psa3 ($p \leq 0.01$) (Figure 2). Following this screen, significant candidate avirulence effector knockout strains were further tested by plate count and qPCR quantification, which corroborated these results (Figures 3A, 3B).

Pathogenicity screening on *A. arguta* AA07_03 determined that Psa3 ΔsEEL carries at least one avirulence effector. To identify which sEEL effector(s) triggers resistance, segmented effector knockouts within the sEEL were generated. Psa3 Δ*hopAW1* lacks *hopAW1* while Psa3 Δ*tEEL* lacks the neighbouring *hopBB1-2*, *hopAF1*, *hopAO2* and *hopBB1-1* (Figure 1). Pathogenicity assays demonstrated that growth of Psa3 Δ*hopAW1* was significantly higher than WT Psa3 and similar to Psa3 Δ*sEEL* (Figure 3C, 3D). In contrast, Psa3 Δ*tEEL* was not significantly different from WT Psa3 (Figure 3C, 3D). This suggests that the individual deletion of *hopAW1* is sufficient to partially release host recognition and further suggests that none of the effectors in the tEEL trigger resistance on AA07_03.

Co-bombardment of the GUS reporter gene with *hopAW1*, *hopF2*, *hopZ5* and *avrRpm1* resulted in a decrease in GUS activity on *A. arguta* AA07_03 in comparison to the control (empty vector), indicating that these candidate avirulence effectors trigger a hypersensitive response in *A. arguta* (Figure 4).

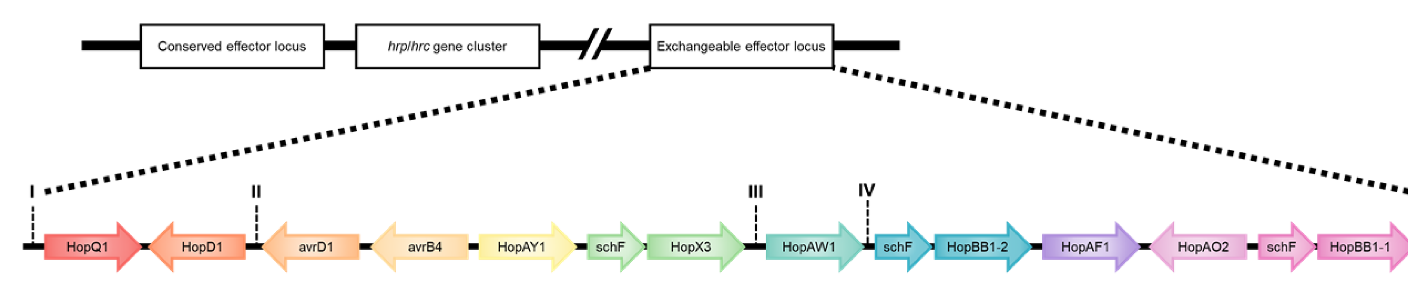

Figure 1. Schematic of the Psa3 V-13 tripartite pathogenicity island. The Psa3 V-13 tripartite pathogenicity island contains the conserved effector locus (CEL), the hrp/hrc gene cluster, and the exchangeable effector locus (EEL). The extended EEL (xEEL) region spans (I) to (V), the full EEL (fEEL) spans from (II) to (V), the short EEL (sEEL) spans from (III) to (V), and the tiny EEL (tEEL) spans from (IV) to (V).

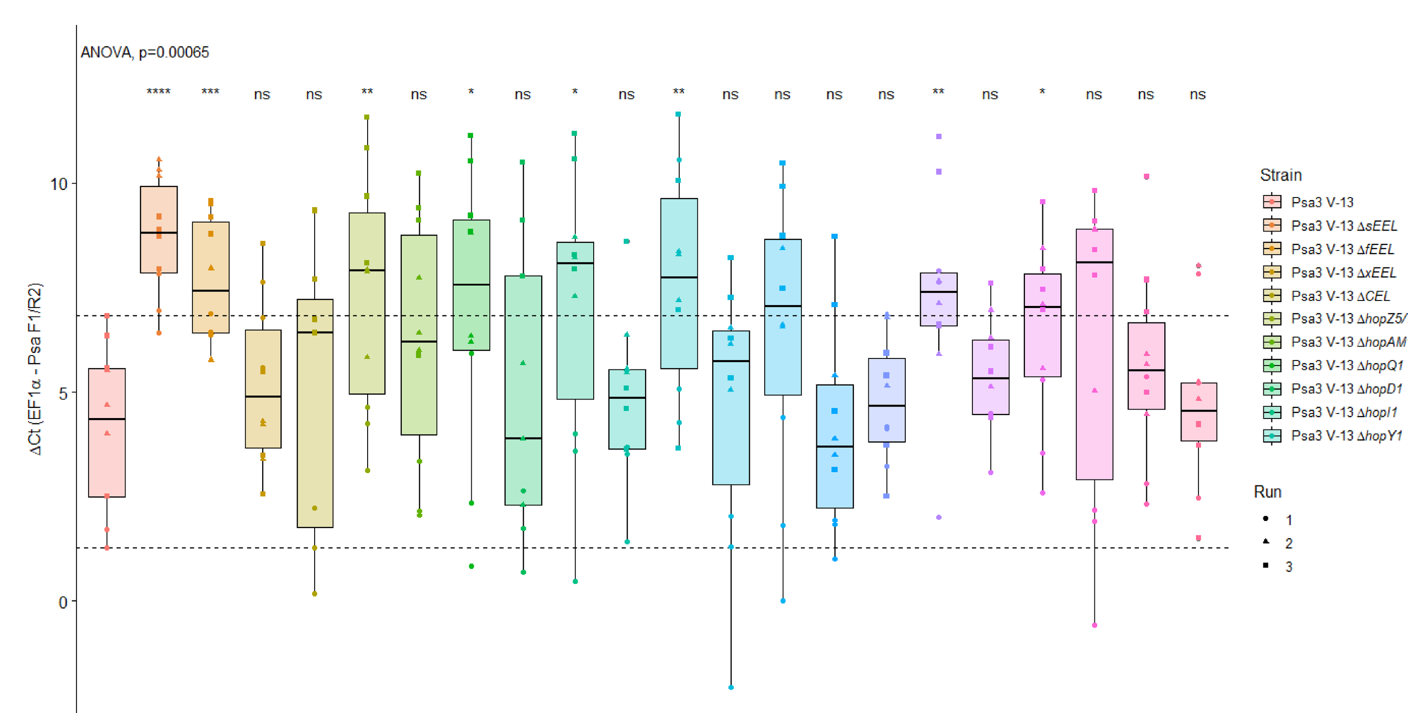

Figure 2. Pathogenicity assay of Psa3 effector knockout strains on *A. arguta* AA07_03. *A. arguta* AA07_03 kiwifruit plantlets were flood inoculated at approximately 106 CFU/mL. Bacterial pathogenicity was quantified relative to Psa3 using the ΔCt analysis method. Asterisks indicate statistically significant differences (Student's t-test) between the indicated strain and WT Psa3, where p≤0.05 (*), p≤0.01 (**), and p≤0.001 (***). This experiment was repeated three times.

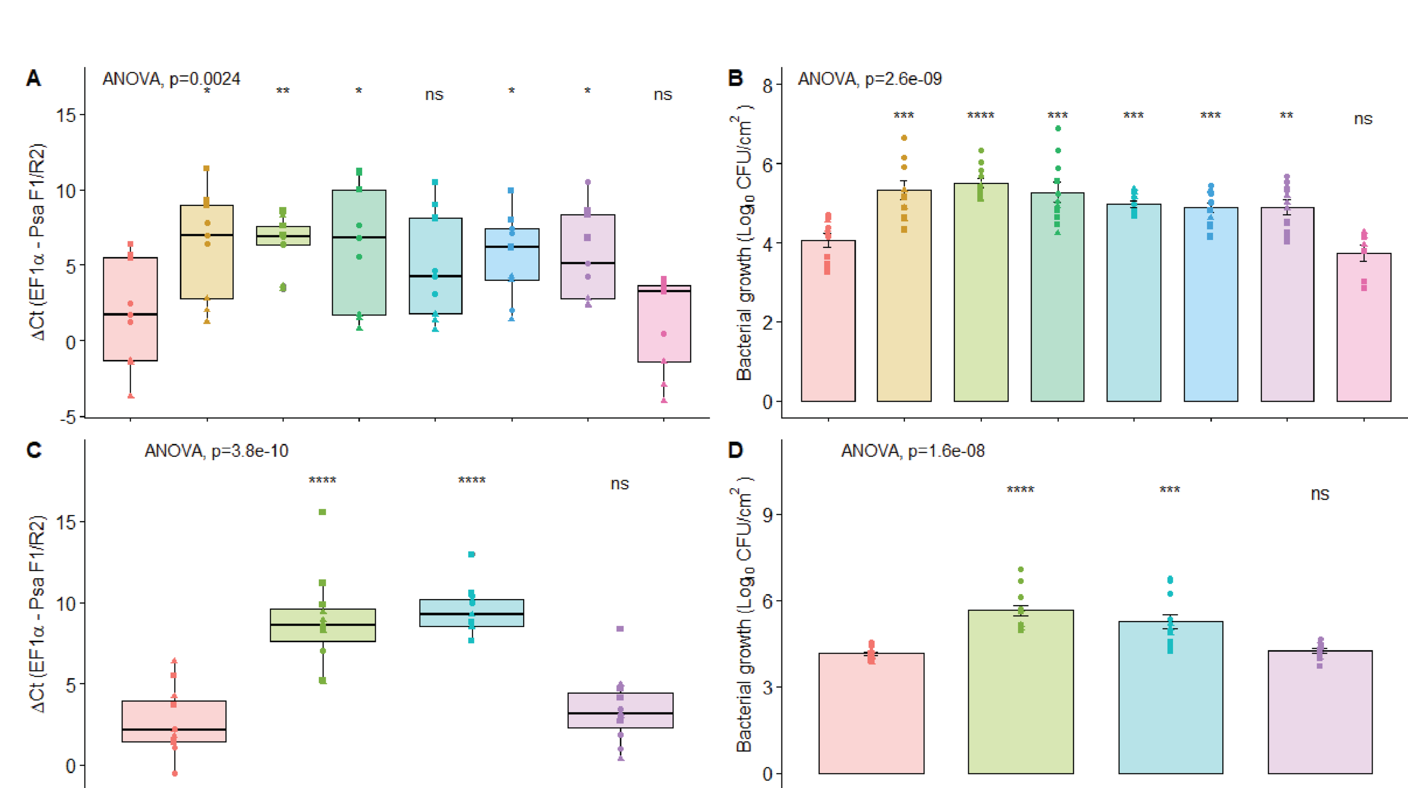

Figure 3. Pathogenicity assay of candidate Psa3 avirulence effector knockout strains and the Psa3 Δ*tEEL* and Δ*hopAW1* strains on *A. arguta* AA07_03. Growth as in Figure 1. Bacterial pathogenicity was quantified relative to Psa3 using qPCR ΔCt analysis (A, C) and plate count quantification (B, D). Asterisks indicate statistically significant differences (Student's t-test) between the indicated strain and WT Psa3, where p≤0.05 (*), p≤0.01 (**), and p≤0.001 (***). Each experiment was repeated 3 times, with similar results.

Conclusions

1. Effector knockouts of avrRpm1, HopF2, HopZ5, and the EEL effector HopAW1 are associated with resistance, as demonstrated through an increase in bacterial growth compared with WT Psa3 in *A. arguta*.
2. avrRpm1, HopF2, HopZ5, and HopAW1 trigger the hypersensitive response in *A. arguta* through biolistic co-bombardment of effectors.

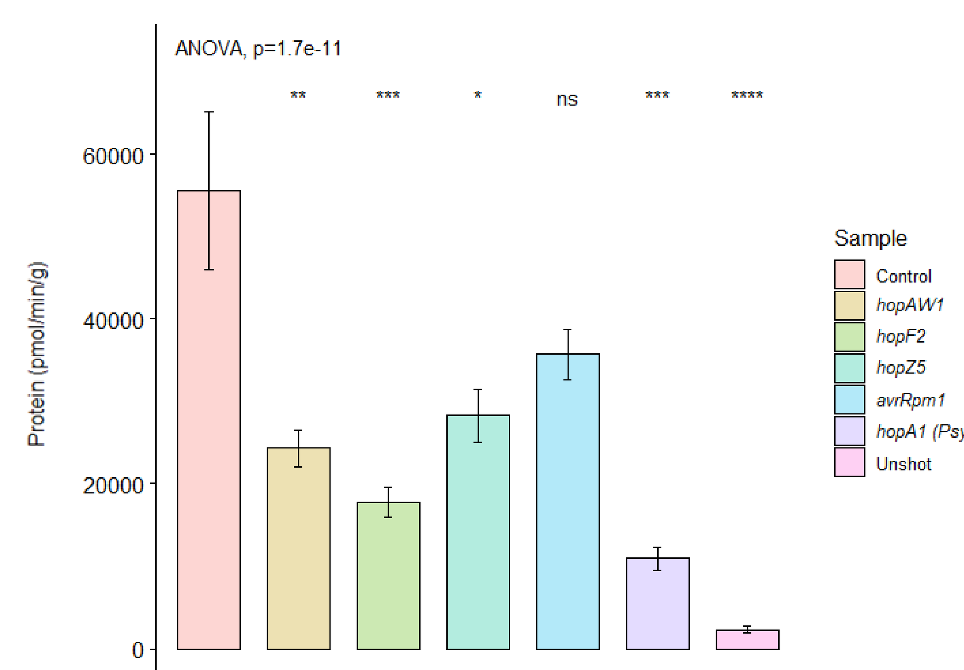

Figure 4. Biolistic transformation demonstrates that candidate Psa3 avirulence effectors trigger an immunity response in *A. arguta* AA07_03. Young leaves of *A. arguta* AA07_03 plantlets were co-bombarded with DNA-coated gold particles carrying pRT99-GUS and pICH86988 with the effector of interest or as an empty vector control. All effectors are derived from Psa3, with the exception of *hopA1* which is derived from *P. syringae* pv. *syringae* strain 61 and was used as a positive control. GUS activity was measured 48 hours after bombardment. Error bars represent the standard errors of the mean (SEM) for three independent biological replicates with six technical replicates each (n=18).

Acknowledgements

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