Rapid Methodologies for Assessing *Pseudomonas syringae* pv. *actinidiae* Colonization and Effector-Mediated Hypersensitive Response in Kiwifruit

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The infection of *Pseudomonas syringae* pv. *actinidiae* in kiwifruit is currently assessed by numerous methodologies, each with their own limitations. Most studies are based on either a laborious method of growth quantification of the pathogen or qualitative assessments by visual scoring following stem or cutting inoculation. Additionally, when assessing for resistance against specific pathogen effectors, confounding interactions between multiple genes in the pathogen can make mapping resistance phenotypes nearly impossible. Here, we present robust alternative methods to quantify pathogen load based on rapid bacterial DNA quantification by PCR, the use of *Pseudomonas fluorescens*, and a transient reporter eclipse assay for assessing resistance conferred by isolated bacterial avirulence genes. These assays compare well with bacterial plate counts to assess bacterial colonization as a result of plant resistance activation. The DNA-based quantification, when coupled with the *P. fluorescens* and reporter eclipse assays to independently identify bacterial avirulence genes, is rapid, highly reproducible, and scalable for high-throughput screens of multiple cultivars or genotypes. Application of these methodologies will allow rapid and high-throughput identification of resistant cultivars and the bacterial avirulence genes they recognize, facilitating resistance gene discovery for plant breeding programs.

Keywords: *Actinidia arguta*, electrolyte leakage, PDQeX, qPCR, resistance

The plant immune system is central to plant survival when constant pathogen and pest attack threatens host survival and crop productivity. The prevailing concept for how the immune system in plants is structured is a bipartite innate immune system, consisting of a plant cell membrane-associated pattern-triggered immunity (PTI) and an intracellular effector-triggered immunity (ETI). PTI is activated by conserved microbial particles present in the apoplast space, whereas ETI is typically activated by pathogen effector proteins sensed by intracellular receptors that subsequently add to the PTI response (Cook et al. 2015; Jones et al. 2016; Ngou et al. 2021; Yuan et al. 2021). Bacterial pathogens secrete around 30 effectors into plant cells to facilitate infection, with the best-characterized mechanism by which this occurs utilizing a type III secretion system (T3SS). If an effector is recognized by the plant surveillance system, which is comprised of a repertoire of resistance (*R*) genes, ETI is triggered, inducing an avirulence response and is termed an avirulence effector. ETI is often associated with a robust programmed cell death response and production of reactive oxygen species (ROS) that collectively result in a phenotype known as the hypersensitive response (HR) (Zurbriggen et al. 2010). Identification of the genetic determinants that trigger ETI in a resistant plant and introgressing them into commercially attractive susceptible plants is the ultimate goal of a plant disease-resistance breeding program. This requires a methodology for robustly assessing disease establishment and the resistance mechanisms preventing this in plant hosts.

For *Pseudomonas syringae* pv. *actinidiae*, the causal pathogen of kiwifruit bacterial canker, several different assays have been reported to assess disease in planta (Hoyte et al. 2015; McCann et al. 2013; Wang et al. 2019). However, it is difficult to distinguish resistance (typically robust gene-for-gene responses) from tolerance (typically a quantitative trait) or susceptibility due to a reliance on symptomology, laborious plant tissue manipulation, a time-consuming phenotyping process, variability associated with minor alterations in environmental conditions, or a combination of these, using these reported assays (Tahir et al. 2019).

The *Arabidopsis thaliana*–*Pseudomonas syringae* pathosystem has been widely utilized as a model for plant-bacterial interactions. The *P. syringae* pv. *tomato* DC3000 strain has been used for visual evaluation of disease symptoms, in vivo pathogen counting methods, or fluorescence tracking and quantification, as well as high-throughput quantitative PCR (qPCR)-based methodologies with high consistency between experiments (Brouwer et al. 2003; Fan et al. 2008; Ross and Somssich 2016; Whalen et al. 1991).

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The qPCR-based method utilizes primers specific to the pathogen and a normalizing reference gene (typically of plant origin) to quantify the amount of pathogen DNA compared with plant DNA in an extracted sample (Brouwer et al. 2003; Ross and Somssich 2016). Unlike visual symptomology and pathogen tracking, in vivo–pathogen counting is accurate and is able to distinguish even small changes in colonization by the pathogen, but it is labor intensive and time consuming. In contrast, qPCR-based methodologies are accurate, rapid, and have the advantage of long-term storage of material following harvest of infected plant tissue not possible for in vivo–pathogen counting, allowing comparisons of large sample sets (Ross and Somssich 2016). qPCR-based methodologies for *P. syringae* pv. *actinidiae* have recently been developed (Gallelli et al. 2014), with modifications for normalization being added to improve quantification between different cultivars (Barrett-Manako et al. 2021).

While the aforementioned assays developed for the *Arabidopsis*–*P. syringae* pathosystem are translatable to other crop plant systems and their cognate pathogens, the complexity of numerous *R* genes in a plant recognizing multiple genes in the pathogen can confound *R*-gene discovery. Thus, the first step to identifying a plant *R* gene to be utilized in a breeding program is often isolating the avirulence effector, to avoid confusing interplay between that effector and others that may be present in the pathogen as well as to overcome redundancy issues (Thomas et al. 2009; Vleeshouwers and Oliver 2014). To be able to discover and exploit avirulence effectors as a tool in breeding, a rapid and robust screening technology must be developed and deployed (Dangl et al. 2013).

In this respect, a number of transient assay systems for isolated effector expression have been adapted to a variety of model and crop plants to streamline *R* gene discovery. Transient expression of genes using *Agrobacterium tumefaciens* or viruses have been the predominant way to express putative avirulence effectors in resistant plants to rapidly screen for resistance (Du et al. 2014; Rietman et al. 2012). The requirement of the plant to trigger an HR is critical to this methodology but may not always be produced in response to an avirulence effector (Gassmann 2005; Jayaraman et al. 2017; Laflamme et al. 2020). In addition, transient expression screens are achieved through syringe infiltration of an *Agrobacterium tumefaciens* strain, which may be difficult for some plant species and needs to be optimized for each species (Hayta et al. 2019; Jelly et al. 2014; Wroblewski et al. 2005; Zhao et al. 2019). Alternatively, biolistic bombardment of plant tissue using coexpression of a reporter gene with a putative avirulence gene has been used to check for HR reducing expression of the reporter gene, a reporter eclipse assay (Gopalan et al. 1996; Jia et al. 2000; Mindrinos et al. 1994). Reporter eclipse assays are currently underutilized in analysis of resistance because of technical issues regarding reproducibility and fine-tuning assay parameters but remain relevant, particularly for multiple-construct transformations (Agrawal et al. 2005).

Previously, *P. syringae* pv. *actinidiae* infection in tissue culture–grown plantlets was quantified for pathogen growth in planta following a flooding inoculation method (mimicking natural infection) as well as stab inoculations (McAtee et al. 2018; McCann et al. 2013). These assays, based on axenically grown plantlets in tissue culture, are reproducible but are nevertheless laborious and time consuming. Recently, a robust methodology for qPCR-mediated quantification of *P. syringae* pv. *actinidiae* infection was described for kiwifruit (Barrett-Manako et al. 2021; Gallelli et al. 2014). Sampling and DNA extraction issues were noted as limitations. Here, we build on previous work to report several optimized higher-throughput assays for pathogen quantification and avirulence determination in tissue culture–grown kiwifruit plantlets, including in comparison with classical plate assays. We develop two novel approaches that can be applied to different cultivars of kiwifruit and can be deployed for accurate and

rapid determination of avirulence presence in the pathogen critical to resistance breeding programs.

RESULTS

Kiwifruit cultivars demonstrate wide variation in their response to *P. syringae* pv. *actinidiae* biovar 3 (*Psa3*).

To assess the variability in the response of different commercially grown kiwifruit cultivars, four selected cultivars grown as plantlets were flood-inoculated with *Psa3*. Photographs taken at 50 days postinfection show that they varied phenotypically from highly susceptible (*Actinidia chinensis* var. *chinensis* Hort16A), moderately susceptible (*A. chinensis* var. *chinensis* Zesy002), tolerant (*A. chinensis* var. *deliciosa* Hayward), and resistant (*A. arguta* AA07_03) (Fig. 1A). The disease symptomology of these tissue culture–grown plantlets reflected closely field- and glasshouse-grown plants for these cultivars (Supplementary Fig. S1) (Hoyte et al. 2015). To assess whether the symptoms reflected the level of colonization by *Psa3* in the host, in-planta growth assays were conducted at 6 and 12 days postinfection (Fig. 1B; Supplementary Fig. S2). Surprisingly, despite large variations in symptomology, even early during the infection process, no difference in in-planta growth was noted for Hort16A compared with Zesy002 and only a small but significant difference was noted for Hayward. In contrast, AA07_03 plants showed significantly reduced in-planta growth by *Psa3*. This was also in line with previously reported resistance in *A. arguta* and tolerance seen for *A. chinensis* var. *deliciosa* (Donati et al. 2020; Nardozza et al. 2015; Wang et al. 2019). These findings indicated that, while symptomology was ideal for studying differences in tolerance (measured through production of deleterious symptoms on the plant), it was neither the most appropriate nor the quickest assay method for assessing genetic resistance in kiwifruit (a lack of pathogen colonization as well as a lack of disease symptoms). Notably, though, this traditional approach to assessing resistance was achieved in 2 weeks, with significant limitations on the number of plant genotypes (6 to 12) able to be assessed per run because of labor requirements and time restrictions (Table 1).

To increase the throughput of assessing resistance in infected plants, the quantification of *Psa3* colonization of the host tissues was also undertaken, using a novel DNA extraction method (PDQeX), which simplified and streamlined extraction (Stanton et al. 2019), coupled to previously reported qPCR-based detection (Barrett-Manako et al. 2021). This coupled approach of PDQeX-qPCR for plants sampled at 7 days postinoculation (dpi) reproduced the resistance in AA07_03 seen by traditional growth assays (Fig. 1C). This infection-sampling PDQeX-qPCR approach reduced assessment times to half that of the traditional plating approach, with the added benefit of allowing around 24 individual plant genotypes to be assessed per run (Table 1) and is an improvement on the previous throughput possible (Barrett-Manako et al. 2021).

To further improve throughput of screening, an HR assay measuring electrolyte leakage (conductivity) was developed for *Psa3*, to test for cell death in response to the pathogen. Unfortunately, kiwifruit is not amenable to syringe-based infiltration and required vacuum-infiltration to achieve consistent levels of leaf infection. The use of vacuum-infiltration and measurement of electrolyte leakage would speed up resistance assessment times to approximately 2 days postinfection (Table 1). Using vacuum-infiltration of *Psa3* and its T3SS nonexpressing mutant ($\Delta hrcC$) into the four kiwifruit cultivars resulted in some early (6 to 24 h) increase in electrolyte leakage indicating HR (Fig. 1D). However, while consistent late increases (24 to 48 h) for wild-type *Psa3* suggested normal function for its T3SS, vis-à-vis electrolyte leakage associated with effector-mediated virulence functions, it also indicated that a clear HR-associated leakage was less obvious (with a high

rate of leakage indicating HR, particularly comparing the most susceptible and most resistant genotypes, Hort16A and AA07_03). The small early electrolyte leakage was consistent irrespective of the level of *Psa3* inoculum used for this assay (10^7 to 10^9 CFU/ml). Indeed, deployment of traditional ROS detection assays to detect the secondary oxidative burst during HR also

did not indicate a clear difference between the most susceptible (Hort16A) and most resistant (AA07_03) plant genotypes (Supplementary Fig. S3), despite testing showing that AA07_03 mounted a significant defense-gene expression response against *Psa3*, unlike Hort16A (Supplementary Fig. S4). The reason, at this stage, for the lack of a clear HR response was unclear,

Fig. 1. PDQeX-quantitative PCR (qPCR) is better than symptomology or electrolyte leakage for quantifying *Pseudomonas syringae* pv. *actinidiae* ICM18884 (*Psa3*) infection in commonly grown kiwifruit cultivars. **A**, Symptom development in *Actinidia chinensis* var. *chinensis* cultivar Hort16A, *A. chinensis* var. *chinensis* Zesy002, *A. chinensis* var. *deliciosa* Hayward, and *A. arguta* AA07_03 plantlets at 50 days postinoculation (dpi) with *Psa3*. *Psa3* was flood-inoculated at approximately 10^5 CFU/ml into plantlets and photographs were taken of a single representative pottle displaying typical disease lesion symptoms, if present. **B**, *Psa3* growth in multiple kiwifruit cultivars determined by classical in-plant growth quantification by serial dilution and plating. *Psa3* was flood-inoculated as described in A, and bacterial growth was determined 0, 6, and 12 dpi. Error bars represent standard error of the mean from four pseudobiological replicates. Asterisks indicate results of a two-tailed Student's *t* test between the selected treatment and susceptible Hort6A; one asterisk (*) indicates $P < 0.05$ and three (***) $P < 0.001$. The experiment was conducted three times with similar results. **C**, *Psa3* growth in multiple kiwifruit cultivars determined by PDQeX-qPCR. *Psa3* was flood-inoculated as described in A, and bacterial presence in leaf samples was quantified by qPCR for the *Psa3* intergenic transcribed spacer region normalized to *AcEF1α* for 7 dpi. Error bars represent standard error of the mean from four pseudobiological replicates. Letters indicate results of a one-way analysis of variance (ANOVA) and Tukey's honest significant difference (HSD) post hoc test ($P < 0.01$), shared letters indicate no significant difference. The experiment was conducted three times with similar results. **D**, Measurement of hypersensitive response (HR) by electrolyte leakage. Leaf discs from plantlets shown in A were vacuum-infiltrated with *Psa3* inoculum at approximately 10^8 CFU/ml, and electrical conductivity due to HR-associated electrolyte leakage was measured at selected timepoints over 48 h. The curves are generated from the combination of three independent runs of this experiment. Error bars represent the standard errors of the means calculated from the three experimental runs, each with five pseudobiological replicates. Letters indicate results of a one-way ANOVA and a Tukey's HSD post hoc test for each timepoint, with shared letters indicating no statistical significance ($P < 0.01$).

Table 1. Comparison of assay parameters for screening resistance in kiwifruit plants

Assay	Assessment	Samples per run	Time to completion (days)
In planta growth and plating	Bacterial colonization of host	6 to 12	14
PDQeX-qPCR	Bacterial colonization of host	6 to 24	7
Electrolyte leakage (<i>Pseudomonas fluorescens</i>)	Hypersensitive response	24 to 48	2
Reporter eclipse (biolistic)	Hypersensitive response	6 to 12	2

including whether *Psa3* was capable of suppressing the HR while still triggering plant immunity.

***Psa3*-delivery of avirulence effectors triggers resistance in Hort16A but not HR.**

To assess whether kiwifruit was capable of producing a resistance response typically seen in model plants (*Arabidopsis thaliana* and *Nicotiana benthamiana*), *Psa3* was first transformed with plasmid vectors carrying known avirulence effectors recognized in these model plants (Bisgrove et al. 1994; Century et al. 1995; Kunkel et al. 1993; van Dijk et al. 2002). The effectors chosen were *avrRpt2* from *P. syringae* pv. *tomato* T1 (representing *P. syringae* phylogroup 1), *hopA1* from *P. syringae* pv. *syringae* 61 (representing *P. syringae* phylogroup 2), *avrPphB* from *P. syringae* pv. *phaseolicola* 1448A (representing *P. syringae* phylogroup 3), and *avrRpm1* from *P. syringae* pv. *maculicola* M2 (since it is an ortholog of an effector present in *Psa3*). The transformed *Psa3* strains were then tested in susceptible Hort16A plants for reduced in-planta growth and lack of disease symptom production as measures of resistance in response to the introduced effectors.

Infection of Hort16A plants resulted in in-planta growth and associated plant disease symptoms identical to wild-type *Psa3* for both *Psa3* + *avrPphB* and *Psa3* + *avrRpm1* (Fig. 2A and B; Supplementary Fig. S5). However, for both *Psa3* + *avrRpt2* and *Psa3* + *hopA1*, a strong reduction in in-planta growth and symptomatology suggested both of these effectors were recognized by Hort16A plants. The in-planta growth and symptomatology testing results were also confirmed by PDQeX-qPCR testing, indicating that both *AvrRpt2* and *HopA1* are avirulence effectors recognized by Hort16A (Fig. 2C). Notably, these effector-carrying strains could not trigger early HR-associated electrolyte leakage despite the very strong reductions in virulence detected through the traditional in-planta growth and disease symptomatology assays, similar to *Psa3* in AA07_03 (Supplementary Fig. S6).

Novel assays discover avirulence effectors recognized by Hort16A and AA07_03.

To assess whether the lack of a clear HR response was due to *Psa3*-mediated interference or whether kiwifruit plants were atypical in their ability to trigger an HR, *P. fluorescens* Pf0-1 with and without a T3SS were transformed with the plasmids bearing these four effectors and were tested for electrolyte leakage. Interestingly, when delivered by *P. fluorescens* (T3S), both *AvrRpt2* and *HopA1* triggered large increases in electrolyte leakage, in contrast to delivery by *P. fluorescens* (wild type [WT]) for these effectors or *AvrPphB* (Fig. 3A). Surprisingly, *P. fluorescens* (T3S) + *avrRpm1* also triggered strong electrolyte leakage, which was, in fact, stronger than the responses seen for *AvrRpt2* and *HopA1*. This suggested that *AvrRpt2* and *HopA1* are both strong avirulence effectors that trigger immunity but not HR when delivered by *Psa3*. In contrast, *AvrRpm1* failed to trigger both immunity and HR when delivered by *Psa3* but triggered a strong HR when delivered individually by *P. fluorescens*. This further validated the use of *P. fluorescens* as a tool for rapidly screening for resistance in kiwifruit, allowing for testing a larger number of plants (12 to 24) but in a significantly reduced timeframe (2 days) (Table 1).

To confirm the results of the isolated effector delivery system, a transient expression system via biolistic bombardment was developed in parallel for kiwifruit that utilized a coexpression of an effector of interest together with a reporter gene for detecting HR. This reporter eclipse system measured the *uidA* (β -glucuronidase [GUS]) reporter gene activity in leaf tissues cobombarded with the putative avirulence effector, and the level of the HR inversely correlated to the level of the GUS activity. Recent findings demonstrated that PTI activation was required to prime for ETI and associated HR (Ngou et al. 2021; Yuan et al. 2021).

Expression of some nucleotide-binding leucine-rich receptor (NLR) genes, such as *NRG1*, is induced upon activation of PTI (Brendolise et al. 2018), thus, a pretreatment of the plant tissue with an *Agrobacterium* sp. was included in our reporter eclipse system to induce PTI. Using this primed reporter eclipse assay, coexpression of *AvrRpt2* and *HopA1* in Hort16A leaves significantly reduced expression of the GUS reporter compared with that when coexpressed with empty vector or *AvrPphB* (Fig. 3B). Interestingly, coexpression with *AvrRpm1* also resulted in a significant reduction of reporter expression, suggesting that it was triggering HR in Hort16A leaves.

To examine if these three effectors are also recognized in AA07_03, *P. fluorescens* (T3S) and *P. fluorescens* (WT), delivery of the four effectors was also tested for electrolyte leakage. In this *Psa3*-resistant host, only *AvrRpt2* and *HopA1* resulted in an HR response with electrolyte leakage and *AvrRpm1* failed to produce a strong HR-associated phenotype (Fig. 4A). Using the primed reporter eclipse assay to confirm this result produced identical findings, suggesting that unlike Hort16A, AA07_03 plants only recognized *HopA1* and *AvrRpt2* expression (Fig. 4B). Taken together, this suggested that Hort16A and AA07_03 share recognition of *AvrRpt2* and *HopA1* but differ in their recognition of *AvrRpm1*.

DISCUSSION

Assessing disease resistance in kiwifruit during infection by *Psa3* is currently undertaken symptomologically (which can be unreliable) or through quantification of bacterial colonization by traditional plate-counting methods (accurate and reliable but low-throughput). This is particularly cumbersome because of the need for immediate processing of harvested samples, making the entire process time-consuming and laborious. Furthermore, different cultivars may have distinct leaf morphologies, thus the available colonization capacity of the plant may naturally vary. This latter issue would require an internal normalization process to facilitate adjustment for variation between cultivars but is unavailable for classical growth assays.

DNA-based detection as an alternative to classical infection quantification has been deployed for plant-pathogen studies in other pathosystems but has not yet been deployed in the *P. syringae* pv. *actinidiae*–kiwifruit system until recently (Abdullah et al. 2018; Barrett-Manako et al. 2021; Brouwer et al. 2003; Gallelli et al. 2014; Narusaka et al. 2009; Ross and Somssich 2016; Weßling and Panstruga 2012). The use of nucleic acid extraction tools for DNA-based detection has typically been the rate-limiting step for this approach. However, the use of the high-quality, rapid DNA extraction methodology (PDQeX) in our work has significantly accelerated this component and is validated here for relative quantification studies for the first time (Stanton et al. 2019). Furthermore, because of the higher sensitivity of the qPCR assay (detection accomplished over twofold increments compared with 10-fold increments with classical plating assays), a shorter period of assessment (7 dpi) can be utilized, further speeding up the process of assessing pathogen colonization of the plant host.

The use of transient assays and isolated effector delivery assays in model plants for *Psa3* has been reported recently but has not, until now, been deployed consistently for kiwifruit (Brendolise et al. 2017; Choi et al. 2017; Jayaraman et al. 2017). Taken together, a number of the assays demonstrated herein are both efficacious and high-throughput for quantifying both the pathogen growth (PDQeX-qPCR) and plant response to the presence of an HR-triggering avirulence effector (electrolyte leakage and reporter eclipse). The transient biolistic approach serves as a stand-in for classical *Agrobacterium*-medium transient assays (agroinfiltration) that typically examine HR and HR-like responses in plants, which are recalcitrant to agroinfiltration-type syringe-infiltration

experiments (Lacroix and Citovsky 2020; Ueki et al. 2013). The biolistic approach also allows organelle targeting, does not require the discovery of a coevolved *Agrobacterium* strain, and is often significantly more rapid in application than other transient

expression methods (Lacroix and Citovsky 2020). However, the biolistics approach has only moderate throughput, as each sample requires significant handling, and thus, it is not an ideal first tool for screening large numbers of plants (Table 1) but instead is useful as

A

B

C

Fig. 2. *Actinidia chinensis* var. *chinensis* Hort16A recognizes effectors AvrRpt2_{PtoT1} and HopA1_{Psy61} when delivered by *Pseudomonas syringae* pv. *actinidiae* ICMP 18884 (*Psa3*). **A**, Growth of *Psa3* variants in Hort16A plantlets was determined by in-plant growth quantification by serial dilution and plating. *Psa3* wild type, $\Delta hrcC$ mutant, or *Psa3* carrying putative avirulence effectors *avrRpm1*_{PmaM2}, *avrRpt2*_{PtoT1}, *avrPphB*_{Pph1448A}, or *hopA1*_{Psy61} were flood-inoculated, at approximately 10⁶ CFU/ml, into plantlets and bacterial growth was determined 0, 6, and 12 days postinoculation (dpi). Error bars represent standard error of the mean from four pseudobiological replicates. Asterisks indicate results of a two-tailed Student's *t* test between the selected treatment wild type; three asterisks (***) indicate *P* < 0.001. The experiment was conducted three times with similar results. **B**, Symptom development in Hort16A plantlets at 50 dpi after inoculation with strains shown in A. Photographs were taken of a single representative pottle displaying typical disease lesion symptoms, if present. **C**, *Psa3* growth in multiple kiwifruit cultivars determined by PDQeX-quantitative PCR (qPCR). *Psa3* strains were flood-inoculated as described in A, and bacterial presence in leaf samples was quantified by qPCR for the *Psa3* intergenic transcribed spacer region normalized to *AcEF1α* at 7 dpi. Error bars represent standard error of the mean from four pseudobiological replicates. Letters indicate results of a one-way analysis of variance and Tukey's honest significant difference post hoc test (*P* < 0.01), shared letters indicate no significant difference. The experiment was conducted three times with similar results.

a heterologous system free of HR-like elicitation occasionally associated with recognition of the type III secretion system itself (Clarke et al. 2014, 2010).

In contrast, the HR-associated electrolyte leakage assays are eminently suitable for high-throughput screens of multiple cultivars and F₁/F₂ mapping populations from crosses. However, to

be useful, these screens need to be verified via a pathogen infection assay (e.g., PDQeX-qPCR) for the deconvolution of HR from the effect of other effectors that seek to block HR and side effects of other virulence-promoting activities (Vleeshouwers and Oliver 2014). Numerous *P. syringae* effectors have been identified that are capable of suppressing the HR triggered by an avirulence

Fig. 3. *Actinidia chinensis* var. *chinensis* cultivar Hort16 recognizes effectors AvrRpt2_{PtoT1}, HopA1_{Psy61}, and AvrRpm1_{PmaM2} when delivered in isolation or during transient expression. **A**, Measurement of hypersensitive response (HR) by electrolyte leakage. Leaf discs from Hort16A plantlets were vacuum-infiltrated with *P. fluorescens* Pf0-1 wild type (WT) or type III secretion system carrying mutant (T3S) carrying empty vector (EV) or putative avirulence effector genes *avrRpm1*_{PmaM2}, *avrRpt2*_{PtoT1}, *avrPphB*_{Pph1448A}, or *hopA1*_{Psy61} inoculum at approximately 10⁸ CFU/ml, and electrical conductivity due to HR-associated electrolyte leakage was measured at selected timepoints over 48 h. Error bars represent the standard errors of the means calculated from five biological replicates. **B**, Putative avirulence effectors, described in A, cloned on binary vector constructs or corresponding empty vector (EV) were coexpressed with a β -glucuronidase (GUS) reporter construct, using biolistic bombardment and priming in Hort16A plantlets. The GUS activity was measured 48 h after DNA bombardment. Error bars represent the standard errors of the means for three independent biological replicates with six technical replicates each ($n = 18$). Letters indicate results of a one-way analysis of variance and Tukey's honest significant difference post hoc test ($P < 0.01$), shared letters indicate no significant difference.

gene (Choi et al. 2017; Guo et al. 2009). This is clearly the case for HR in kiwifruit, in particular, because *Psa3* delivery of in-planta bacterial growth-restricting effectors (AvrRpt2 and HopA1) did not lead to detectable HR (Supplementary Fig. S5).

The results obtained with AvrRpm1 in this study typify the need for deconvoluting HR-inducing effectors from HR-suppressing effectors, since Hort16A plants were able to recognize and trigger a strong electrolyte leakage response to this effector when delivered

by *P. fluorescens* and reporter eclipse during transient expression (Fig. 3). However, pathogen growth did not show an avirulence-associated pathogen growth restriction (Fig. 2). This was not the case for AvrRpt2 or HopA1, both of which had strong pathogen growth suppression and HR. The likelihood of an AvrRpm1-associated artifact is reduced, since the same *P. fluorescens* (T3S) strain delivery of AvrRpm1 failed to trigger an HR response in AA07_03, suggesting an authentic host genotype-specific

Fig. 4. *Actinidia arguta* AA07_03 recognizes effectors AvrRpt2_{PtoT1} and HopA1_{Psy61} but not AvrRpm1_{PmaM2} when delivered in isolation or during transient expression. **A**, Measurement of hypersensitive response (HR) by electrolyte leakage. Leaf discs from AA07_03 plantlets were vacuum-infiltrated with *P. fluorescens* Pf0-1 wild type (WT) or type III secretion system carrying mutant (T3S), empty vector (EV), or putative avirulence effectors *avrRpm1*_{PmaM2}, *avrRpt2*_{PtoT1}, *avrPphB*_{Pph1448A}, or *hopA1*_{Psy61} inoculum at approximately 10^8 CFU/ml, and electrical conductivity due to HR-associated electrolyte leakage measured at selected timepoints over 48 h. Error bars represent the standard errors of the means calculated from five biological replicates. **B**, Putative avirulence effectors described in A cloned on binary vector constructs or corresponding empty vector (EV) were coexpressed with a β -glucuronidase (GUS) reporter construct, using biolistic bombardment and priming in AA07_03 plantlets. The GUS activity was measured 48 h after DNA bombardment. Error bars represent the standard errors of the means for three independent biological replicates with six technical replicates each ($n = 18$). Letters indicate results of a one-way analysis of variance and Tukey's honest significant difference post hoc test ($P < 0.01$), shared letters indicate no significant difference.

HR in Hort16A (Fig. 4). It is possible that, in the case of AvrRpm1, because of its similarity to an existing *Psa3* effector (AvrRpm1_{*Psa3*}), members of the large effector repertoire in *Psa3* are able to block HR and growth restriction (Sawada and Fujikawa 2019). Indeed, *P. syringae* pv. *actinidiae* strains appear to have a larger than expected repertoire of putative RIN4-targeting and HopF-related effectors, including AvrRpm1, AvrRpm2, HopF4b (a.k.a. HopF2), HopX3, HopZ3, HopBB1, and HopBN1 (McCann et al. 2017, 2013; Sawada and Fujikawa 2019). These effectors may, independently or in combination, act to suppress HR triggered by other effectors targeting the same pathways. It is therefore intriguing that AvrRpt2, another RIN4-targeting effector, is still able to trigger both HR (in isolation) and growth restriction, despite triggering a weaker HR than AvrRpm1 in Hort16A plants. Alternatively, the presence of several demonstrated HR-suppressing effectors in *Psa3* may be able to counteract avirulence triggered by AvrRpm1 (Choi et al. 2017). These HR-suppressing effectors displayed avirulence effector-specific HR suppression ability. In contrast, the HR against AvrRpt2 and HopA1 are not suppressed because *Psa3* does not carry orthologous effectors in its repertoire, and thus, has not evolved the ability to suppress immunity triggered by these two effectors. Nevertheless, the identification of HR and immunity suppressors against recognition of AvrRpm1 (specifically the ortholog from *P. syringae* pv. *maculicola* M2) may render any immunity against the ortholog of this effector from *Psa3* less useful for breeding efforts, highlighting the need for multipronged approaches to discovering effectors in *Psa3* that trigger immunity in resistant kiwifruit. Thus, it appears that electrolyte leakage (or biolistic reporter eclipse assays or both) are useful for determining effectors that, in isolation, are able to trigger an HR, but they need to be checked with in-planta pathogen growth assays to determine if the accompanying HR results in resistance in the host plant.

The further use of a defense-associated reporter gene (*WRKY70a*) to assess whether phenotypes observed are authentic defense responses was also utilized (Supplementary Fig. S4). Interestingly, *Psa3* Δ *hrcC* mutant was also able to trigger a defense response in *A. arguta* to a level similar to that by WT *Psa3*. This is not unexpected, since both PTI and ETI pathways appear to be convergent on immune signaling and ETI activation by WRKY70 appears to require PTI (Knoth et al. 2007; Ngou et al. 2021; Yuan et al. 2021; Zhou et al. 2018). Notably, however, while *Psa3* did not trigger significant *AcWRKY70* expression in Hort16A as expected, Hort16A also did not appear capable of detecting the *Psa3* Δ *hrcC* mutant and this may partially explain the extreme susceptibility of this cultivar to *Psa3* infection (Supplementary Fig. S4).

A major hurdle in breeding disease resistance is the apparently quantitative nature of one or both disease severity and resistance in segregating mapping populations. ETI is typically conferred by a single, dominant, intracellular NLR protein recognizing the action of a single avirulence effector (Dangl et al. 2013). Multiple non-redundant NLRs recognizing multiple effectors required for pathogen virulence would likely provide durable disease resistance. However, these NLRs may be difficult to map in a breeding population using the pathogen itself, as they would present as a complex quantitative phenotype, particularly with the interplay of multiple effectors and their associated virulence functions (Corwin and Kliebenstein 2017). By using cloned effectors in isolation of other effectors from the pathogen, the process of mapping corresponding resistance loci can be accelerated, as disease resistance scoring is likely to be simple and individual effector recognition is most likely conferred by a single dominant locus. Testing isolated effectors for recognition via a high-throughput platform, instead of relying on pathogen virulence, identification of one or more corresponding NLRs by mapping using this approach, and identifying NLR-associated molecular markers is the most efficacious approach to accelerate breeding new resistant cultivars. Such

effector-assisted breeding approaches are currently at the forefront of nontransgenic resistance breeding programs around the world (Vleeshouwers and Oliver 2014; Zhang and Coaker 2017). Effector-assisted resistance strategies have been successfully deployed against potato late blight, rice blast, and bacterial blight in cassava (Bart et al. 2012; Ning et al. 2020; Vleeshouwers et al. 2008). Introgression of major *Psa3* disease resistance loci into commercial cultivars can be facilitated in kiwifruit breeding programs using a similar approach.

The resistance offered by *A. arguta* in response to *Psa3* is a promising source for future analysis and possible deployment. *A. arguta* has proved durably resistant and largely disease-free in the field, despite identification of some *P. syringae* pv. *actinidiae* in isolated sampling from *A. arguta* vines in orchards (McCann et al. 2017; Vanneste et al. 2014). Using an effector-guided strategy to discover avirulence effectors from *Psa3* would allow screening of hybrid mapping populations generated from *A. arguta* crossed with commercially attractive *A. chinensis* var. *chinensis* or *A. chinensis* var. *deliciosa* species to select lines that could provide durable resistance to *Psa3*. The simplicity of an efficient, rapid, high-throughput screening approach enables this goal to be realized in a realistic and efficient manner.

MATERIALS AND METHODS

Bacterial strains and plasmids.

P. syringae pv. *actinidiae* ICMP 18884 biovar 3 strain (*Psa3*, also referred to as *P. syringae* pv. *actinidiae* V13 or NZ13 [McCann et al. 2013]) and *P. fluorescens* Pf0-1 strains (Thomas et al. 2009) were streaked from glycerol stocks onto lysogeny broth (LB) agar (Bertani 1951) supplemented with antibiotics (all from Sigma Aldrich). *Psa3* was supplemented with nitrofurantoin at 12.5 µg/ml, cephalexin at 40 µg/ml, and either kanamycin at 50 µg/ml or gentamicin at 25 µg/ml; *P. fluorescens* was supplemented with chloramphenicol at 10 µg/ml, tetracycline (only for T3S) at 5 µg/ml, and either kanamycin at 50 µg/ml or gentamicin at 25 µg/ml. Petri plates were sealed and grown for 48 h at 22°C. Overnight shaking liquid cultures inoculated from plates were grown in LB supplemented with appropriate antibiotics and incubated at 22°C with shaking at 200 rpm.

pVSP61 (AvrPphB, AvrRpt2, or AvrRpm1) or pML123 (HopA1) plasmids (Bisgrove et al. 1994; Century et al. 1995; Kunzel et al. 1993; van Dijk et al. 2002) carrying avirulence effectors were transformed into *Psa3* or *P. fluorescens* strains using rapid electrocompetent cell preparation methodologies described earlier (Choi et al. 2006; Mesarich et al. 2017) and were transformed using a BioRad Gene Pulser Xcell electroporation system (BioRad), using the “*P. aeruginosa*” program settings, and were recovered for 1 h in LB before plating on selective media. Positive transformants were confirmed by gene-specific colony PCR.

Plant material and in-planta growth assay.

Actinidia spp. plantlets of *A. chinensis* var. *chinensis* Hort16A, *A. chinensis* var. *chinensis* Zesy002 (also called Gold3), *A. chinensis* var. *deliciosa* Hayward, or *A. arguta* AA07_03 were obtained from Multiflora Laboratories. Plantlets were grown from shoot callus tissues in 400 ml-lidded plastic ‘pottles’ on half-strength Murashige and Skoog (MS) agar, with three to five plantlets per pot. Plantlets were grown in a climate-controlled room at 20°C with a 16-h-light and 8-h-dark cycle and were used when they were 2 to 4 months old.

Psa3 pathogenicity was assessed using an in-planta flooding assay, as established by McAtee et al. (2018). Briefly, overnight-grown *Psa3* shaking liquid cultures were centrifuged at 5,000 × *g* for 3 min, supernatant was decanted, and pellets were resuspended and diluted in 10 mM MgSO₄ to 10⁶ CFU/ml (optical density at 600 nm [OD₆₀₀] adjusted to 0.004) with Silwet

L-77 (Lehle Seeds) (0.0025% vol/vol). Kiwifruit plantlets were inoculated by flooding with the *Psa3* cell suspension for 3 min, fully submerging the plantlets. The inoculum was poured off the plantlets, and the pottles were resealed with lids and were kept under plant growth conditions until sampled.

In planta bacterial growth was assessed as previously described (Jayaraman et al. 2020). Briefly, four leaf discs per pseudobiological replicate (with four replicates per treatment, per timepoint), randomly sampled using a 1-cm diameter cork-borer from three plants in a single pottle, were harvested at 2 h (day 0), day 6, and day 12 postinoculation. Each treatment timepoint was sampled from an independent pottle. Leaf discs were surface-sterilized, were placed in Eppendorf tubes containing three sterile stainless-steel ball bearings and 350 μ l of 10 mM MgSO_4 , and were macerated in a Storm 24 Bullet Blender (Next Advance) for two bursts of 1 min each at maximum speed. A 10-fold dilution series of the leaf homogenates was made in sterile 10 mM MgSO_4 , until a dilution of 10^{-8} , and was plated as 10- μ l droplets on LB medium amended with antibiotics. Petri plates were sealed with Parafilm (Sigma Aldrich) and were incubated for 2 days at 20°C before the leaf area (colony-forming units per square centimeter) was ascertained from dilutions. Three completely independent experimental runs were conducted for each in-planta growth assay, with treatment and sampling order for each strain randomized for every run and a representative run selected for depiction. Statistical analysis was done by Student's *t* test between control (wild-type *Psa3*) and variants. To observe pathogenic symptoms on the plants, infected pottles were kept for 50 dpi and photographs were taken of pottles. Infection severity was qualitatively assessed based on typical symptoms, i.e., necrotic leaf spots, chlorotic haloes, leaf death, and plant death. Each symptomology assessment was conducted on independent batches of plants (one pottle per treatment) at least three times, with treatment and sampling order for each strain randomized for every run.

DNA extraction and qPCR.

The PDQeX extraction platform (MicroGEM) was used to perform single-step DNA extraction in under 30 min (Stanton et al. 2019). Genomic DNA was extracted from infected leaf homogenate samples (as prepared for in-planta growth assays prior to serial dilution) at 7 dpi. Leaf homogenate (20 μ l) was added to 80 μ l of the master mix containing GREEN+ buffer, reaction enhancer, PrepGEM, and Histosolv (according to kit specifications) in a phytoGEM extraction tube, and extraction run under a modified 'Plant' program on a PDQeX nucleic acid extractor (MicroGEM). The 'Plant' program protocol used was 52°C for 10 min, 75°C for 10 min, 95°C for 2 min, then cooling to 30°C.

Real-time qPCR was carried out on an Illumina Eco real-time PCR platform, using SsoFast EvaGreen Supermix (BioRad) with 2 μ l of PDQeX template DNA. qPCR was performed over 40 cycles using *P. syringae* pv. *actinidiae* intergenic transcribed spacer (ITS) (target) and *AcEF1 α* (reference) primers and protocol outlined by Barrett-Manako et al. (2021) but with a lower melting temperature of 57°C. qPCR results were reported as relative expression $\log_{10}$ values of $2^{(\text{Ct}_{PsaITS} - \text{Ct}_{AcEF1\alpha})}$ for each sample with means and standard errors calculated for four pseudobiological replicates. This infection-sampling PDQeX-qPCR experiment was conducted three times on independent batches of plants. Samples were statistically analyzed for significance by analysis of variance (ANOVA) followed by a Tukey's honest significant difference (HSD) post hoc test.

Vacuum infiltration and electrolyte leakage assay.

P. fluorescens (T3S or WT) carrying empty vector or effector constructs were streaked from glycerol stocks onto LB agar plates with antibiotic selection, were grown for 2 days at 22°C, and were restreaked on fresh agar media, and were allowed to grow

overnight. Bacteria were then harvested from plates, were resuspended in 10 mM MgCl_2 , and were diluted to required $\text{OD}_{600} = 1$ (approximately 10^8 CFU/ml). Vacuum-infiltrations were carried out using a pump and glass bell. Leaves were harvested from the tissue culture tubs and were submerged in 30 ml of bacterial inoculum. The vacuum was run until bubbles were rapidly forming. The vacuum valve was then shut and the air slowly let back in. The infiltration was repeated a second time for those leaves not fully infiltrated and any remaining non-infiltrated leaves were removed, as determined by visual examination. For each treatment, leaf discs (6 mm diameter) were harvested from the uniformly vacuum-infiltrated leaf area and were washed in distilled water for 1 h. Six discs were placed in 3 ml of water, and conductivity was measured over 48 h, using a LAQUAtwin EC-33 conductivity meter (Horiba). The standard errors of the means were calculated from five pseudobiological replicates. Data for each timepoint was analyzed by ANOVA followed by a Tukey's HSD post hoc test. The entire experiment was conducted three times on independent batches of plants.

Constitutive-expression binary vector cloning.

Each effector sequence was PCR-amplified (primers in Supplementary Table S1) as a single module with flanking *BsaI* site-containing primers, using Platinum *Taq* HiFi polymerase (Thermo Fischer). Amplified PCR products were ligated with *Eco53kI*-digested (*avrPphB*, *avrRpm1*, and *hopA1*) or *SmaI*-digested (*avrRpt2*) entry vector pICH41021. Ligated constructs were verified by Sanger sequencing. The *shcA* chaperone for *hopA1* was not included in the module amplified. Each of the effector modules was assembled into the constitutive-expression binary vector pICH86988 (under the cauliflower mosaic virus 35S promoter and tobacco mosaic virus Ω leader) with either a C-terminal yellow fluorescent protein (*avrPphB* and *hopA1*) or 3xFLAG tag (*avrRpm1* and *avrRpt2*), using the Golden Gate assembly method (Engler et al. 2008).

Biolistic co-bombardment reporter eclipse assay.

Fresh young leaves of *A. chinensis* Hort16A or *A. arguta* AA07_03 [10 mm (W) $\times$ 20 mm (L)] were excised from 4-week-old in vitro-grown plantlets. Two hours prior to bombardment, three leaves were placed in the center of a circular 15-mm Whatman No. 2 filter paper disc on solidified MS medium Petri plates before bombardment. Plasmid DNA was prepared using Qiagen plasmid midi kits (Qiagen) according to manufacturer instructions.

Particle bombardment was carried out using the particle inflow gun made by KiwiScientific (Whangarei), based on the design by Finer et al. (1992). DNA-coated gold particles (1.0 μ m diameter [Bio-Rad]) were prepared as follows. A 50- μ l aliquot of gold suspension containing 5 mg of gold particles in distilled water was mixed with 10 μ g of GUS plasmid pRT99-GUS (Töpfer et al. 1988) and 10 μ g of pICH86988 empty vector (Weber et al. 2011) or vector containing the cloned effector of interest, 20 μ l of 100 mM spermidine, and 50 μ l of 2.5 M CaCl_2 . The solution was vortexed vigorously for 2 min and was incubated at room temperature for 10 min. The supernatant was carefully removed to leave a 35- μ l final volume for bombardment.

For each bombardment, 5 μ l of a well-mixed particle preparation was loaded onto a syringe filter carrier and the bombardment was performed using the following conditions: shot distance = 14 cm from the carrier to the target shelf, helium pressure = 300 KPa, pulse time = 30 ms, chamber vacuum = -90 KPa. Target leaves were held (abaxial side uppermost) under a stainless-steel mesh (100 μ m) and were bombarded once. Six bombardments were performed for each particle preparation, and each experiment was carried out in triplicate. After bombardment, the leaves were immersed in a 20-ml suspension of *Agrobacterium tumefaciens*

GV3101 (OD₆₀₀ adjusted to 0.6) for 30 s, after which they were transferred onto solid MS medium.

After 48 h of incubation in the light at room temperature, the leaf tissue was frozen in liquid nitrogen and was ground at 4 m/s for 20 s with metal beads, using a Bead Ruptor Elite (Omni International, Inc.). The frozen tissue powder (approximately 300 mg) was then mixed by vigorous vortexing with 1 ml of GUS extraction buffer (50 mM sodium phosphate buffer [pH 7.0], 10 mM EDTA, 0.1% Triton X-100 [vol/vol], 0.1% N-lauryl sarcosine [wt/vol], 10 mM β -mercaptoethanol, 1% of polyvinylpyrrolidone [wt/vol], 20% methanol [vol/vol], and one Complete ULTRA Mini Protease inhibitor cocktail tablet for every 10 ml of extraction buffer (all from Sigma Aldrich). The crude extract was centrifuged at 18,000 $\times g$ for 15 min at 4°C, and the supernatant was collected and assayed. The protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories), following manufacturer instructions. The GUS activity was determined using 100 μ l of leaf extract with methylumbelliferyl- β -glucuronide as the substrate, following the method described by Hunter and Watson (2008). The amount of 4-methylumbelliferyl (MU) product was measured in a Tecan Safire2 fluorescence microplate reader (Tecan). For each bombardment, triplicate samples (technical replicates) were assayed and expressed in terms of pmol of MU produced per minute per gram of total protein. The means and standard errors were calculated from 18 replicates conducted across three independent experimental runs. Data for each treatment was stacked from all three runs and was analyzed by ANOVA followed by a Tukey's HSD post hoc test.

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