



Macroscopic and microscopic visualization of *Pseudomonas syringae* pv. *tabaci* infection using complementary bioluminescent and fluorescent reporter systems

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Abstract

The development of bioluminescent and fluorescent reporter systems has enabled visualization of plant-pathogenic bacterial infection dynamics, providing insights into detailed mechanisms of infection and potential applications for disease control. *Pseudomonas syringae* pv. *tabaci* 6605 (*Pta*) possesses quorum sensing systems and produces fluorescent pigments, which make it difficult to directly apply commonly used reporter systems. In this study, we first constructed a homologous recombination-based genomic insertion system for stable chromosomal integration of reporter genes in *Pta*. Using this system, we established a LuxCDABE bioluminescent system driven by the *nptII* promoter, which successfully enabled macroscale tracking of bacterial populations during infection. We then attempted to construct an sGFP fluorescent reporter using the same *nptII* promoter. However, the endogenous autofluorescence of *Pta* masked the sGFP signal, making quantitative detection difficult. To overcome this autofluorescence problem, we screened constitutively highly expression promoters based on transcriptome data and identified two effective promoters suitable for fluorescent monitoring of *Pta* infection. Together with the LuxCDABE system, this integrated platform provides comprehensive spatiotemporal analysis of virulence mechanisms and plant defense responses in the *Pta* pathosystem, with potential applicability to other plant-pathogenic bacteria.

Keywords *Pseudomonas syringae* pv. *tabaci* · LuxCDABE · sGFP-reporter

Introduction

Understanding the infection behavior of plant-pathogenic bacteria is essential for developing strategies to control plant diseases. Characterizing the spatiotemporal dynamics of bacterial infection in plants, including initial invasion, colonization, proliferation, and systemic movement, remains a key challenge in understanding plant disease. Recent advances in fluorescence and confocal microscopy,

together with the availability of diverse fluorescent protein reporters and bioluminescent tagging systems, now allow real-time visualization of plant-pathogenic bacteria during infection (Wang et al. 2007; Fan et al. 2008; Hupp et al. 2019; Matsumoto et al. 2022).

Visualization of plant-pathogenic bacteria during infection requires a monitoring system tailored to the target organism. Constructing such a system involves several design choices. The reporter gene can be maintained on a plasmid or integrated into the genome. Promoters vary in strength and expression patterns. The reporter itself may be based on bioluminescence or fluorescent proteins. Bioluminescence-based reporters do not require external excitation and produce minimal background signals, making them advantageous for sensitive, population-level quantification, but lack the spatial resolution required for single-cell observation. In contrast, fluorescent reporters enable high-resolution single-cell imaging but are susceptible to background

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Table 1 Bacterial strains and plasmids

Bacterial strain/plasmid	Relevant characteristics	Reference or source
<i>Escherichia coli</i>		
DH5α	<i>F⁻ λ-ø80dLacZ ΔM15 Δ(lacZYA-argF)U169 recA1endA1 hsdR17(rK-mK+) supE44 thi-1 gyrA relA1</i>	Takara, Kyoto, Japan
S17-1	<i>thi pro hsdR hsdR hsdM⁺ recA(chr::RP4-2-Tc::Mu-Km::Tn7)</i>	Schäfer et al. 1994
<i>P. syringae</i> pv. <i>tabaci</i> (Pta)		
Isolate 6605	Wild type isolated from tobacco; Nal ^r	Shimizu et al. 2003
<i>Pta ΔhrcC</i>	Isolate 6605 ΔPta6605_RS21955; Nal ^r	Marutani et al. 2005
<i>Pta</i> WT:: <i>lux</i>	<i>PnptII::luxCDABE</i> integrated into the intergenic region between Pta6605_RS27155 and Pta6605_RS27160 on the <i>Pta</i> chromosome; Nal ^r	In this study
<i>Pta ΔhrcC::lux</i>	<i>PnptII::luxCDABE</i> integrated into the intergenic region between Pta6605_RS27155 and Pta6605_RS27160 on the <i>Pta</i> chromosome; Nal ^r	In this study
<i>Pta</i> pDSK-PnptII::sgfp	<i>Pta</i> WT carrying pDSK-PnptII::sgfp; Nal ^r , Km ^r	In this study
<i>Pta</i> pDSK-PsdhC::sgfp	<i>Pta</i> WT carrying pDSK-PsdhC::sgfp; Nal ^r , Km ^r	In this study
<i>Pta</i> pDSK-PraiA::sgfp	<i>Pta</i> WT carrying pDSK-PraiA::sgfp; Nal ^r , Km ^r	In this study
<i>Pta</i> pDSK-PfliC::sgfp	<i>Pta</i> WT carrying pDSK-PfliC::sgfp; Nal ^r , Km ^r	In this study
<i>Pta</i> pDSK-PprkA-like::sgfp	<i>Pta</i> WT carrying pDSK-PprkA-like::sgfp; Nal ^r , Km ^r	In this study
<i>Pta</i> pDSK-PglA::sgfp	<i>Pta</i> WT carrying pDSK-PglA::sgfp; Nal ^r , Km ^r	In this study
<i>Pta</i> pDSK-Phyp::sgfp	<i>Pta</i> WT carrying pDSK-Phyp::sgfp; Nal ^r , Km ^r	In this study
<i>Pta</i> pDSK-PgroES::sgfp	<i>Pta</i> WT carrying pDSK-PgroES::sgfp; Nal ^r , Km ^r	In this study
<i>Pta</i> pDSK-Psbp::sgfp	<i>Pta</i> WT carrying pDSK-Psbp::sgfp; Nal ^r , Km ^r	In this study
<i>Pta</i> pDSK-GFPuv	<i>Pta</i> WT carrying pDSK-GFPuv; Nal ^r , Km ^r	In this study
<i>Pta</i> PnptII::sgfp	<i>PnptII::sgfp</i> integrated into the intergenic region between Pta6605_RS27155 and Pta6605_RS27160 on the <i>Pta</i> chromosome; Nal ^r	In this study
<i>Pta</i> PglA::sgfp	<i>PglA::sgfp</i> integrated into the intergenic region between Pta6605_RS27155 and Pta6605_RS27160 on the <i>Pta</i> chromosome; Nal ^r	In this study
<i>Pta</i> Phyp::sgfp	<i>Phyp::sgfp</i> integrated into the intergenic region between Pta6605_RS27155 and Pta6605_RS27160 on the <i>Pta</i> chromosome; Nal ^r	In this study
<i>Pta</i> PgroES::sgfp	<i>PgroES::sgfp</i> integrated into the intergenic region between Pta6605_RS27155 and Pta6605_RS27160 on the <i>Pta</i> chromosome; Nal ^r	In this study
Plasmids		
pAKlux2	pBBR1MCS-4-based plasmid carrying the <i>luxCDABE</i> operon; Amp ^r	Karsi A, Lawrence ML. <i>Plasmid</i> . 2007
pK18mobsacBN	<i>NotI</i> site inserted between <i>PstI</i> and <i>HindIII</i> site of MCS in pK18; Km ^r	Kashihara et al. 2022
pK18IP	pK18mobsacBN-based plasmid designed for chromosomal integration between Pta6605_RS27155 and Pta6605_RS27160; Km ^r	In this study
pK18IP-PnptII::luxCDABE	pK18IP possessing <i>luxCDABE</i> operon under <i>nptII</i> promoter; Km ^r	In this study
pK18IP-PnptII::sgfp	pK18IP possessing <i>sgfp</i> under <i>nptII</i> promoter; Km ^r	In this study
pK18IP-PgroES::sgfp	pK18IP possessing <i>sgfp</i> under Pta6605_RS06075 promoter; Km ^r	In this study
pK18IP-Phyp::sgfp	pK18IP possessing <i>sgfp</i> under Pta6605_RS15520 promoter; Km ^r	In this study
pK18IP-PglA::sgfp	pK18IP possessing <i>sgfp</i> under Pta6605_RS18010 promoter; Km ^r	In this study
pDSK519	Broad-host-range cloning vector; Km ^r	Keen et al. 1988
pDSK-GFPuv	pDSK519 possessing <i>gfpuv</i> under <i>psbA</i> promoter; Km ^r	Wang et al. 2007
pDSK-PnptII::sgfp	pDSK519 possessing <i>sgfp</i> under <i>nptII</i> promoter; Km ^r	In this study
pDSK-PsdhC::sgfp	pDSK519 possessing <i>sgfp</i> under Pta6605_RS18005 promoter; Km ^r	In this study
pDSK-PraiA::sgfp	pDSK519 possessing <i>sgfp</i> under Pta6605_RS22890 promoter; Km ^r	In this study
pDSK-PfliC::sgfp	pDSK519 possessing <i>sgfp</i> under Pta6605_RS09935 promoter; Km ^r	In this study
pDSK-PprkA-like::sgfp	pDSK519 possessing <i>sgfp</i> under Pta6605_RS25480 promoter; Km ^r	In this study
pDSK-PglA::sgfp	pDSK519 possessing <i>sgfp</i> under Pta6605_RS18010 promoter; Km ^r	In this study
pDSK-Phyp::sgfp	pDSK519 possessing <i>sgfp</i> under Pta6605_RS15520 promoter; Km ^r	In this study

Table 1 (continued)

Bacterial strain/plasmid	Relevant characteristics	Reference or source
pDSK-PgroES::sgfp	pDSK519 possessing <i>sgfp</i> under Pta6605_RS06075 promoter; Km ^r	In this study
pDSK-Psbp::sgfp	pDSK519 possessing <i>sgfp</i> under Pta6605_RS15075 promoter; Km ^r	In this study

Plasmids and strains generated in this study. Detailed descriptions include the vector backbone, promoter source, reporter gene, antibiotic resistance markers, and (for chromosomal integrants) the integration site. The candidate genes used as promoter sources are listed in Table 2. *Amp^r* ampicillin resistant, *Km^r* kanamycin resistant, *Nal^r* nalidixic acid resistant, Five-digit annotation indicates the database source for *Pta*. The accession number indicates the gene number in the Pseudomonas Genome Database

autofluorescence. Combining both approaches therefore provides complementary insights into bacterial behavior at different scales. The optimal combination of these elements differs among bacterial species and strains. The impact of reporter systems on bacterial fitness must also be considered. Plasmid maintenance and constitutive expression of fluorescent proteins can impose a metabolic burden, potentially reducing growth and overall fitness (Bienick et al. 2014). The severity of these effects varies among bacterial species and strains. In some cases, the fitness cost is negligible (Wang et al. 2007), while in others it can be substantial. This variability underscores the need to optimize and validate reporter systems for each target bacterium.

We have been conducting research on plant–microbe interactions using *Pseudomonas syringae* pv. *tabaci* 6605 (*Pta*) as a model organism. Although *Pta* possesses high motility, it also produces fluorescent pigments (Taguchi et al. 2010), which have posed a challenge for fluorescence-based quantification and microscopic observation. Wang et al. (2007) previously reported fluorescent monitoring of *Pta* using a GFPuv-based system. However, the effects of reporter expression on bacterial virulence were not examined in that study, and further optimization of reporter systems for *Pta* remains necessary. Furthermore, to understand infection behavior, a constitutive promoter that remains relatively stable and detectable across a wide range of bacterial densities is required. Since *Pta* possesses a quorum sensing (QS) mechanism, gene expression can fluctuate significantly in response to changes in bacterial density during the infection process (Kawakita et al. 2012). Therefore, QS-independent gene expression was considered an effective strategy for promoter selection.

In this study, we aimed to establish a multilayered imaging platform combining luminescent and fluorescent reporters to visualize the infection process in *Pta*. Although effective reporter integration systems have been developed for other plant-pathogenic bacteria (Matsumoto et al. 2022), these systems rely primarily on electroporation for transformation, which are not readily applicable to *Pta*. We therefore established a gene insertion system targeting a specific locus in the *Pta* genome and constructed a luminescent reporter system using LuxCDABE (Karsi and Lawrence 2007; Matsumoto et al. 2022). To construct a fluorescent reporter

system, we utilized previously reported transcriptome data to select promoters that exhibit constitutively high expression independent of QS and established a fluorescent observation system at the single-cell level. These tools enable the analysis of infection dynamics ranging from the macroscale to the microscale.

Materials and methods

Bacterial strains

Pseudomonas amygdali pv. *tabaci* 6605, hereafter referred to as *P. syringae* pv. *tabaci* 6605 (*Pta*), was used as the bacterial pathogen in this study. The *Pta* was routinely cultured on King's B (KB) agar medium supplemented with nalidixic acid (50 µg mL⁻¹, final concentration) at 27 °C. *Escherichia coli* strains DH5α and S17-1 were propagated in Luria–Bertani (LB) medium at 37 °C with appropriate antibiotics when required. Bacterial strains used in this study are listed in Table 1.

Plant growth conditions

Nicotiana tabacum cv. Xanthi and *Nicotiana benthamiana* plants were cultivated at 28 °C under a long-day photoperiod (16 h light/8 h dark). For the flood inoculation assay, surface-sterilized *N. tabacum* cv. Xanthi seeds were germinated on Murashige and Skoog (MS) medium supplemented with 1% sucrose, 0.8% agar and MS vitamin supplements (3 mg L⁻¹ thiamine hydrochloride, 5 mg L⁻¹ nicotinic acid, and 0.5 mg L⁻¹ pyridoxine hydrochloride). Ten days after germination, seedlings were transferred to MS medium containing 0.01% sucrose, 0.8% agar, and MS vitamin supplements and incubated for an additional 2–3 days prior to bacterial inoculation. For infiltration assays, plants were grown in pots containing a 1:1 (v/v) mixture of vermiculite and Supermix A soil (Sakata Seed Corporation, Yokohama, Japan) and maintained for 4–5 weeks before use.

Selection of constitutive expression promoters

Genes with constitutively highly expression were screened using published microarray datasets (Kawakita et al. 2012; Taguchi and Ichinose 2013). We used previously published Roche NimbleGen microarray data for *Pta* wild-type (WT), $\Delta aefR$, Δvfr , and $\Delta mexT$ mutants. These data had been normalized using the Roust Multichip Average (RMA) method in the original analyses (Irizarry et al. 2003). Based on normalized signal intensity value, expression values were ranked, and candidate genes were selected according to their median expression levels. The expression-based ranking was performed using R (version 4.5.2). Promoter names were assigned based on the annotation of the corresponding genes. For genes with no annotated function, names were assigned based on the closest homolog identified by BLAST search (e.g., *prkA-like*). Genes with no identifiable homolog were designated as hypothetical protein (*hyp*).

Vector construction and transformation

The genome sequence of *Pta* (Matsui et al. 2021) was examined to identify intergenic regions lacking predicted ORFs, which yielded a contiguous region of 4,275 bp located approximately 1 kb upstream of RS27155. The flanking genomic regions were amplified by PCR using primer pairs HMP2343/HMP2344 and HMP2345/HMP2346 and introduced into the *NotI* and *EcoRI* sites, respectively, of pK18*mobsacBN* (Kashihara et al. 2022) using NEBuilder (New England Biolabs, Ipswich, MA, USA). We designated the resulting plasmid as the pK18*mobsacBN*-*Pta* integration platform (pK18IP).

For chromosomal reporter constructs, the *nptII* promoter was amplified from pDSK519 (hereafter pDSK) (Keen et al. 1988), fused to *luxCDABE* amplified from pAKlux2 (Karsi and Lawrence 2007) and inserted into the *BamHI* site of pK18IP using NEBuilder. The *nptII* promoter was also amplified by PCR from pDSK, fused to *sgfp* amplified from pGWB5, and inserted into the *BamHI* site of pK18IP using NEBuilder. The *gltA* promoter was amplified by PCR from the *Pta* genome, fused to *sgfp* amplified from pGWB5 (Nakagawa et al. 2007), and inserted into the *BamHI* site of pK18IP using In-Fusion Snap Assembly Master Mix (Takara, Shiga, Japan). The *PgroES::sgfp* and *Phyp::sgfp* fusion fragments were amplified by PCR from pDSK-*PgroES::sgfp* and pDSK-*Phyp::sgfp* respectively, and inserted into the *BamHI* site of pK18IP using In-Fusion Snap Assembly Master Mix to generate the corresponding reporter constructs.

For plasmid-based reporter constructs, the *nptII* promoter and individual candidate constitutive promoter regions were amplified from pDSK and *Pta* genomic DNA, respectively.

Each promoter was fused with *sgfp* amplified from pGWB5 (Nakagawa et al. 2007) and inserted into the *EcoRI* site of pDSK using In-Fusion Snap Assembly Master Mix. pDSK-*GFPuv* plasmid was used as a positive control (Wang et al. 2007).

pK18IP-derived and pDSK-derived plasmids were verified by Sanger sequencing and introduced into *Pta* by conjugation as previously described (Kuroe et al. 2025). After conjugation, pK18IP-transconjugants carrying pK18IP-derived constructs were subjected to sucrose counter-selection to obtain double-crossover recombinants, and successful integration was confirmed by PCR. Primer sets used for vector construction and integration confirmation are listed in Table S1.

Measurement of luminescence

The assay was performed as described previously (Matsumoto et al. 2022), with minor modifications. Bioluminescence measurements were performed using a plate luminometer (TriStar2 S LB942 multimode Reader, Berthold GmbH). To evaluate the relationship between bacterial concentration and bioluminescence intensity, bacterial suspensions of *Pta* WT::*lux* and *Pta* $\Delta hrcC$::*lux* were adjusted to optical densities at 600 nm (OD₆₀₀) ranging from 0.000002 to 0.2 with sterile dH₂O. Aliquots (200 μ l) were transferred to a white light-reflecting 96-well plate (SUMITOMO BAKELITE CO., LTD., Japan), and relative luminescence units (RLU) were measured. Colony-forming units (CFU) were determined in parallel to assess the correlation between CFU and RLU.

To confirm comparable luminescence levels between strains under inoculation conditions, suspensions of dH₂O, *Pta* WT without the reporter construct, *Pta* WT::*lux*, and *Pta* $\Delta hrcC$::*lux* were adjusted to OD₆₀₀ = 0.002, and bioluminescence was measured.

To measure bioluminescence from *Pta* present in infected leaf tissue, two leaf discs (4 mm diameter) were punched using a biopsy punch and placed in a white light-reflecting 96-well plate. Leaf discs were floated on 200 μ l of sterile dH₂O in each well. The plate containing bacterial suspensions or leaf discs in each well was placed in the sample drawer of the plate luminometer and kept in the dark by closing the lid for 10 min to reduce background signal. Then, the luminescence of each sample was measured for 10 s. Bioluminescence was detected using a ChemiDoc Touch Imaging System (Bio-Rad Laboratories, Hercules, CA, USA). Seedlings grown on agar plates were imaged in chemiluminescence mode. Bright-field images were acquired using the Ponceau S preset mode.

Inoculation test

The flood assay was performed as previously described (Ishiga et al. 2011; Tumewu et al. 2020). A bacterial suspension prepared in sterile dH₂O containing Silwet L-77 (Biomedical Sciences, Tokyo, Japan) was dispensed into the plate containing 2-week-old *N. tabacum* cv. Xanthi seedlings. After the bacterial suspension was removed by decantation, plates containing inoculated plants were sealed and incubated at 22 °C under 16 h light/8 h dark conditions. Each experiment was repeated at least three times. After inoculation, the bacterial population was measured as CFU on KB plates.

Infiltration assays were performed on *N. benthamiana* leaves. Bacterial suspensions ($OD_{600} = 0.02$) prepared in sterile dH₂O were infiltrated into leaves using a needleless syringe. Inoculated plants were incubated at 22 °C under 16 h light/8 h dark conditions, and bacterial populations were determined as CFU on KB plates.

For fluorescence time-lapse observation and video acquisition, a detached leaf from a 2-week-old *N. tabacum* cv. Xanthi seedling was placed abaxial side down onto a drop of bacterial suspension ($OD_{600} = 0.2$). Fluorescence imaging was initiated immediately after contact between the leaf surface and the bacterial suspension. Images were acquired using a fluorescence microscope (Olympus IX70 inverted microscope equipped with a DP75 camera).

Measurement of fluorescence

Fluorescence of the bacteria was quantified using a plate reader (TriStar2 S LB942 multimode Reader, Berthold GmbH). Bacterial suspensions were adjusted to $OD_{600} = 0.2$ with sterile dH₂O, and 200 µl aliquots were transferred to a black 96-well plate (SUMITOMO BAKELITE CO., LTD., Japan). The plate was placed in the sample drawer of the plate reader and kept in the dark by closing the lid for 10 min to reduce background signal. Fluorescence was measured in endpoint mode using an excitation filter at 485/14 nm and an emission filter at 535/25 nm, with a counting time of 0.5 s per well. Sterile dH₂O was used as a negative control.

In vitro growth assay

For in vitro growth assays, overnight cultures of *Pta* strains carrying pDSK-*promoter::sgfp* fusions were adjusted to $OD_{600} = 0.1$ in KB medium and dispensed into a 96-well plate. Plates were incubated at 28 °C with shaking at 180 rpm, and bacterial growth was monitored by measuring OD_{595} using an iMark microplate reader (Bio-Rad Laboratories) at the indicated time points. Three independent

experiments were performed with three technical replicates per experiment.

Microscopic observation

Fluorescence imaging was performed using an Olympus IX70 inverted microscope equipped with a DP75 camera and NIBA filter set (excitation 470–490 nm, emission 515–550 nm). Images were acquired using CellSens Standard software (Evident, Tokyo, Japan) with identical exposure times and gain settings for all strains; specific conditions are noted in the figure legends. Confocal imaging was performed using an Olympus FV1200/IX83 laser-scanning microscope with a 473-nm laser for excitation and detection at 497–570 nm.

Statistical analysis

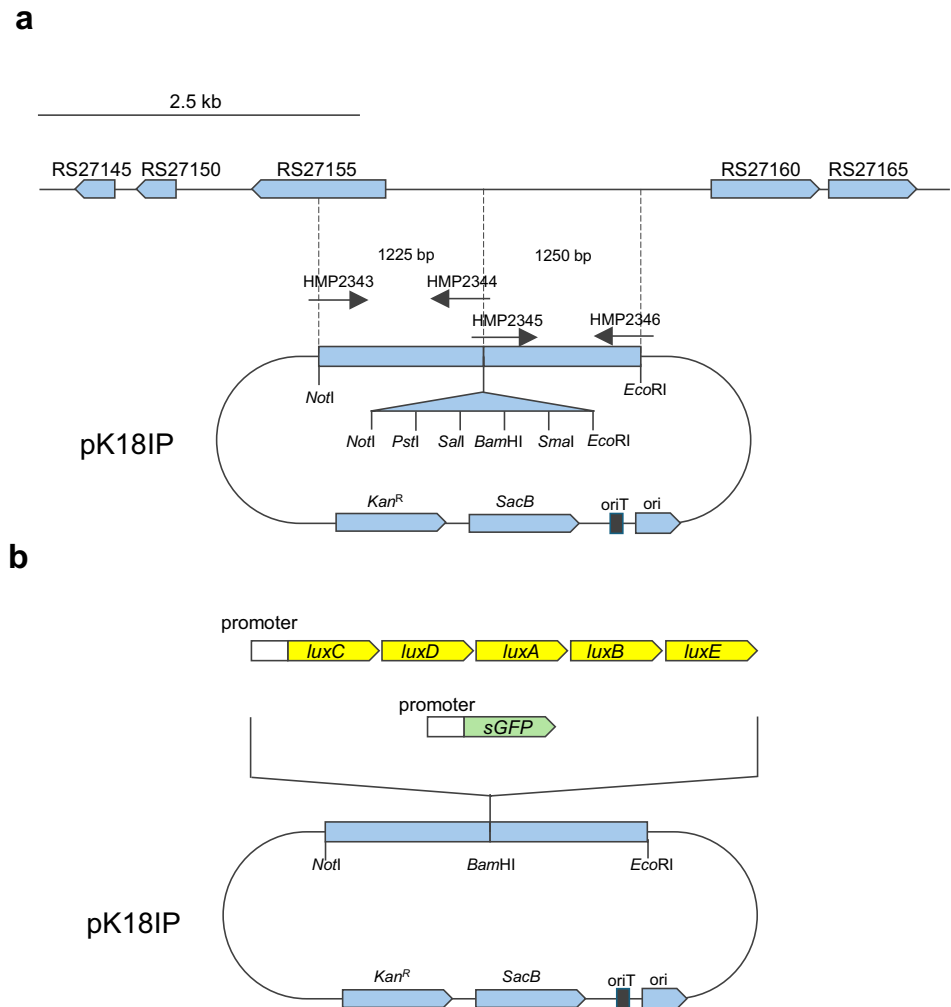
Statistical analyses were performed using one-way ANOVA followed by Tukey's honestly significant difference (HSD) test for log₁₀-transformed RLU data, the Kruskal-Wallis test followed by Dunn's multiple comparisons test with Benjamini–Hochberg method, or the two-tailed Wilcoxon rank-sum test, as indicated in the figure legends. All statistical procedures utilized the R and RStudio (version 2025.09.2).

Results

Construction of a genome integration plasmid vector pK18IP

To minimize potential plasmid loss and copy-number-dependent effects, we constructed chromosomally integrated bioluminescent and fluorescent reporters. Because random chromosomal insertion can disrupt endogenous genes, we screened the *Pta* genome for intergenic regions lacking predicted open reading frames (ORFs) and designed constructs to insert reporter cassettes into these sites. This analysis identified a contiguous 4,275 bp genomic region that entirely lacked annotated ORFs. Based on this locus, we constructed a genome integration plasmid derived from pK18*mobsacBN*, designed to insert *promoter-luxCDABE* or *promoter::sgfp* cassettes specifically into this locus (designated pK18*mobsacBN::Pta* integration platform; pK18IP). Homologous recombination using this vector enables site-specific integration of the reporter construct into the selected genomic locus (Fig. 1). Following integration, the kanamycin resistance marker and the *sacB* gene can be eliminated through sucrose counterselection. The *nptII* promoter was selected for constitutive expression of *luxCDABE* and *sgfp* in our reporter constructs, as it has been successfully used

Fig. 1 Construction of the pK18IP vector. **a** Schematic representation of the pK18IP vector. For introduction of reporter constructs into a genomic region lacking open reading frames between Pta6605_RS27155 and Pta6605_RS27160 in the *Pta* genome, the corresponding genomic regions were amplified using primer pairs HMP2343/HMP2344 and HMP2345/HMP2346. The amplified fragments were cloned into the *NotI* and *EcoRI* sites of pK18IP using NEBuilder. **b** Cloning of *promoter::reporter* fragments into pK18IP

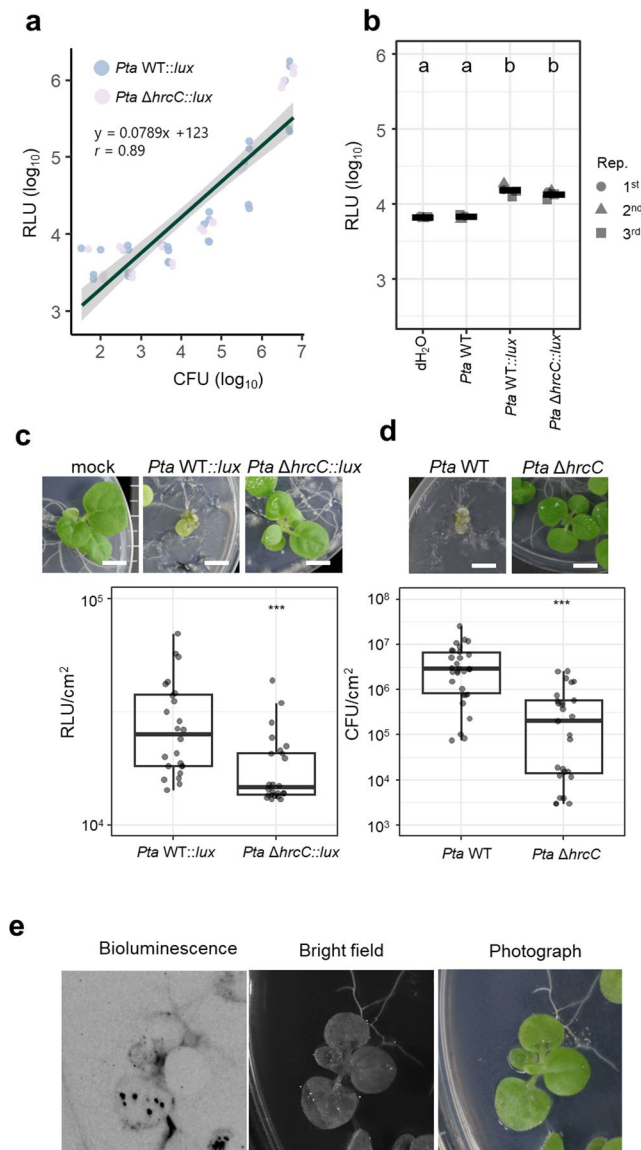


in bioluminescent reporter systems for *P. syringae* (Matsumoto et al. 2022). Using this system, we fused the *nptII* promoter to *luxCDABE* and introduced the constructs into the chromosomes of *Pta* WT and *Pta* $\Delta hrcC$ (defective in the type III secretion system) (Marutani et al. 2005). *Pta* $\Delta hrcC$ is defective in type III effector secretion and thus largely fails to cause disease symptoms. *Pta* $\Delta hrcC$ was included in this study to investigate how the absence of effector delivery affects bacterial behavior during infection in comparison with *Pta* WT. For the fluorescent reporter system, the *nptII* promoter was fused to *sgfp* and cloned into both pK18IP (for chromosomal integration) and pDSK519 (for plasmid-based expression) to compare reporter performance under single-copy and multi-copy conditions. These constructs were introduced into *Pta* WT and served as baseline references for subsequent comparisons with other candidate promoters.

Validation of a LuxCDABE-based luminescent reporter system in *Pta*

To evaluate the LuxCDABE-based reporter system, we examined the relationship between bioluminescence intensity (relative light units, RLU) and bacterial cell counts *in vitro* using *Pta* WT and *Pta* $\Delta hrcC$ strains carrying *PnptII::luxCDABE* (hereafter referred to as *Pta* WT::*lux* and *Pta* $\Delta hrcC$::*lux*, respectively). The combined data from *Pta* WT::*lux* and *Pta* $\Delta hrcC$::*lux* showed a moderate correlation between bioluminescence intensity (RLU) and colony-forming units (CFU) ($r = 0.89$, $p < 0.001$; Fig. 2a). In our calibration measurements, bioluminescence signals were detectable above the background level at OD₆₀₀ values of approximately 0.002 and higher. We therefore used this cell density as the lower limit for quantitative analysis in the present study.

Before inoculation, bacterial suspensions were adjusted to OD₆₀₀ = 0.002 and RLU values were measured to confirm comparable luminescence levels between *Pta* WT::*lux* and *Pta* $\Delta hrcC$::*lux*. Both strains exhibited similar RLU values,



which were significantly higher than those of the *Pta* WT strain without the reporter construct and the dH₂O control (Fig. 2b). This experiment was performed to confirm that bioluminescence levels were comparable between strains at the cell density used in Fig. 2a (OD₆₀₀ = 0.002).

To assess whether the integration of the *luxCDABE* cassette influences virulence or bacterial proliferation *in planta*, flood inoculation assays were performed using *Pta* WT::*lux* and *Pta* Δ*hrcC*::*lux* strains. The *Pta* WT::*lux* induced severe disease symptoms, whereas symptom development was markedly reduced in the *Pta* Δ*hrcC*::*lux* (Fig. 2c). These phenotypes were consistent with those observed following inoculation with the respective parental strains, suggesting that the introduction of *luxCDABE* under the *nptII* promoter does not substantially affect bacterial virulence (Fig. 2d). In addition, bacterial proliferation following flood inoculation was monitored by bioluminescence imaging. Signals

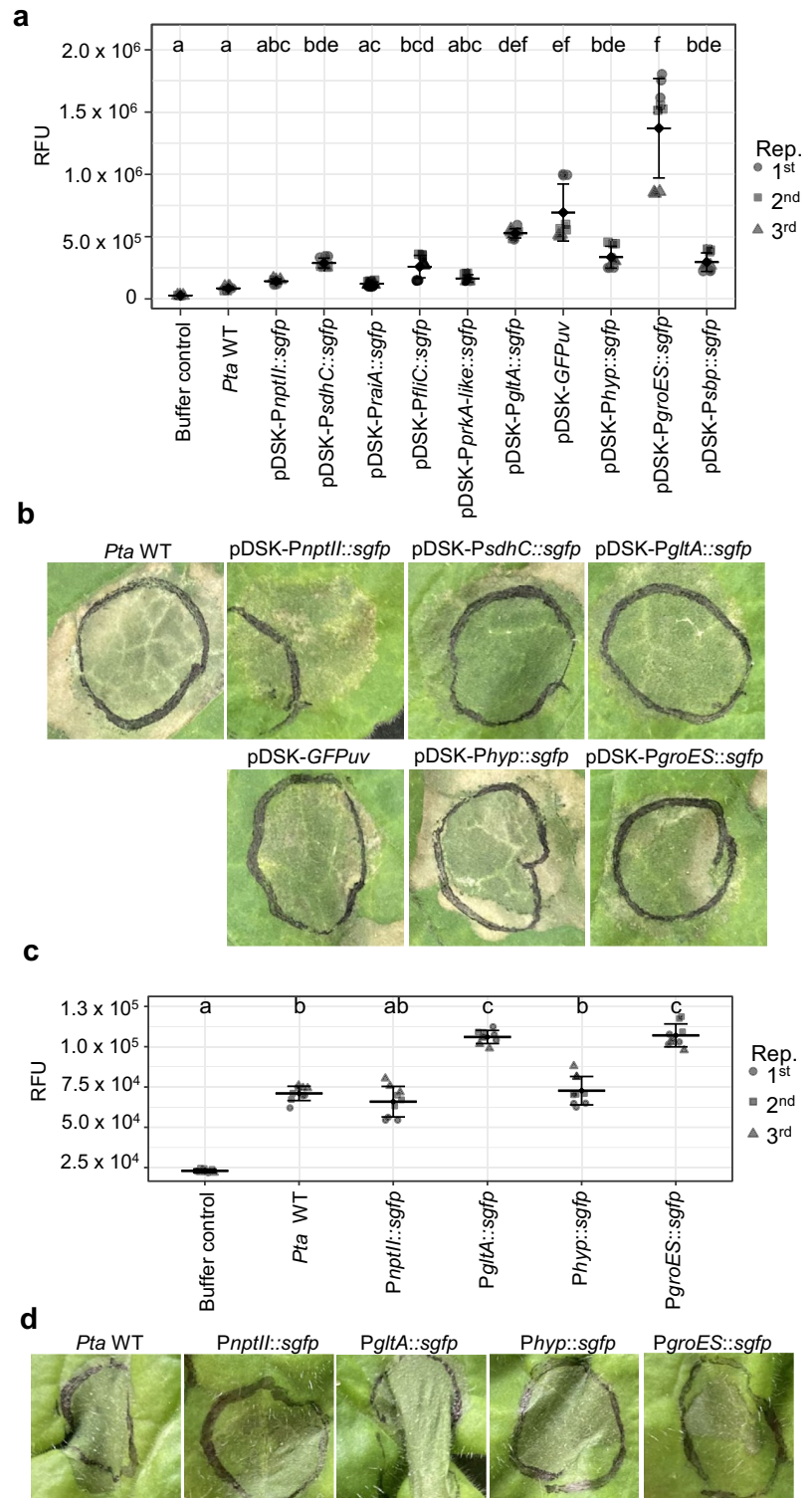
Fig. 2 Characterization of *Pta* with insertion of *luxCDABE*. **a** Correlation between colony-forming units (CFU) and bioluminescence (RLU) in *Pta* WT::*lux* (blue) and *Pta* Δ*hrcC*::*lux* (purple). Bacterial suspensions were adjusted to OD₆₀₀ values ranging from 0.000002 to 0.2. Each data point represents an individual technical replicate. Data from both strains were combined to generate a single regression line. The regression equation and coefficient of determination (*r*) are shown above the plot. **b** Bioluminescence (RLU) was measured for dH₂O and bacterial suspensions (*Pta* WT without the reporter construct, *Pta* WT::*lux*, and *Pta* Δ*hrcC*::*lux*) adjusted to OD₆₀₀ = 0.002 prior to inoculation. Data are presented as log₁₀-transformed RLU values. Different point shapes indicate independent experiments. Crossbars indicate mean values. Different letters indicate significant differences determined by one-way ANOVA followed by Tukey's honestly significant difference (HSD) test (*p* < 0.05). **c**, **d** Disease symptoms and bacterial growth of plants inoculated with *Pta* WT, *Pta* Δ*hrcC*, *Pta* WT::*lux* and *Pta* Δ*hrcC*::*lux* are shown. Representative images of plants inoculated with each strain are shown in the upper panels. *Pta* strains (OD₆₀₀ = 0.002) were inoculated into 2-week-old *N. tabacum* cv. Xanthi through the flood assay. Disease development was observed at 6 days post-inoculation (dpi) (Bar = 1 cm). Mock-treated (dH₂O) was used as a control. Bacterial growth at 3 dpi was quantified as RLU in (c) and CFU in (d). Boxes show upper and lower quartiles of the data, and black lines represent the medians. Each dot represents individual data points pooled from three independent experiments (*n* = 24). Differences in CFU and RLU between two strains were evaluated using the two-tailed Wilcoxon rank-sum test (*** *p* < 0.001). **e** Bioluminescence detection of *Pta* WT::*lux* (OD₆₀₀ = 0.002) in *N. tabacum* cv. Xanthi leaves at 3 dpi. Images of the same inoculated leaf were acquired under three different imaging modes. Bioluminescence image capturing *lux*-derived luminescence signals with a luminescence imager. Visible light image of the same leaf acquired with the luminescence imager. Color photograph taken with a digital camera. Leaves were inoculated with a bacterial suspension at OD₆₀₀ = 0.002 by flood inoculation

indicative of bacterial growth were detected at the sites showing disease symptoms (Fig. 2e). Collectively, these results demonstrate that the experimental system based on pK18IP functions effectively in *Pta*.

Selection and evaluation of constitutive promoters for fluorescent reporter construction in *Pta*

Because *PnptII*::*luxCDABE* was functional in *Pta*, we next attempted to construct sGFP-expressing *Pta* to enable visualization of bacterial behavior during the infection process at the single-cell level. Since the fluorescence intensity of *PnptII*::*sgfp* was weak and showed no difference from *Pta* WT (Fig. 3a and Supplementary Fig. 4), we searched for an alternative promoter that would induce stronger expression in *Pta*. Since bacterial density is low during early infection and quorum sensing (QS) is not yet activated, the reporter promoter needed to function independently of cell density and QS. To identify promoters suitable for constitutive reporter expression, we compared expression patterns among *Pta* WT and three mutants with distinct regulatory defects. *Pta* Δ*aeiR* is deficient in AHL-mediated QS (Kawakita et al. 2012), *Pta* Δ*vfr* is impaired in virulence regulation but retains AHL-mediated QS (Taguchi and

Fig. 3 Characterization of relative fluorescence units and virulence of *Pta* sGFP reporter strains harboring plasmid-borne or genomic constructs. **a** Relative fluorescence units (RFU) of *Pta* sGFP reporter strains harboring plasmid-borne constructs. Bacterial suspensions were adjusted to $OD_{600} = 0.2$ prior to RFU measurement. Each dot represents an individual data point from three independent experiments. *Pta* WT was included as a non-sGFP control. Different letters indicate statistically significant differences between groups (Kruskal–Wallis test followed by Dunn's multiple comparisons test with Benjamini–Hochberg correction, $p < 0.05$). **b** Virulence assay of *Pta* sGFP reporter strains harboring plasmid-borne constructs. Bacterial suspensions were adjusted to $OD_{600} = 0.02$ and infiltrated into *N. benthamiana* leaves using a needleless syringe. Photographs were taken at 7 dpi. Three independent experiments were performed with similar results. **c** RFU of *Pta* sGFP reporter strains harboring genomic constructs. Bacterial suspensions were adjusted to $OD_{600} = 0.2$ prior to RFU measurement. Each dot represents an individual data point from three independent experiments. *Pta* WT was included as a non-sGFP control. Different letters indicate statistically significant differences between groups (Kruskal–Wallis test followed by Dunn's multiple comparisons test with Benjamini–Hochberg correction, $p < 0.05$). **d** Virulence assay of *Pta* sGFP reporter strains harboring genomic constructs. Bacterial suspensions were adjusted to $OD_{600} = 0.02$ and infiltrated into *N. benthamiana* leaves using a needleless syringe. Photographs were taken at 7 dpi. Three independent experiments were performed with similar results



Ichinose 2013), and *Pta* $\Delta mexT$ lacks a LysR-type transcriptional regulator involved in multidrug efflux pump regulation, but retains WT-like AHL production and virulence under laboratory conditions (Kawakita et al. 2012; Sawada et al. 2018). We hypothesized that the promoter regions of genes exhibiting consistently high expression across

Pta WT and three mutant strains with distinct regulatory defects ($\Delta aefR$, Δvfr , and $\Delta mexT$) would serve as reliable candidates for stable reporter expression. For each dataset (5,172 probes), genes were ranked by their normalized expression values, and the median rank across the four datasets was calculated (Table S2). Genes consistently ranked

among the top approximately 10 transcripts (top ~0.2%) were selected as candidate promoters capable of consistent high-level expression (Table 2 and Supplementary Fig. 1). Promoter regions upstream of these candidate genes were cloned into pDSK. For genes within operons, the promoter upstream of the entire operon was cloned, and the constructs were introduced into *Pta* (Table 1). Fluorescence intensity measurements showed that the pDSK-*PgroES::sgfp* strain exhibited the strongest fluorescence, approximately 2.0-fold higher than that of the positive control pDSK-*GFPuv* (Fig. 3a). The pDSK-*PglTA::sgfp* strain showed significantly higher fluorescence than the pDSK-*PnptII::sgfp* strain in *Pta* (Fig. 3a). The pDSK-*PsdhC::sgfp*, pDSK-*Phyp::sgfp*, and pDSK-*Psbp::sgfp* strains also showed higher mean fluorescence than the pDSK-*PnptII::sgfp* strain, although

Table 2 Gene expression ranking of *Pta* WT, $\Delta aefR$, Δvfr , and $\Delta mexT$ mutant by microarray analysis

SEQ_ID	<i>Pta</i> 6605	median_raw	Promoter name	Description
PSPPH_4078	RS06075	60,776	<i>PgroES</i>	co-chaperonin GroES
PSPPH_1090	RS22890	60,730	<i>PraiA</i>	HPF/RaiA family ribosome-associated protein, <i>raiA</i>
PSPPH_3392	RS09935	58,664	<i>PfliC</i>	Flagellin, <i>fliC</i>
PSPPH_2596	RS15075	53,254	<i>Psbp</i>	sugar ABC transporter substrate-binding protein, <i>sbp</i>
PSPPH_1121	RS22735	47,330		PhoH-like protein
PSPPH_1977	RS18000	45,897	<i>PsdhC</i>	succinate dehydrogenase, hydrophobic membrane anchor protein, <i>sdhD</i>
PSPPH_2509	RS15520	43,302	<i>Phyp</i>	hypothetical protein
PSPPH_1974	RS18010	35,924	<i>PglTA</i>	type II citrate synthase, <i>glTA</i>
PSPPH_4711	—	35,636	—	hypothetical protein
PSPPH_0629	RS25480	34,127	<i>PprkA-like</i>	PrkA family serine protein kinase

Seq_ID indicates the *Pph1448A* gene ID used in the microarray; *Pta* indicates the corresponding accession number. median_raw represents the median of microarray raw signal intensities derived from four strains. ND indicates that no homolog corresponding to the Seq_ID was identified. The top 10 genes by expression are shown. —, no homolog identified by BLAST search

these differences were not statistically significant (Fig. 3a). In contrast, the fluorescence of the pDSK-*PraiA::sgfp* and pDSK-*PfliC::sgfp* strains remained weak (Fig. 3a).

Because strong GFP expression could potentially affect growth, we assessed bacterial proliferation *in vitro*. pDSK-*Phyp::sgfp* strain showed a slight delay in growth during the late phase in the *in vitro* growth assay, while no substantial effects on *in vitro* growth were observed for most other strains (Supplementary Fig. 2). We examined whether GFP expression affected virulence by monitoring symptom development after syringe infiltration into *N. benthamiana* leaves. Symptom development was slightly attenuated in the pDSK-*GFPuv*, pDSK-*PglTA::sgfp*, and pDSK-*PgroES::sgfp* strains as compared with *Pta* WT (Fig. 3b). Although pDSK-*Phyp::sgfp* showed a slight effect on growth *in vitro* (Supplementary Fig. 2), symptoms development was at the same level as *Pta* WT, and bacterial growth *in planta* was not affected (Supplementary Fig. 3). Despite the reduced symptom development, bacterial multiplication *in planta* did not differ significantly among strains compared with *Pta* WT (Supplementary Fig. 3). These results suggest that the hypothetical protein (*Pta*6605_RS15520) promoter may be useful for monitoring bacterial infection without compromising virulence.

Comparison of fluorescence levels between genomic and plasmid-borne sGFP constructs

When pDSK-*PgroES::sgfp* and pDSK-*GFPuv* were introduced into *Pta*, the disease symptoms showed a delay in onset. We hypothesized that this phenotype was influenced by plasmid copy number. To verify this, we constructed a *promoter::sgfp* fusion and integrated it into the *Pta* genome using the pK18IP system. Following genomic insertion of *PgroES::sgfp*, *PglTA::sgfp*, and *Phyp::sgfp*, fluorescence intensity was reduced compared to their plasmid-borne counterparts (Fig. 3a and c). *PgroES::sgfp* and *PglTA::sgfp* still exhibited higher fluorescence intensity than *PnptII::sgfp*. Furthermore, no significant difference in disease progression was observed in any of the *promoter::sgfp* genomic insertion strains compared to the *Pta* WT (Fig. 3d).

Analysis of the behavior of *Pta* during the early stages of infection

To investigate the feasibility of fluorescent-based observation during the *Pta* infection process, we first attempted fluorescence microscopy using a *Pta* strain in which *PgroES::sgfp* was integrated into the chromosome, reasoning that chromosomal integration would provide stable single-copy expression. Although bacterial cells expressing the plasmid-based fluorescent reporter could be visualized by

fluorescence microscopy, fluorescence of the genome-integrated reporter strains was insufficient to detect individual bacterial cells (data not shown). To enhance signal intensity through increased gene copy number, we next used *Pta* strains carrying pDSK-PgroES::sgfp and pDSK-Phyp::sgfp. To evaluate whether individual bacterial cells could be visualized by fluorescence microscopy, bacterial suspensions of these two strains were examined on glass slides. Although *Pta* pDSK-Phyp::sgfp produced weaker fluorescence compared with *Pta* pDSK-PgroES::sgfp, both strains allowed visualization of individual bacterial cells by fluorescence microscopy on glass slides (data not shown).

Based on these results, we next performed live imaging to monitor bacterial behavior during the infection process. For fluorescence time-lapse observation, the abaxial surface of *N. tabacum* cv. Xanthi seedlings was inoculated by drop contact with a bacterial suspension, and bacterial behavior

was examined under a fluorescence microscope at 0–20 min after inoculation. Interestingly, the *Pta* pDSK-PgroES::sgfp strain began to accumulate around open stomata as early as 5 min after inoculation, and by 10 min, clear accumulation of bacteria in the vicinity of open stomata was consistently observed (Fig. 4 and Supplementary Fig. 5). This observation was reproduced in three independent experiments, suggesting that *Pta* rapidly initiates chemotactic behavior toward open stomata upon contact with the leaf surface. In contrast, when inoculated onto leaves, detection of individual *Pta* pDSK-Phyp::sgfp cells was not possible due to high background fluorescence from the leaf tissue (data not shown).

At three days post-inoculation (dpi), the pDSK-PgroES::sgfp strain and pDSK-Phyp::sgfp strains were clearly detected in tissues surrounding stomata (Fig. 5a). In addition, confocal microscopy revealed individual

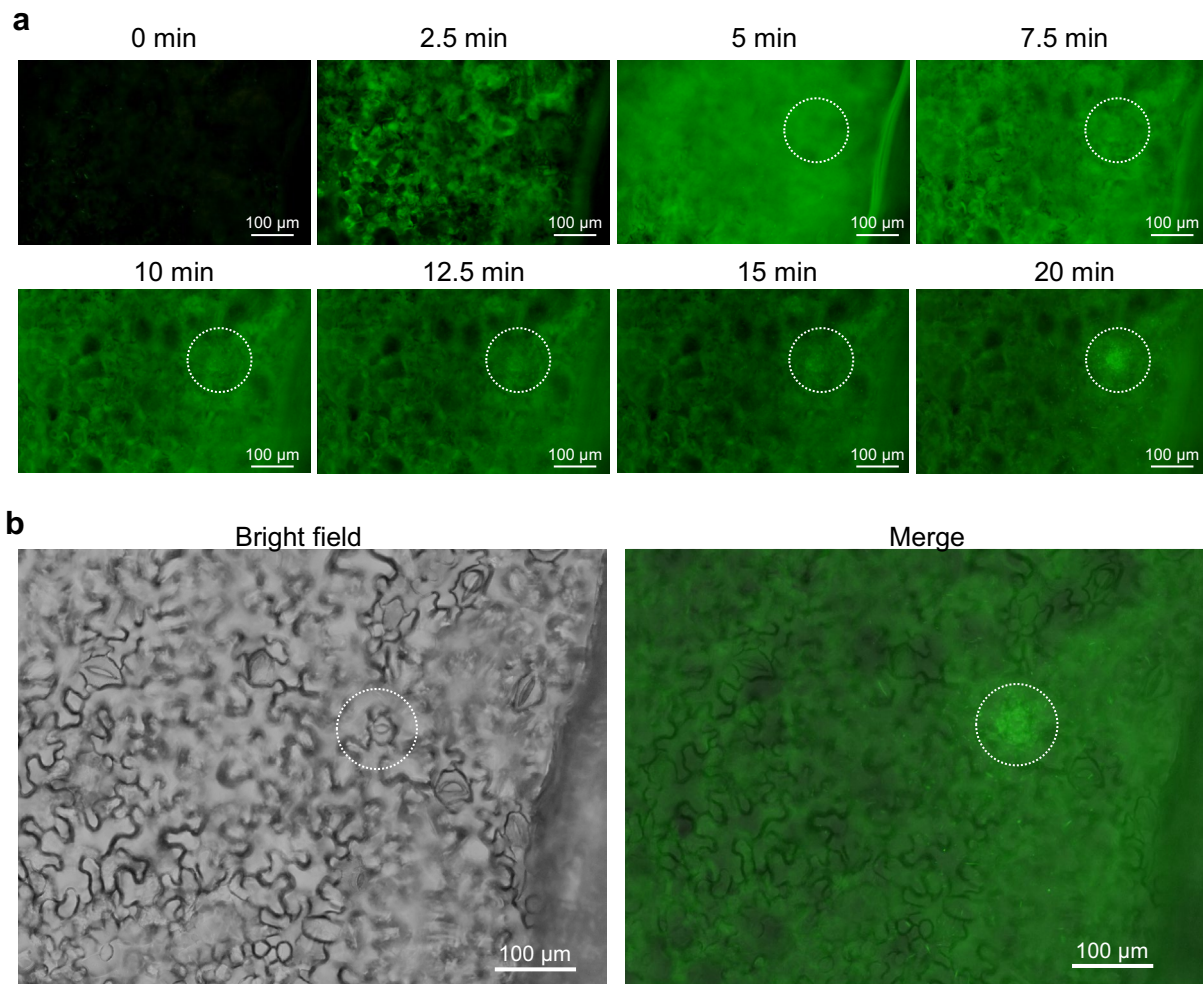


Fig. 4 Time-lapse visualization of bacterial accumulation around a stoma. **a** Time-lapse fluorescence microscopy images showing accumulation of *Pta* pDSK-PgroES::sgfp around a stoma on *Nicotiana tabacum* cv. Xanthi leaves. The abaxial leaf surface was placed onto a bacterial suspension, and images were captured at 0, 2.5, 5, 7.5, 10,

12.5, 15, and 20 min after contact with the bacterial suspension. The dashed circle indicates the stoma around which bacterial accumulation was observed over time. **b** Merged bright-field and fluorescence image corresponding to the 20 min time point in (a), showing that bacteria accumulated preferentially around the stoma

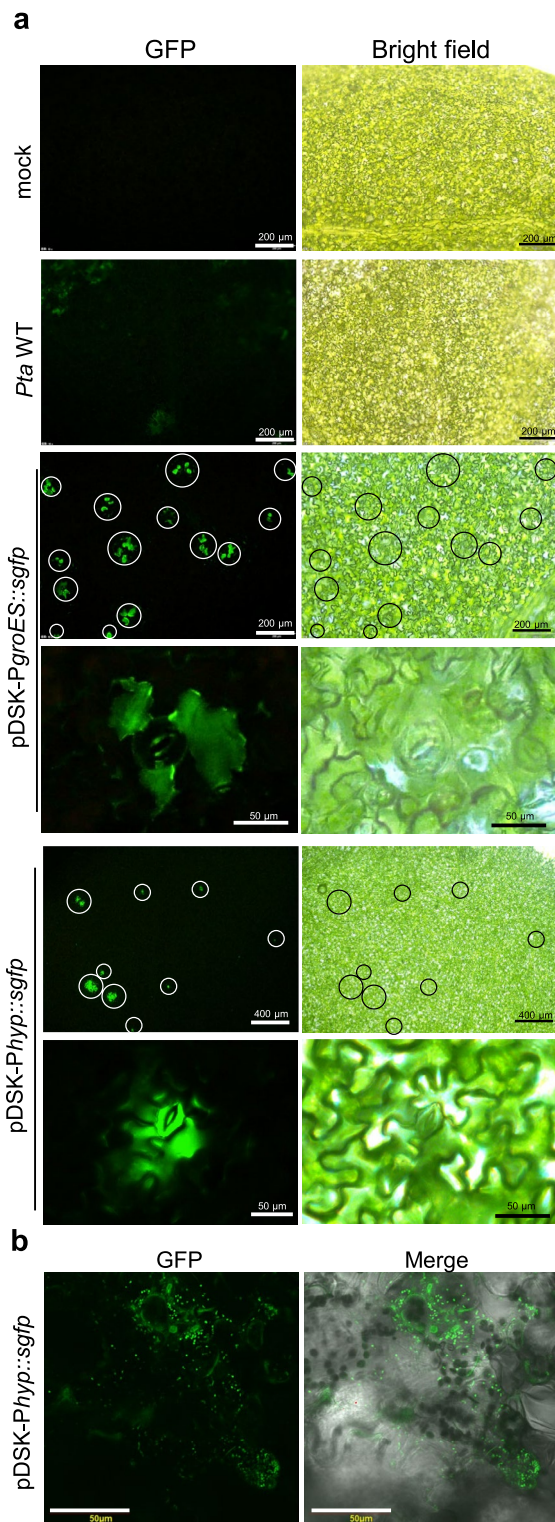


Fig. 5 Fluorescence and confocal microscopy of *Pta* sGFP reporter strains in *Nicotiana tabacum* cv. Xanthi leaves during infection. **a** Fluorescence microscopy images of leaves inoculated with mock, *Pta* WT, *Pta* pDSK-PgroES::sgfp and *Pta* pDSK-Phyp::sgfp at 3 dpi. All images were acquired under identical conditions with automatic exposure settings. Fluorescent signals detected in cells surrounding stomata are indicated by circles. **b** Confocal microscopy images of *N. tabacum* cv. Xanthi leaves inoculated with *Pta* pDSK-Phyp::sgfp. Individual fluorescent bacterial cells are visible within the leaf tissue

bacterial cells at infection sites in the pDSK-Phyp::sgfp strain (Fig. 5b). These results demonstrate that multicopy plasmid-based expression is essential for generating fluorescence signals above the autofluorescence background of *Pta*, and that plasmid-borne reporter strains are suitable for fluorescence-based observation of the infection process.

Discussion

In this study, we developed an imaging platform to visualize the infection process of *Pta* at both macro- and micro-scale. We established a gene insertion system at a specific genomic locus and constructed a LuxCDABE reporter system. Matsumoto et al. (2022) developed a versatile Tn7-based tool for integrating *luxCDABE* into plant-pathogenic bacteria and demonstrated its utility in several species including *P. syringae* pv. *tomato* DC3000. However, their system relies primarily on electroporation for transformation, and *Pta* is not amenable to electroporation-based genetic manipulation. We therefore constructed an alternative gene insertion system targeting a specific genomic locus in *Pta* via conjugation and successfully established a LuxCDABE luminescent reporter that showed stable expression without affecting bacterial fitness. Constructing a fluorescent reporter using the same genomic insertion system proved difficult due to the strong autofluorescence of *Pta*. To overcome this, we selected promoters exhibiting consistently high expression based on transcriptome data and utilized a multicopy plasmid, thereby establishing a fluorescent observation system at the single-cell level.

The *nptII* promoter functioned adequately for luminescence detection but failed to produce detectable sGFP fluorescence above *Pta* WT autofluorescence levels (Fig. 3a, c, and Supplementary Fig. 4). The fact that luminescence was detectable under the same promoter strongly suggests that *Pta* autofluorescence, rather than insufficient promoter activity, masks the sGFP signal. Bacterial autofluorescence interfering with GFP detection has been reported even in *E. coli* (Mihalcescu et al. 2015), and this problem is more severe in *Pseudomonas* species due to the spectral overlap between pyoverdine and GFP (Schlechter et al. 2021). *Pta* also produces pyoverdine, which functions as a virulence factor (Taguchi et al. 2010), underscoring the need for a strategy to achieve signal intensity exceeding autofluorescence.

To address this, we utilized microarray data from the Δ aeFR, Δ yfr, and Δ mexT mutants to select promoters that are consistently expressed at high levels independent of QS (Kawakita et al. 2012; Taguchi and Ichinose 2013), and identified two promoters suitable for fluorescence-based observation. The *groES* promoter provides signal intensity sufficient for detecting individual bacterial cells even at low

cell densities, making it particularly useful for observing the initial stages of infection when bacterial population on the leaf surface are still sparse. However, *groES* promoter is associated with notable reduction in virulence, likely reflecting a fitness cost imposed by high level expression of the fluorescent protein. Metabolic trade-offs between heterologous protein expression and virulence have been reported in other bacterial pathogens. For example, resource allocation between virulence factor production and bacterial proliferation in *Ralstonia solanacearum* (Peyraud et al. 2016) and fitness costs associated with virulence gene expression in *Salmonella enterica* serovar Typhimurium (Sobota et al. 2022). The precise mechanism underlying virulence attenuation in *Pta* remains to be elucidated. Notably, bacterial proliferation *in planta* was not significantly affected, suggesting that the fitness cost primarily impacts disease progression rather than colonization capacity. Since tabtoxin production plays a key role in symptom development in *Pta* (Kunishi et al. 2026), the metabolic burden of constitutive fluorescent protein expression may preferentially affect toxin-related pathways, although this remains to be investigated. The *Pta6605_RS15520* promoter, with minimal virulence impact, is better suited for observing infection behavior under near-wild type conditions at stages when bacterial populations have increased sufficiently to be detected with this promoter. Although the molecular function of *Pta6605_RS15520* remains unknown, its consistently high expression level suggests that it may be involved in cellular maintenance. Replacing sGFP with brighter fluorescent proteins such as sfGFP (Pédélecq et al. 2006) or Venus (Nagai et al. 2002) in the hypothetical protein promoter construct would be an effective strategy to enhance detection sensitivity without compromising virulence.

This platform enables both multilayered analysis of the infection process. The LuxCDABE reporter allows non-invasive, macroscale tracking of bacterial population dynamics throughout infection without the need for external excitation. At the microscale, *PgroES::sgfp* enables sensitive detection of individual cells at low densities, while *Phyp::sgfp* permits observation under near-native conditions at higher bacterial densities. Together, these three systems cover different spatial scales and detection sensitivities, providing a comprehensive view of the infection process from initial colonization to disease development. Although direct spatial comparison between the bioluminescence and fluorescence systems at the single-cell level was not feasible due to the lack of low-light imaging instrumentation required for microscopic bioluminescence detection, fluorescent reporters showed similar bacterial localization around stomata at 3 dpi (Fig. 5a), supporting the reliability of the fluorescence-based observations.

By replacing the stable high-level expression promoters used in this study with promoter of interest, the system can also be adapted to monitor the expression of specific genes during infection. These tools should prove useful for analyzing virulence mechanisms and plant defense responses in the *Pta* pathosystem. Our system, which enables observation of individual cells during natural infection from the leaf surface, provides a complementary approach for investigating such heterogeneous behavior.

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Declarations

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

Ethical approval This study does not involve any experiments with animals.

Human and animal rights This article does not contain any studies with human participants or animals performed by any of the authors.

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