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BACTERIAL AND PHYTOPLASMA DISEASES

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

Takako Ishiga[1] · Nanami Sakata[1] · Viet Tru Nguyen[1] · Yasuhiro Ishiga[1]

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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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✉ Yasuhiro Ishiga
ishiga.yasuhiro.km@u.tsukuba.ac.jp

[1] Faculty of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan

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. 2008). To establish a robust assay to study Psa and kiwifruit interactions, in the present study, we developed a rapid and reliable flood-inoculation method using kiwifruit seedlings that had been grown in Murashige and Skoog (MS) medium for 4 weeks. We also demonstrated that this method is suitable to study Psa virulence mechanisms.

Materials and methods

Plant materials and growth conditions

Kiwifruit (*Actinidiae delisiosa*) cv. Hayward plants were used for all experiments. Kiwifruit seeds (100–200) were surface-sterilized in 70% (v/v) ethanol for 5 min in a Falcon tube (50 ml), then in 5% (v/v) sodium hypochlorite (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) containing 0.1% (v/v) Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) for 1 h. Seeds were then washed with sterile distilled H_2O at least four times and incubated in water at 4 °C overnight. Surface sterilization and washing were repeated, and seeds were germinated on one-half strength MS medium (FUJIFILM Wako Pure Chemical Corporation) containing 1% (w/v) sucrose and Gamborg vitamins (Sigma-Aldrich) solidified with 0.3% (w/v) Phytagel (Sigma-Aldrich) in deep Petri plates (100 mm × 25 mm). Four-week-old kiwifruit seedlings were grown at 24 °C with a light intensity of 150–200 μmol/(m^2 sec) and 12 h light/12 h dark before use. Six-week-old kiwifruit plants were grown in Berger BM2 Germinating Mix soil (Berger, Saint-Modeste, QC, Canada) at 24 °C with a light intensity of 200 μmol/(m^2 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 H_2O, and the bacterial cell densities at 600 nm (OD_{600}) were measured using a JASCO V-730 spectrophotometer (JASCO, Tokyo, Japan).

Bacterial inoculation

A flood-inoculation method that we developed previously (Ishiga et al. 2017) to infect *Arabidopsis* seedlings with *P. syringae* pathogens was modified to develop a kiwifruit seedling inoculation method conducive to observing disease symptoms. Briefly, 40 ml of bacterial suspension (OD_{600} of 0.2) in sterile distilled H_2O containing 0.025% (v/v) Silwet L-77 (OSI Specialties Inc., Danbury, CT, USA) 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 with 3 M Micropore 2.5 cm surgical tape (3 M, St. Paul, MN, USA) and incubated at 22 °C with a light intensity of 150–200 μmol/(m^2 sec) and 12 h light/12 h dark. Symptoms were observed at 1- and 2-weeks post-inoculation (wpi). In each experiment, more than six plants were evaluated, and each experiment was repeated at least three times.

For spray inoculation, 6-week-old kiwifruit plants were sprayed to runoff with a bacterial suspension (OD_{600} of 0.2) in sterile distilled water containing 0.025% Silwet L-77. The plants were then incubated 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

Table 1 Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristics	Reference or source
E. coli strain		
S17-1	Thi pro hsdR-hsdM + recA [chr::RP4-2-Tc::Km::Tn7]	Schäfer et al. (1994)
P. syringae pv. *actinidiae* (Psa)		
Psa1	Psa biovar 1	MAFF 613022
Psa1 GFPuv	Psa1 containing pDSK-GFPuv	This study
Psa3	Psa biovar 3	MAFF 212115
Psa3 *hrcN* mutant	Insertion *hrcN*::mini-Tn5, Km^r, Cm^r	This study
Psa3 GFPuv	Psa3 containing pDSK-GFPuv	This study
Psa5	Psa biovar 5	MAFF 212056
Psa5 GFPuv	Psa5 containing pDSK-GFPuv	This study
Psa6	Psa biovar 6	MAFF 212133
Psa6 GFPuv	Psa6 containing pDSK-GFPuv	This study
P. syringae pv. tomato (*Pst*)		
DC3000	*Pst* DC3000 wild-type, *Arabidopsis* and tomato pathogen	Buell et al. (2003)
Plasmid		
pBSLC1	Transposon vector constructed by ligation of pBSL118 and pHSG396 at *Eco*RI site, Amp^r, Km^r, Cm^r	Sawada et al. (2018)
pDSK-GFPuv	Vector containing constitutive *psbA* promoter and *GFPuv1* gene inserted into pDSK519, Km^r	Wang et al. (2007)

Amp^r ampicillin resistance, Cm^r chloramphenicol resistanc, Km^r kanamycin resistance, Rif^r rifampicin resistance

2 wpi. Inoculated seedlings or leaves were collected, and the inoculated plants were weighed. Seedlings or leaves were then surface-sterilized with 10% H_2O_2 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 OD_{600} 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

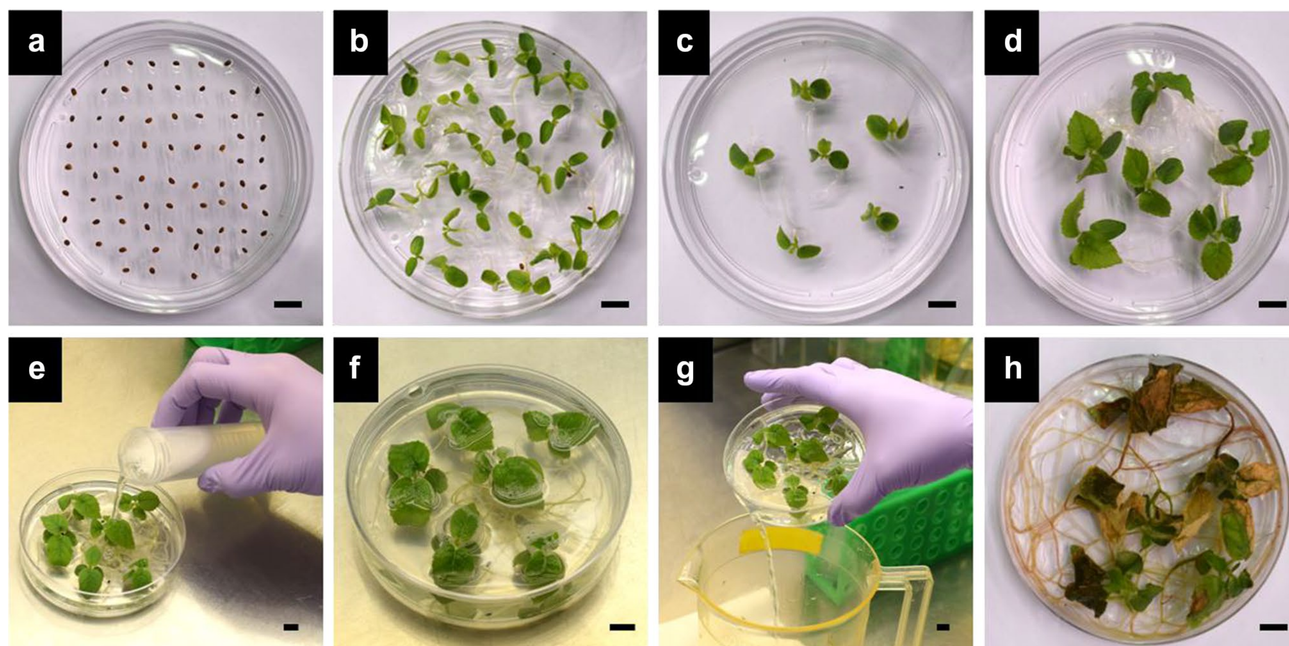

Fig. 1 Flood-inoculation assay of kiwifruit seedlings with *Pseudomonas syringae* pv. *actinidiae* biovar 3 (Psa3). **a** Seeds planted on one-half strength MS plates. **b** Two-week-old seedlings. **c** Two-week-old seedlings transferred to new one-half strength MS plates. **d** Four-week-old kiwifruit seedlings ready for flood inoculation. **e** Preparation of inoculum. **f** Flood inoculation by pouring the Psa3 suspension onto plates. **g** Removal of bacterial suspension by decantation. **h** Disease phenotypes 1 week post inoculation. Scale bars show 1 cm

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 (OD_{600} 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

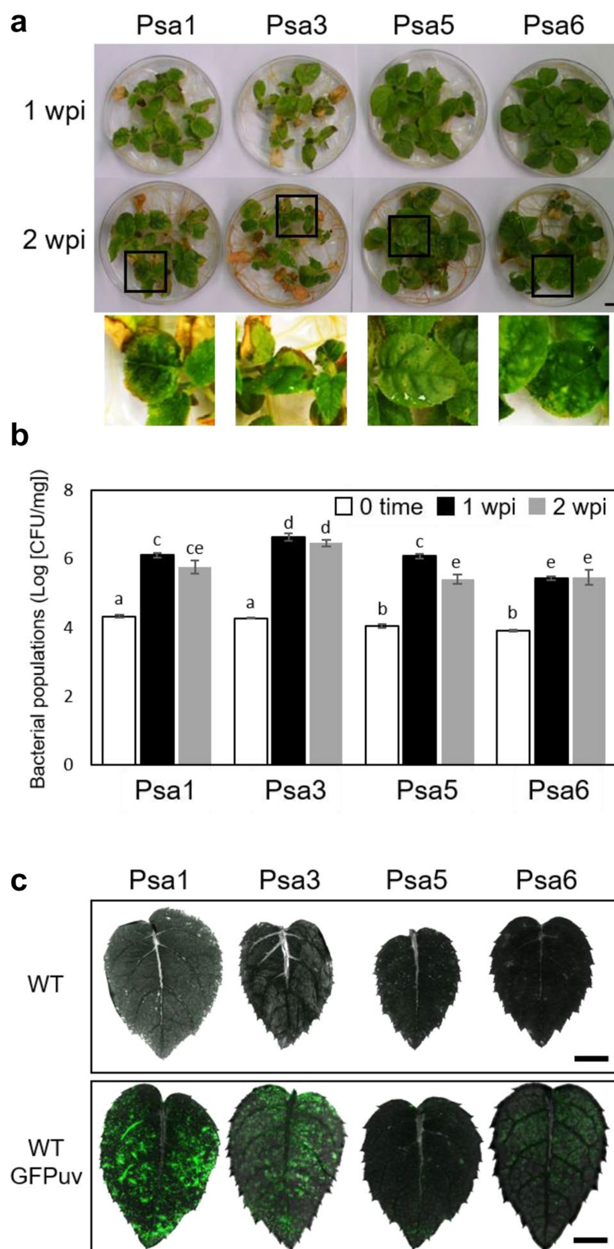

Fig. 2 Flood-inoculation assay to analyze interactions between *Pseudomonas syringae* pv. *actinidiae* (Psa) biovars 1, 3, 5, and 6 and kiwifruit seedlings. **a** Disease phenotype of kiwifruit seedlings 1 and 2 weeks post-inoculation (wpi) with suspensions (OD_{600} of 0.2) of Psa biovars containing 0.025% Silwet L-77. Close-up of typical symptoms in black square at 2 wpi. Scale bars, 1 cm. **b** Populations of Psa biovars in kiwifruit seedlings at 0, 1, and 2 wpi. Vertical bars indicate the standard errors for three independent experiments. Significant differences ($P<0.05$) are symbolized by different letters. **c** Visualization of Psa biovars labeled with green fluorescent protein variant GFPuv in the leaves of kiwifruit seedlings at 6 days after flood-inoculation with at 5×10^7 CFU/ml Psa biovars. The whole leaf was examined using a Keyence BZ-X 800 fluorescent stereomicroscope (Keyence, Osaka, Japan). Scale bars, 5 mm

seedlings and leaves (Fig. 3a and b). 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

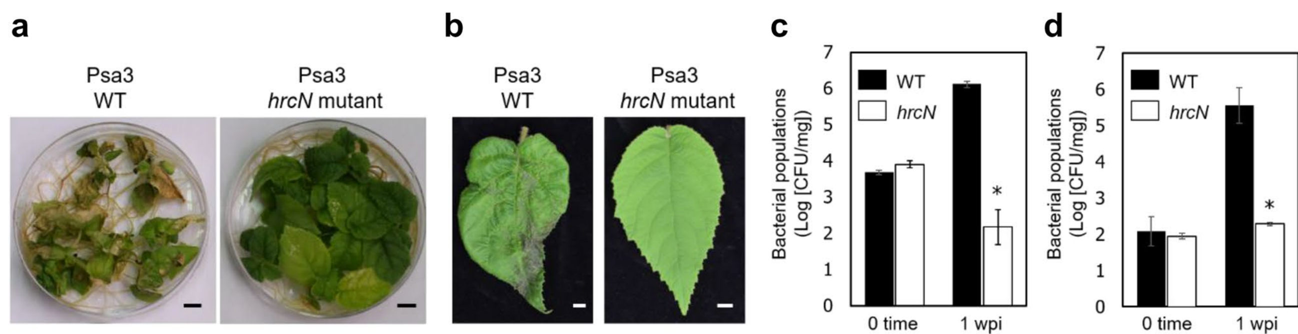

Fig. 3 Seedling flood-inoculation assay to analyze virulence factors in *Pseudomonas syringae* pv. *actinidiae* biovar 3 (Psa3). **a** Disease phenotype of kiwifruit seedlings 1 week after flood inoculation with a bacterial suspension (OD_{600} of 0.2) of wild-type Psa3 or T3SS *hrcN* mutant. Scale bars, 1 cm. **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 5×10^7 CFU/ml. Scale bars, 1 cm. **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 ($*P<0.01$). **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 ($*P<0.01$)

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. *P. syringae* pathovars are known to have different lifestyles (Lindow and Brandl 2003; Xin and He 2013). The Psa observation system with GFPuv, which we developed in the present study, will help us spatiotemporally analyze colonization by Psa biovars and understand the Psa infection processes.

The T3SS is a key virulence component of *P. syringae*, because *hrp*/*hrc* mutants that block the T3SS completely eliminate virulence against susceptible host plants (Lindeberg et al. 2012; Xin and He 2013). We demonstrated that a T3SS deficient Psa *hrpN* mutant impaired virulence not only in kiwifruit seedlings, but also in soil-grown plants (Fig. 3), indicating that the T3SS has an important role in Psa3 virulence. In addition, several studies reported that each Psa strain has T3SEs that are conserved among biovars as well as a unique set (Fujikawa and Sawada 2016, 2019; McCann et al. 2013; Zhao et al. 2019). However, the molecular function of the T3SEs in Psa is largely unknown. Therefore, it is important to investigate the molecular basis of the T3SEs in the virulence of Psa biovars, and the flood-inoculation assay will be a robust method.

We also demonstrated that the expression of Psa3 genes encoding the T3SS and T3SEs was induced in planta compared to culture conditions in both flood- and spray-inoculated plants (Fig. 4i–l). Several transcriptome studies have been performed using different *P. syringae* pathovars including *P. syringae* pv. *syringae* (*Pss*) B728a, *P. syringae* pv. *tomato* (*Pst*) DC3000, and Psa3 (McAtee et al. 2018; Nobori et al. 2018; Yu et al. 2013). Yu et al. (2013) showed that expression of genes encoding the T3SS and T3SEs in *Pss* B728a was not strongly induced in the apoplast. In contrast, these genes were greatly upregulated in planta in comprehensive analyses of the expression profiles of *Pst* DC3000 and Psa3 (McAtee et al. 2018; Nobori et al. 2018). One reason to explain the differences in the T3SS and T3SEs expression profiles in planta among *P. syringae* pathovars is their different lifestyles. *Pss* is known as a particularly successful epiphyte, whereas *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 arerequired 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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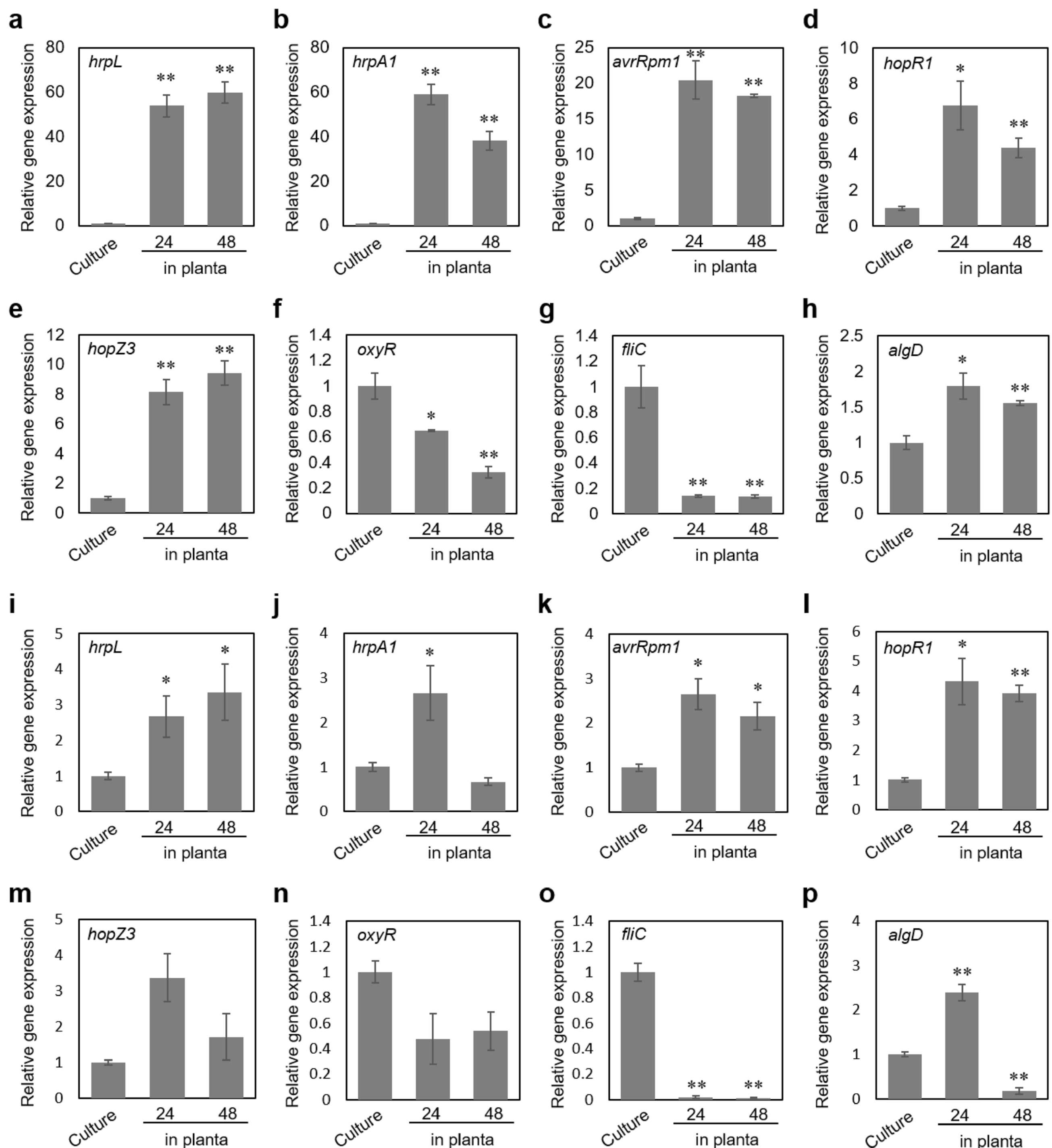

Fig. 4 Expression profiles of genes involved in the virulence of *Pseudomonas syringa*e pv. *actinidiae* biovar 3 (Psa3) at 24 and 48 h after growth in liquid LB broth (culture) or after flood- or spray-inoculation of kiwifruit plants. Psa3 inoculum suspension had OD_{600} of 0.2. Total RNA was extracted 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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