BACTERIAL AND PHYTOPLASMA DISEASES

Flood inoculation of seedlings on culture medium to study interactions between *Pseudomonas syringae* pv. *actinidiae* and kiwifruit

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Abstract
Bacterial canker of kiwifruit caused by *Pseudomonas syringae* pv. *actinidiae* (Psa) is a serious threat to kiwifruit production. Highly virulent strains of Psa biovar3 (Psa3) have spread rapidly to kiwifruit production areas worldwide. Therefore, there is an urgent need to develop critical management strategies for bacterial canker based on dissecting the interactions between Psa and kiwifruit. Here, we developed a rapid and reliable flood-inoculation method using kiwifruit seedlings grown on Murashige and Skoog medium. This method has several advantages over inoculation of conventional soil-grown plants. We demonstrated the utility of a kiwifruit seedling assay to study the virulence of Psa biovars and Psa3 virulence factors, including the type III secretion system (T3SS). Kiwifruit seedlings inoculated with Psa3 developed severe necrosis within 1 week, whereas those inoculated with a T3SS-deficient *hrcN* mutant of Psa3 did not. This method was also useful for analyzing expression profiles of genes involved in Psa3 virulence during infection, and revealed that the expression of genes encoding the T3SS and type III secreted effectors were strongly induced in planta. Our results indicate that the T3SS has an important role in Psa3 virulence, and the flood-inoculation assay using kiwifruit seedling is suitable for analyzing Psa and kiwifruit interactions.

Keywords *Pseudomonas syringae* pv. *actinidiae* · Kiwifruit · Flood-inoculation · Type III secretion system · Type III secreted effectors

Introduction

*Pseudomonas syringae* induces a variety of symptoms such as leaf spots, cankers, galls, wilt, and blights on different plants, and can be classified into more than 50 pathovars (pv.) based on their host plant specificities and disease symptoms (Mansfield et al. 2012; Xin and He 2013). *P. syringae* pv. *actinidiae* (Psa), causal agent of bacterial canker of kiwifruit, is an economically important pathogen worldwide. First reported in Japan, Psa causes severe damage to kiwifruit plants and decreases yields under optimal conditions (Scortichini et al. 2012; Takikawa et al. 1989). Psa is organized into five biovars including biovar 1, 2, 3, 5, and 6 based on biochemical, pathogenicity, and molecular characteristics of the Psa strains (Fujikawa and Sawada 2016, 2019). The emergence of Psa3 strains with enhanced virulence was reported in 2008 in Italy, and a pandemic spread rapidly to kiwifruit production areas worldwide (Scortichini et al. 2012). Major control strategies include chemical treatments such as copper fungicides and antibiotics (Cameron and Sarojini 2013), but they are associated with potential risks such as induction of Psa resistance strains, phytotoxicity, and chemical residues in fruits (Nakajima et al. 2002; Serizawa et al. 1989; Vanneste and Voyle 2003). Breeding for resistance is another control strategy, but cultivar development is slow (Kisaki et al. 2019). Therefore, alternative management strategies based on the dissection of the dynamic interactions between Psa and kiwifruit are urgently needed.

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Plants have been inoculated with *P. syringae* using syringe pressure infiltration, vacuum infiltration, and sprays and dips to investigate the molecular basis of plant–pathogen interactions (Katagiri et al. 2002). Syringe pressure infiltration is the most commonly used method (Kisaki et al. 2019). Conventional soil-grown plant inoculation assays have included stem puncture inoculation, syringe pressure infiltration, and spray inoculation were used to study the interactions between Psa and kiwifruit (Bartoli et al. 2015; Gao et al. 2016; Kisaki et al. 2019). However, to functionally investigate the molecular interactions between plants and *P. syringae*, the inoculation assay should mimic natural infection and be suitable for high-throughput assays. *P. syringae* has two lifestyles, an epiphytic phase on the plant leaf surface and an endophytic phase in the apoplastic space (Melotto et al. 2008; Xin and He 2013). The bacterium generally enters host tissues through natural openings such as stomata and multiplies in the apoplast to cause disease in nature (Lindow and Brandl 2003).

In previous studies, we developed a flood-inoculation method to investigate the interactions of plants with *P. syringae pv. tomato* DC3000 (Ishiga et al. 2011; Uppalapati et al. 2015) but the inoculation assay should mimic natural infection and be suitable for high-throughput assays. *P. syringae* pathogen was modified to develop a kiwifruit inoculation assay conducive to observing disease symptoms. Briefly, 40 ml of bacterial suspension (OD600 of 0.2) in sterile distilled H2O containing 0.025% (v/v) Silwet L-77 was dispensed onto a plate containing 4-week-old kiwifruit seedlings, and the plates were incubated for 2–3 min at room temperature. After the bacterial suspension was removed by decantation, plates containing inoculated plants were sealed in growth chambers at approximately 100% RH for the first 24 h, then at approximately 70% RH for the rest of the experiment. The inoculated plants were observed for 1 wpi for symptom development.

To determine the bacterial growth in kiwifruit seedlings or leaves, we measured the internal bacterial population at 24 °C with a light intensity of 200 μmol/(m² sec) and 16 h light/8 h dark to use for spray inoculation.

### Bacterial strains and growth conditions

All bacterial strains and plasmids are shown in Table 1. *Pseudomonas syringae* pv. *actinidiae* biovar 1 (Psa1; MAFF 613022), biovar 3 (Psa3; MAFF 212115), biovar 5 (Psa5; MAFF 212056), and biovar 6 (Psa6; MAFF 212133) were a gift from NARO Genebank, Ibaraki, Japan. Psa biovars were used as the pathogenic strains to inoculate kiwifruit plants. The Psa3 *hrcN* mutant defective in the type III secretion system (T3SS) was generated by conjugation with *Escherichia coli* S17-1, which possesses pBSLC1 (Sawada et al. 2018; Schäfer et al. 1994), and was used as a reduced-virulence mutant of Psa3. The pDSK-GFPuv vector was introduced into each Psa biovar by electroporation (Wang et al. 2007). All Psa strains were grown at 28 °C on King’s B (KB) (King et al. 1954) agar. For inoculation, Luria–Bertani (LB) (Sambrook et al. 1989) broth was used to grow bacterial cultures from plates for 18 h at 28 °C. Before inoculation, bacteria were suspended in sterile distilled H2O, and the bacterial cell densities at 600 nm (OD600) were measured using a JASCO V-730 spectrophotometer (JASCO, Tokyo, Japan).
2 wpi. Inoculated seedlings or leaves were collected, and the inoculated plants were weighed. Seedlings or leaves were then surface-sterilized with 10% H2O2 for 3 min, then washed three times with sterile distilled water. Plants were then homogenized in sterile distilled water, and the diluted samples were plated onto solid KB agar. Bacterial colony forming units (CFU) were normalized as CFU/mg using the total mass of the inoculated plants. The bacterial population at 0 day was estimated using leaves harvested 1 h post-inoculation (hpi). The bacterial populations were evaluated in three independent experiments.

Real-time quantitative RT-PCR

For expression profiles of Psa3 genes in culture or during infection, we incubated Psa3 in LB broth for 3 h or flood-inoculated kiwifruit plants for 24 and 48 h. For expression profiles in culture conditions, Psa3 was grown in LB broth for 24 h, then adjusted to OD600 of 0.1 with fresh LB broth and grown for 3 h to investigate the expression profiles during the exponential phase, not the stationary phase. Total RNA was extracted from kiwifruit seedlings by an ultrasonication method as described (Yu et al. 2013). Briefly, 12 plants were collected, immediately submerged in 5 ml of RNAlater Stabilization Solution (Thermo Fisher Scientific, Waltham, MA, USA), sonicated for 7 min, then plants were removed from the solution. The bacterial cells in the suspension were harvested by centrifugation at 12,000 rpm for 2 min, and cell pellets were used for subsequent purification. Total RNA was extracted using Reliaprep (Promega, Madison, WI, USA) and the manufacturer’s protocol. Two micrograms of total RNA was treated with gDNA Remover (TOYOBO, Osaka, Japan) to eliminate genomic DNA, and the DNase-treated RNA was reverse transcribed using the ReverTra Ace qPCR RT Master Mix (TOYOBO). The cDNA (1:20) was then used for qRT-PCR that was performed using the primers shown in supplementary Table S1 with THUNDERBIRD® SYBR qPCR Mix (TOYOBO) on a Thermal Cycler Dice Real Time System (TaKaRa, Kusatsu, Japan). Three genes encoding fructose-bisphosphate aldolase, chromosome participating protein ParA, and a TetR family transcriptional regulator were used for normalizing the results (McAtee et al. 2018).

Results

Flood inoculation of seedlings to study Psa–kiwifruit interactions

For standardizing the seedling flood-inoculation assay and testing whether Psa multiplies and causes disease symptoms similar to adult plants grown in soil, 4-week-old kiwifruit seedlings (containing two to four leaves except for the cotyledon) were grown on MS plates. Around 80–100 seeds were planted on each MS plate (Fig. 1a), which germinated
2 weeks after planting when exposed to light (Fig. 1b). Six small seedlings were transferred to a new plate (Fig. 1c), and after 2 weeks of continuous growth, kiwifruit seedlings were flood-inoculated with a bacterial suspension (OD600 of 0.2) of Psa (Fig. 1d–g). We observed severe disease symptoms within 3 days, and severe necrosis by 1–2 wpi with Psa3 (Fig. 1h).

To further assess the utility of the seedling flood-inoculation assay, we next inoculated kiwifruit seedlings with Psa biovars including Psa1, Psa3, Psa5, and Psa6. Psa1- and Psa3-inoculated seedlings showed necrosis within 1 wpi, and severe disease symptoms at 2 wpi (Fig. 2a). Among the Psa biovars, Psa3 caused the most severe symptoms (Fig. 2a). Symptoms on Psa5-inoculated seedlings developed slower during the first compared to those on Psa1- and Psa3-inoculated seedlings, but developed typical symptoms such as leaf spots at 2 wpi (Fig. 2a). Typical disease symptoms such as leaf spots can be seen in magnified images of Psa1-, Psa3-, and Psa5-inoculated seedlings at 2 wpi (Fig. 2a, bottom row). On the other hand, symptoms on seedlings-inoculated with Psa6 were only observed at 2 wpi (Fig. 2a). Mild symptoms such as chlorosis without leaf spots can be seen in the magnified image of Psa6-inoculated seedlings. These results suggest that Psa6 shows the least virulence among the Psa biovars (Fig. 2a, bottom row). Consistent with symptom development caused by the different biovars, Psa3 populations were the highest compared to those of Psa1, Psa5, and Psa6 (Fig. 2b), and Psa6 populations were the lowest. We also observed bacterial colonies in kiwifruit seedlings by labeling the Psa biovars with the GFPuv protein. Interestingly, the bacterial colonies were clearly observed in Psa1- and Psa3-inoculated seedlings at 6 days post inoculation (Fig. 2c). To evaluate the utility of the kiwifruit seedling-flood inoculation assay for studying nonhost interactions, we inoculated kiwifruit seedlings with P. syringae pv. tomato strain DC3000 (Pst DC3000). Pst DC3000 did not induce HR cell death in the healthy-looking inoculated seedlings by 1 wpi (Supplementary Fig. S1a). Pst DC3000 bacterial populations were also lower than those of Psa3 (Supplementary Fig. S1b). Together, these results suggest that the kiwifruit seedling-flood inoculation assay is a reliable method to study Psa disease progression and to evaluate in planta bacterial growth.

**Kiwifruit seedling flood-inoculation assay to study a Psa virulence mutant**

To evaluate the utility of the seedling flood-inoculation assay for the study of Psa3 virulence factors, we flood- or spray-inoculated kiwifruit seedlings or plants with wild-type Psa3 and a Type III secretion system (T3SS) deficient hrcN mutant. Wild-type Psa3 caused severe symptoms not only on seedlings but also on mature leaves (Fig. 3a and b). However, severe necrosis was not observed on hrcN mutant-inoculated...
Consistent with disease development, the bacterial populations of the hrcN mutant were approximately 1000-fold lower compared to wild-type Psa3 after both inoculation methods (Fig. 3c and d). These results indicate that the T3SS has important roles in bacterial multiplication, persistence, and disease symptom development of Psa3 in kiwifruit seedlings, and in soil-grown kiwifruit plants. The kiwifruit seedling flood-inoculation assay is suitable for analyzing Psa virulence mutants.

**Flood-inoculation assay of kiwifruit seedlings to study expression profiles of virulence genes during Psa infection**

Pseudomonas syringae virulence genes are expressed during infection (McAtee et al. 2018; Nobori et al. 2018). We thus investigated the expression profiles of these genes in wild-type Psa3 using RT-qPCR and gene-specific primer sets according to a previous study (McAtee et al. 2018; Supplementary Table S1). We carried out expression analysis using RNAs from flood-inoculated seedlings (Fig. 4a–h) and spray-inoculated plant leaves similar to the method used for Fig. 3 (Fig. 4i–p). Our results demonstrated that expression of the alternative sigma factor hrpL, which controls the expression of T3SS effectors (T3SEs) (Fouts et al. 2002), was clearly induced in planta (in flood- and in spray-inoculated kiwifruit plants) compared to culture in LB broth (Fig. 4a and i). In addition, the expression of Psa-conserved T3SS and T3SEs genes, including hrpA1, avrRpm1, hopR1, and hopZ3 were also induced in planta (Fig. 4j–l), indicating that the T3SS is activated during Psa3 infection. We also investigated other P. syringae virulence genes (Ichinose et al. 2013; Ishiga et al. 2018; Ishiga and Ichinose 2016). The oxyR and fliC transcripts, encoding transcription factors related to oxidative stress (Ishiga and Ichinose 2016) and flagellin (Shimizu et al. 2003), respectively, were reduced in planta compared to levels produced in broth culture (Fig. 4f, g, and o). The expression of algD encoding an alginate biosynthesis enzyme (Yu et al. 1999) was induced in planta (Fig. 4h and p). Overall, we found that expression trends for most genes were the same between the two types of inoculation methods (Fig. 4). Together, our results suggest that the kiwifruit seedling flood-inoculation assay is suitable for analyzing Psa expression profiles during infection.

**Discussion**

Our assessments of the newly developed seedling flood-inoculation assay for kiwifruit (Fig. 1) showed that it is suitable for analyzing the virulence mechanism of Psa biovars (Fig. 2). Our method has several advantages over previous conventional soil-grown plant inoculation assays (Bartoli...
et al. 2015; Gao et al. 2016; Kisaki et al. 2019), including a shorter growth and incubation period, ease of inoculation and handling, uniformity of infection and disease development, and need for less growth chamber space (Fig. 1). This new method is suitable not only for dissecting the dynamic interactions between Psa and kiwifruit, but also for high-throughput screening for Psa virulence mutants, to further develop critical management strategies for bacterial canker of kiwifruit.

We observed Psa colonies in kiwifruit seedlings by labeling with a GFPuv protein. The bacterial colonies were clearly observed in Psa1- and Psa3-inoculated seedlings compared to Psa5- and Psa6-inoculated leaves (Fig. 2c). However, the population size of Psa5 was almost the same as that of Psa1 (Fig. 2b). One reason to explain the differences in the GFPuv intensity and the population size between Psa5 and Psa1 could be differences in colonization sites. \textit{P. syringae} pathovars are known to have different lifestyles (Lindow and Brandl 2003; Xin and He 2013). A putative epiphyte, whereas \textit{P. syringae} \textit{P. syringae} is known as a particularly successful pathovar to explain the differences in the T3SS and T3SEs expression profiles in planta among \textit{P. syringae} pathovars is their different lifestyles. \textit{Pss} is known as a particularly successful epiphyte, whereas \textit{Pst} DC3000 is a weak epiphyte (Lindow and Brandl 2003; Xin and He 2013). A putative epiphytic phase was suggested for Psa3 on asymptomatic flowers and leaves (Gallelli et al. 2011; Stefani and Giovanardi 2011); however, how Psa modulates the epiphytic and endophytic phases and which genes are required for these phases has not been fully ascertained. Understanding Psa lifestyles will be critical to developing new management strategies for bacterial canker of kiwifruit. We believe that the flood-inoculation method will enable us to identify Psa genes required not only for virulence but also for epiphytic fitness and will provide critical clues to develop effective control strategies.

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\textbf{Fig. 3} Seedling flood-inoculation assay to analyze virulence factors in \textit{Pseudomonas syringae pv. actinidiae} biovar 3 (Psa3). \textbf{a} Disease phenotype of kiwifruit seedlings 1 week after flood inoculation with a bacterial suspension (OD$_{600}$ of 0.2) of wild-type Psa3 or T3SS hrcN mutant. Scale bars, 1 cm. \textbf{b} Disease phenotype of leaves 1 week after spray inoculation of 6-week-old kiwifruit plants with suspension of wild-type Psa3 or T3SS hrcN mutant at $5 \times 10^7$ CFU/ml. Scale bars, 1 cm. \textbf{c} Populations of wild-type Psa3 and hrcN mutant at 1 week after flood inoculation of kiwifruit seedlings. Vertical bars indicate standard error for three independent experiments. Asterisks indicate a significant difference from the wild type in a $t$ test (*$P < 0.01$). \textbf{d} Populations of wild-type Psa3 and hrcN mutant in kiwifruit plants at 0 and 1 week after spray inoculation. Vertical bars indicate the standard error for three independent experiments. Asterisks indicate a significant difference from the wild type in a $t$ test (*$P < 0.01$).
Fig. 4 Expression profiles of genes involved in the virulence of *Pseudomonas syringae* pv. *actinidiae* biovar 3 (Psa3) at 24 and 48 h after growth in liquid LB broth (culture) or after flood- or spray-inoculation of kiwifruit plants. Psa3 inoculum suspension had OD$_{600}$ of 0.2. Total RNA was extracted for use in real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR) with gene-specific primer sets. a hrpL, b hrpA1, c avrRpm1, d hopR1, e hopZ3, f oxyR, g fliC, and h algD in flood-inoculated kiwifruit seedlings. i hrpL, j hrpA1, k avrRpm1, l hopR1, m hopZ3, n oxyR, o fliC, and p algD in spray-inoculated kiwifruit. Expression was normalized using *fbp, parA,* and *tetR* (Supplementary Table S1). Vertical bars indicate the standard error for three biological replicates. Asterisks indicate a significant difference from culture cells in a *t* test (*P* < 0.05; ** P < 0.01)
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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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