Chapter 15

Generating Chromosome-Located Transcriptional Fusions to Fluorescent Proteins for Single-Cell Gene Expression Analysis in *Pseudomonas syringae*

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Abstract

The last decade has seen significant effort directed toward the role of phenotypic heterogeneity in bacterial adaptation. Phenotypic heterogeneity usually refers to phenotypic diversity that takes place through nongenetic means, independently of environmental induced variation. Recent findings are changing how microbiologists analyze bacterial behavior, with a shift from traditional assays averaging large populations to single-cell analysis focusing on bacterial individual behavior. Fluorescence-based methods are often used to analyze single-cell gene expression by flow cytometry, fluorescence microscopy and/or microfluidics. Moreover, fluorescence reporters can also be used to establish where and when are the genes of interest expressed. In this chapter, we use the model bacterial plant pathogen *Pseudomonas syringae* to illustrate a method to generate chromosome-located transcriptional gene fusions to fluorescent reporter genes, without affecting the function of the gene of interest.

Key words Phenotypic heterogeneity, Gene expression, Fluorescent reporter genes, Single-cell methods, Fluorescence microscopy, Nongenetic variation, Allelic Exchange

1 Introduction

Bacterial pathogens deploy a multitude of virulence factors to colonize plants and cause disease. Many animal and plant bacterial pathogens relay on type III secretion systems (T3SS) to deliver effector proteins inside the host cell and thus modify cellular processes in order to allow bacterial survival, proliferation and spread [1]. *Pseudomonas syringae* is one of the most studied bacterial plant pathogens [2] that is both a model pathosystem and an increasing economically important pathogen in agriculture, with recent resurgence of old diseases and emergence of new ones [3, 4]. *P. syringae* is a foliar pathogen and its life history is linked to the water cycle, often reaching the leaf surface via rainfall [5]. *P. syringae* enters the

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leaf through natural openings (stomata, hydathodes) [6] or wounds, to reach the intercellular space of the leaf parenchyma, the apoplast, where it replicates. Once within the apoplast, *P. syringae* uses its T3SS to deliver effector proteins into the plant cell cytosol to suppress plant defenses, allowing bacterial colonization [7–9]. Where and when are these factors expressed during the interaction with the host is therefore of relevance for the understanding of the host-pathogen dynamics. Furthermore, recent work from our laboratory has shown that *P. syringae* T3SS genes display phenotypic heterogeneity in their expression, including *hrpL* the gene encoding the main transcriptional activator of the system [10]. Phenotypic heterogeneity refers to phenotypic variation arising within a population living in the same microenvironment through nongenetic mechanisms [11]. Apoplastic populations of *P. syringae* pv. phaseolicola display phenotypically heterogeneous activation of the T3SS genes leading to cell-to-cell differences, which are likewise observed in the homogeneous environment of nutrient-limited culture medium, and are relevant for virulence. This finding is consistent with reports of many virulence genes displaying cell-to-cell expression differences in expression in animal pathogens, such as *Salmonella enterica* [12–15], *Vibrio cholerae* [16], or *Yersinia pseudotuberculosis* [17]. These reports have raised the interest in using single-cell analytic methods to study bacterial behavior. Fluorescence-reporters are often used to analyze single-cell gene expression since they allow the application of techniques such as flow cytometry, fluorescence microscopy and/or microfluidics. In this chapter, we describe a method to generate chromosome-located transcriptional gene fusions to fluorescent reporter genes without affecting the function of the gene of interest, using the model bacterial plant pathogen *P. syringae*.

2 Materials

2.1 Bacterial Growth

1. Bacterial strains: *Pseudomonas syringae* pv. tomato DC3000 [18], Escherichia coli DH5α [19].
2. Lennox Broth (LB) [20], modification of Luria–Bertani [21] with NaCl concentration halved (For 1 L): 10 g tryptone, 5 g yeast extract, and 5 g NaCl and add to 800 mL of $_{d}H_2O$. Fill up to 1 L with $_{d}H_2O$ using a measuring cylinder. Add 10 g of bacteriological agar when necessary. Autoclave at 121 ºC for 20 min. Cool down to a temperature about 50 ºC and add the appropriate antibiotic. Pour about 20 mL of LB agar per 10 cm petri dish.
3. Antibiotic stock solutions: Ampicillin (Amp; 100 mg/mL), Kanamycin (Km; 50 mg/mL). LB was supplemented with either ampicillin (100 μg/mL for E. coli DH5α, 500 μg/mL for

P. syringae strains) or kanamycin (50 μg/mL for E. coli DH5α, 15 μg/mL for *P. syringae* strains).

4. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) stock solution (40 mg/mL in dimethylformamide).
5. TB Buffer: 10 mM HEPES pH 6.7, 15 mM $CaCl_2$, 55 mM $MnCl_2$, 250 mM KCl.
6. 25% glycerol

2.2 Plasmids Constructs

1. Plasmids (Table 1).
2. Total DNA extracted from *P. syringae* using JetFlex DNA Purification Kit (Genomed, Germany).
3. High-Fidelity polymerase system (e.g., Q5 High-Fidelity DNA Polymerase, NEB, UK).
4. dNTP mix: 10 mM each.
5. Specific primers (Table 2).
6. Gel Band Purification Kit.
7. RedSafe.
8. Vector for cloning PCR product (e.g., pGEM-T).
9. Restriction enzymes.
10. T4 DNA ligase and buffer.
11. NanoDrop spectrophotometer or similar.
12. 10 mM $MgCl_2$.
13. Agarose.
14. E. coli DH5α competent cells.

2.3 P. syringae Transformation

1. Sucrose stock solution, 300 mM.
2. Refrigerated microcentrifuge.
3. Electroporation cuvettes (2 mm).
4. Electroporator.

2.4 Southern Blot Analysis

1. One Kb Plus ladder.
2. HCl 0.25 N.
3. Denaturing solution: 1.5 M NaCl, 0.5 M NaOH.
4. Neutralizing solution: 3 M NaCl, 0.5 M Tris–HCl pH 7.
5. 20x SSC: 3 M NaCl, 300 mM Na-citrate.
6. Nylon membrane.
7. Blocking reagent.

   DIG Labeling Mix.

   Anti-Digoxigenin antibody.

   (Disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2^0-(5^0-chloro) tricyclo [3.3.1.1]decan}-4-yl)phenyl phosphate) CSPD.

Table 1
Plasmids used or generated in this work

Plasmid	Description	Antibiotic resistance	Reference
pGEM-T	Cloning vector	Amp	Promega
pGT-*hrpL*-AB	pGemT containing the A + B fragment of *hrpL* with an *EcoRI* site	Amp	This work
pGT-YFP	pGemT derivative carrying the promoterless ORF of *eyfp* and the FRT-*nptII*-FRT cassette flanked by *EcoRI* sites	Amp	This work
pGT-Turquoise2	pGemT derivative carrying the promoterless ORF of *turquoise2* and the FRT-*nptII*-FRT cassette flanked by *EcoRI* sites	Amp, km	This work
pGT-mPlum	pGemT derivative carrying the promoterless ORF of *mplum* and the FRT-*nptII*-FRT cassette flanked by *EcoRI* sites	Amp, km	This work
pGT-mOrange2	pGemT derivative carrying the promoterless ORF of *morange2* and the FRT-*nptII*-FRT cassette flanked by *EcoRI* sites	Amp, km	This work
pGT-mCherry	pGemT derivative carrying the promoterless ORF of *mcherry* and the FRT-*nptII*-FRT cassette flanked by *EcoRI* sites	Amp, km	This work
pGT-GFP$^+$	pGemT derivative carrying the promoterless ORF of *gfp* + and the FRT-*nptII*-FRT cassette flanked by *EcoRI* sites	Amp, km	This work
pFLP2	Contains a flippase gene	Amp	[25]
pKD4	pANTS derivative containing an FRT-flanked kanamycin resistance gene	Amp, km	[26]
miniTn7(Gm) PA1/04/03–eyfp-a	Contains the eYFP ORF	Gm, cm	[27]
pmTurquoise2	Contains the Turquoise2 ORF	Km	[28]
pBAD-mPlum	Contains the mPlum ORF	Amp	Michael Davidson and Roger Tsien (unpublished)
pBAD-mOrange2	Contains the mOrange2 ORF	Amp	Michael Davidson and Roger Tsien (unpublished)
pBAD-mCherry	Contains the mCherry ORF	Amp	Michael Davidson and Roger Tsien (unpublished)
pZEP07	Contains the GFP+ ORF	Cm	[29]

Table 2
Primers used in this work

Name	Sequence	Restriction site[a]
HrpLA1	Attcgccaaatgacggcc	NA
HrpLA2	aatgatcgagGAATTCatcgccattcaggcgaacg	*EcoRI*
HrpLB1	gaatggcgatGAATTCctcgatcatttttctggaaccaac	*EcoRI*
HrpLB2	Tcagaattgtcgagaaaggctg	NA
Prot fluor F	aaGAATTCggagatatacatatggtgagcaagggcg	*EcoRI*
Prot fluor-km R	Ccagcctacacttacttgtacagctcgtcc	NA
GFP+ F	aaGAATTCggagatatacatatgagcaaaggagaagaac	*EcoRI*
GFP+ km R	Ccagcctacacttatttgtagagctcatccatgc	NA
Km-Prot fluor F	Ctgtacaagtaagtgtaggctggagctgc	NA
P2	tcaGAATTCcatatgaatatcctccttag	*EcoRI*
P1	tcaGAATTCgtgtaggctgga	*EcoRI*

[a]*NA* Not applicable. RS in capital letters

3 Methods

To illustrate the method, adapted from one previously developed by our laboratory to generate knockout strains in *P. syringae* [22], we will use the generation of an *hrpL* transcriptional fusion to the fluorophore-encoding gene *mturquoise2* in the model strain *Pseudomonas syringae* pv. tomato DC3000 as an example. Using the same method and primers (Table 2), and just by changing the vectors (Table 1), we also generate fusions to reporter genes encoding alternative fluorophores, namely mOrange2, mPlum, GFP+ and eYFP. The method requires the PCR-based generation of an allelic exchange fluorescent reporter DNA module to be recombined into a specific location within the bacterial chromosome. In our example, the allelic exchange module comprises: (1) the last 500 bps of the *hrpL* coding sequence including the STOP codon, (2) the *mturquoise2* ORF carrying its own ribosomal-binding site (RBS), (3) the *nptII* gene flanked by FRT sequences, and (*iv*) 500 bps immediately downstream the *hrpL* ORF STOP codon. Generation of this module is carried out sequentially.

3.1 Generating the hrpL:: mturquoise2 Allelic Exchange Plasmid

The outline of the steps described in this section is illustrated in Fig. 1.

3.1.1 Primer Design

The insertion point (where the reporter gene would be inserted) should be located 5–10 nucleotides after the STOP codon of the

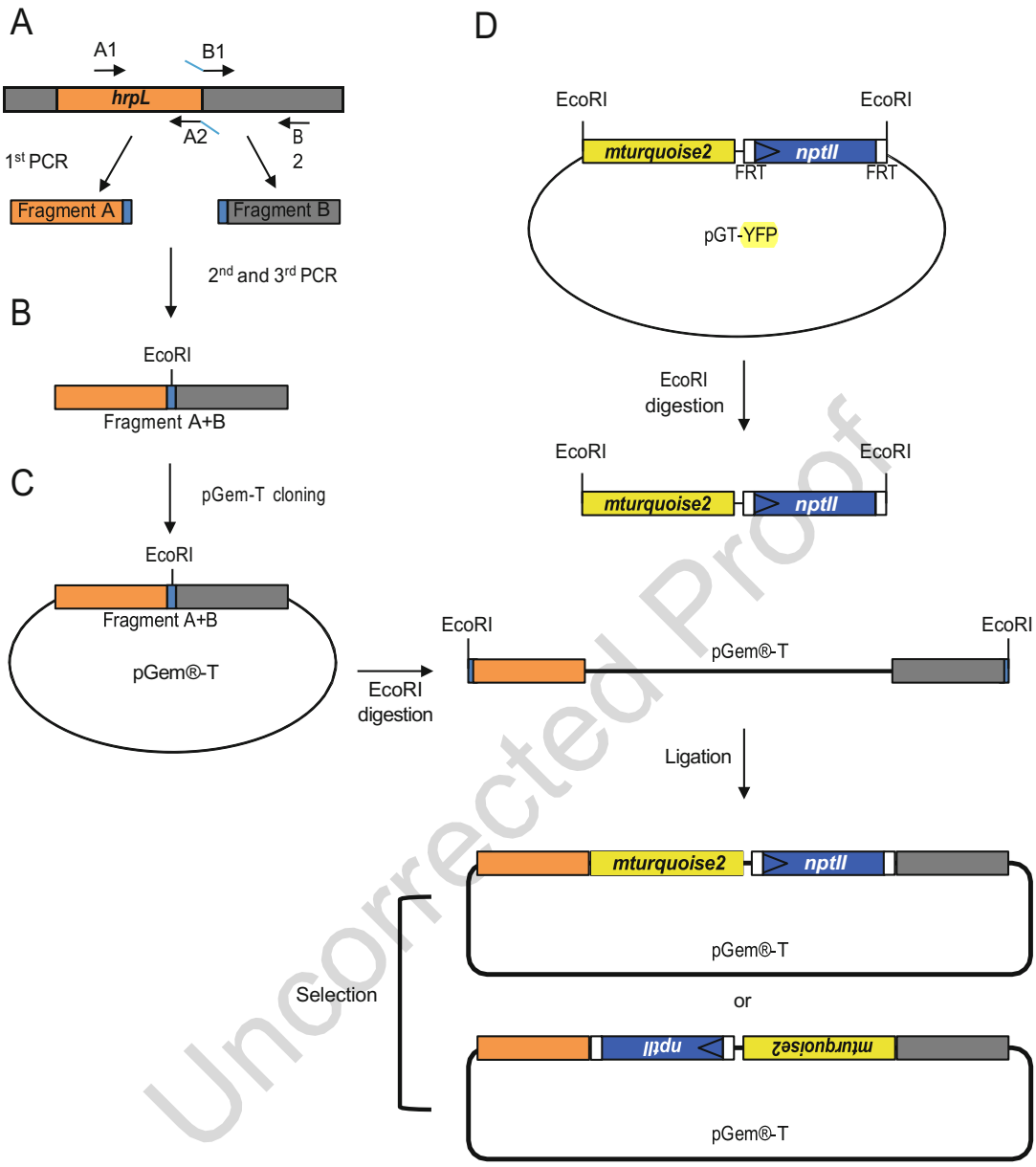

Fig. 1 Generating allelic exchange vectors for transcriptional gene fusions. (a) The last 0.5 Kb including the STOP codon of the target gene and the 0.5 Kb immediately downstream are amplified independently. Primers A2 and B1 share a 20 nucleotide-long homologous sequence at their 5^0 ends, and incorporate a unique restriction site. (b) By using the resulting PCR products as both primers and template, a polymerization is carried out resulting in a joint 1 kb fragment. This is followed by amplification of the newly generated allele using primers A1 and B2. (c) The allele is A/T cloned into pGEM-T. (d) Using the same unique restriction site incorporated into primers A2 and B1, a fragment containing the *mturquoise2* ORF followed by an FRT-flanked *nptII* gene is cloned to generate the allelic exchange module, and the correct orientation is confirmed. This correctly oriented construct represents the allelic exchange vector to be transformed into *P. syringae* to obtain the double recombinants that would have incorporated the transcriptional reporter fused to the target gene of interest

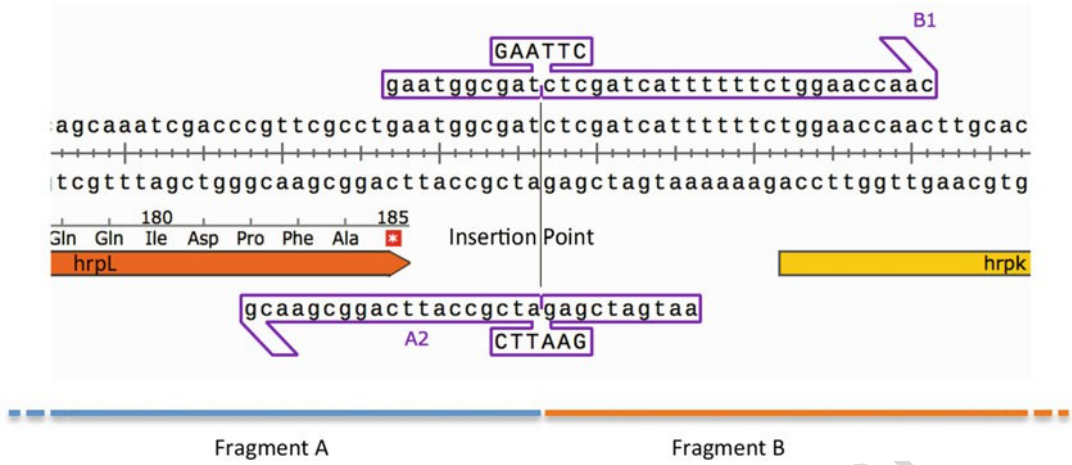

Fig. 2 Sequences and positions of primers A2 and B1 used to amplify fragments A and B to generate the allelic exchange vector used to generate chromosome located *hrpL* transcriptional fusions. Insertion point indicates the position in which, through cloning on the indicated *Eco*RI site, the promoterless reporter ORF would be inserted

gene of interest. We chose nucleotide 8 for the *hrpL:: mturquoise2* construct (Fig. 2). Fragment A in the allelic exchange module should correspond to the 500 bp (approximately) upstream of the insertion point, while fragment B should correspond to the 500 bps downstream. For each fragment, we designed a forward primer (A1 and B1, respectively) and a reverse primer (A2 and B2, respectively). Primers A1 and B2 are regular primers located approximately 500 bps upstream and downstream of the insertion point, respectively. Primers A2 (reverse primer for fragment A) and B1 (forward primer for fragment B) (Fig. 2) should include a restriction enzyme site (in this case a *Eco*RI site) upstream the insertion point, plus an additional ten nucleotide-long overlapping sequence.

3.1.2 Consecutive PCRs

Three consecutive PCR reactions are required to obtain the A + B fragment. This is achieved by following the following five steps:

1. To generate fragments A and B, a 25 μL PCR reaction with the respective primer pails is carried out using DC3000 genomic DNA as template, 0.2 mM dNTP, 0.5 μM of each primer (Table 2) and 0.25 μL Q5 High-Fidelity DNA Polymerase (NEB, UK). The reaction is performed according to the supplier's protocol, starting with an initial 30 s at 98 ºC denaturation step, followed by 35 cycles at 98 ºC for 10 s, 60 ºC (*see* Note 1) for 20 s, and 72 ºC for 30 s; and finishing with a further 2 min at 72 ºC.
2. Resolve the PCR samples by electrophoresis in a 1% agarose gel containing 1x RedSafe. Cut out the bands corresponding to the predicted molecular size (around 500 bp) using a blade and

purify the DNA using a Gel Band Purification Kit (e.g., Illustra GFX PCR DNA and Gel Band Purification Kit, GE Healthcare, Spain), following the manufacturer's instructions.

3. Using equal amounts (10–20 ng) of each of the purified fragments A and B, carry out a second reaction, using with 0.2 mM dNTP and 0.25 μL Q5® High-Fidelity DNA Polymerase, but *without* additional primers or template. Put the mix into a thermocycler and subject it to eight cycles at 98 ºC for 10 s, 60 ºC (*see* Note 1) for 20 ºC and 72 ºC for 45 s, with a final extension for 2 min at 72 ºC.
4. Use 5 μL of the Step 3 reaction as template in a third consecutive PCR, containing 0.2 mM dNTP, 0.5 μM of each primers A1 and B2 and 0.25 μL Q5® High-Fidelity DNA Polymerase. Run the thermocycler using identical cycles and conditions as used in the first PCR—Step 1 (*see* Subheading 3.1.2).
5. Visualize the PCR sample as above by electrophoresis on a 1% agarose gel. Recover the DNA corresponding to the 1 Kb band as described.

3.1.3 pGEM®-T Cloning and Selection

1. Set up the pGEM®-T ligation reaction, using 3.3 μL (50–100 ng) of your purified DNA (*see* Note 2), 0.7 μL of pGEM®-T vector, 1 μL of T4 DNA ligase and 5 μL of 2x Ligase buffer, to a volume of 10 μL. Incubate overnight at 16 ºC.
2. Mix 2 μL of the ligation with 20 μL of E. coli DH5α competent cells and transform following an appropriate protocol (*see* Note 3).
3. Plate the transformation onto LB agar plates supplemented with ampicillin (100 μg/mL) and X-gal (40 μg/mL). Incubate plates at 37 ºC overnight.
4. Pick 2–3 white colonies and grow them overnight in LB broth supplemented with ampicillin (100 μg/mL).
5. Extract the plasmid from the bacteria using a plasmid mini-prep protocol (*see* Note 4). Confirm sequence of the fragment generated (Fragment A + B) by sequencing.

3.1.4 Restriction and Ligation

1. To generate the allelic exchange plasmid cut the pGEM®-T derivative obtained carrying the A + B fragment with an appropriate restriction enzyme (*see* Note 5). Use the same enzyme to cut the plasmid containing the *mturquoise2-nptII* construct (Table 1) (*see* Note 6). Mix 2 μg of each plasmid with 5 μL of 10x buffer, 1 μL of EcoRI (10 U), and $_{dd}H_2O$ up to 50 μL. Incubate the reactions at 37 ºC for 1 h.
2. Analyze the sample by electrophoresis in a 1% agarose gel (*see* Note 7). The expected sizes in this particular example are 4 Kb for the pGEM®-T- fragment A + B digested plasmid and 2.2 Kb for the *mturquoise2-nptII* construct.

3. Purify the bands of the correct sizes as described above. Measure the DNA concentration using a NanoDrop® Spectrophotometer.
4. Set up a ligation reaction as follows: 20 ng of linearized pGEM®-T- fragment A + B, 30 ng of *mturquoise2-nptII* fragment, 1 U T4 DNA ligase, 1x T4 DNA ligase buffer, and $_{dd}H_2O$ up to 10 μL. Incubate the reaction overnight at 16 ºC.
5. Mix 2 μL of the ligation with 20 μL of E. coli DH5α competent cells and transform following an appropriate protocol.
6. Plate the transformation in LB agar plates supplemented with kanamycin (50 μg/mL) (*see* Note 8).

Pick 2–3 colonies and grow them overnight in LB broth supplemented with kanamycin (50 μg/mL). Incubate at 37 ºC overnight. Since at this stage expression of the fluorophores can be driven from a constitutive promoter from the plasmid backbone, for some fluorophores (i.e., mOrange, eYFP, and mPlum) E. coli transformants may appeared colored on the plate (Fig. 3).

Extract the plasmid from the selected clones using a plasmid mini-prep protocol (*see* Note 4). Check the orientation of the *mturquoise2-nptII* insert by restriction endonuclease analysis (*see* Note 9).

3.2 Introducing the Transcriptional Fusions into the *P. syringae* Chromosome

3.2.1 Preparing *P. syringae* Competent Cells

1. Streak from −80 ºC stock the *P. syringae* strain to be transformed onto an LB plate and incubate for 2 days at 28 ºC (*see* Note 10).
2. Scrape all biomass off of the plate and suspend it in 1 mL of chilled 300 mM sucrose. Keep the cells on ice unless otherwise stated.
3. Spin for 2 min at 12500 × *g* at 4 ºC. Discard supernatant and resuspend the pellet into 1 mL of chilled 300 mM sucrose.
4. Repeat step 3 three times.
5. Remove all the supernatant and resuspend the pellet in 100 μL of chilled 300 mM sucrose. The cells are now ready for electroporation (*see* Note 11).

3.2.2 Electroporation of P. syringae

1. Add a maximum of 2 μL of your purified insertion plasmid to the 100 μL of competent cells. Mix by gently pipetting up and down.
2. Place the mix in a cold 2 mm electroporation cuvette and keep on ice.
3. Electroporate with a pulse of 25 kV/cm, and place the cuvette on ice immediately after. Add 1 mL of liquid LB. Mix gently.
4. Transfer the volume into a fresh Eppendorf tube and incubate for 1 h at 28 ºC prior to plating on the selective medium.
5. Incubate the plates at 28 ºC for 48 h.

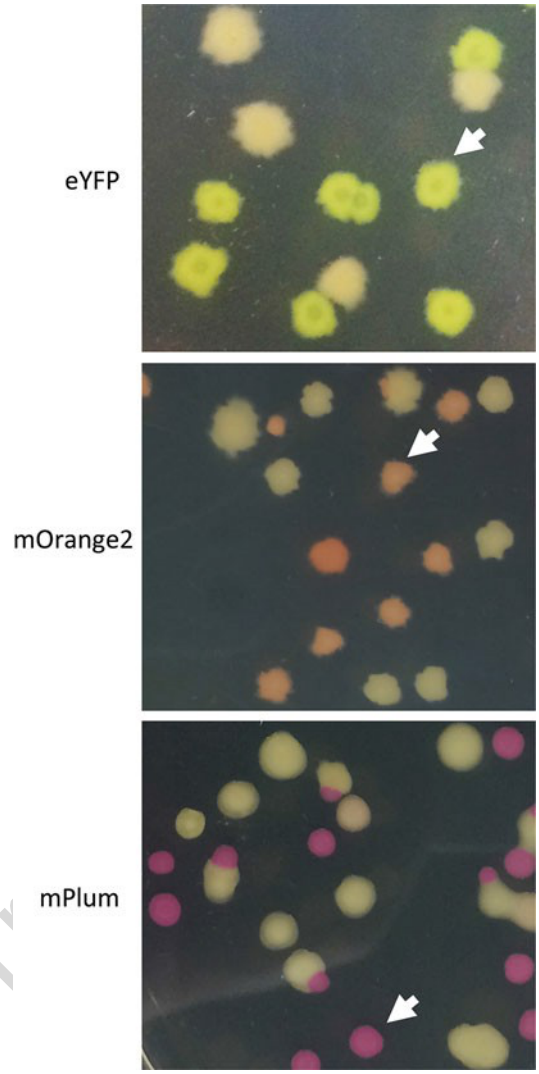

Fig. 3 Images taken from the selection plates used to get *E. coli* transformants carrying the allelic exchange vectors for: '*hrpL::eyfp* (top), *'hrpL::mOrange* (center) and *'hrpL::mPlum* (bottom). Positive transformants colonies appeared colored as indicated by arrowheads

3.2.3 *Clone Selection*

All kanamycin-resistant clones obtained in the previous step would have incorporated the *nptII* gene, however, some would have done so through integrating the whole plasmid (via a single recombination event in one of the homologous 500 pb regions flanking the insertion point), while others would have integrated only the fluorescent reporter module by allelic exchange (via a double recombination event in both the homologous A and B 500 bp regions flanking the insertion point). Only the latter would carry a stable transcriptional fusion of the gen of interest (*hrpL*) to the fluorescent

reporter (*mturquoise2*). These two types of recombinant clones can be differentiated on the basis of their resistance to ampicillin, since only the first type which has the pGEM®-T backbone would be resistant. To check this:

Replicate up to 50 Km^R colonies obtained following the transformation, in three different LB agar plates supplemented with: (1) 15 μg/mL kanamycin, (2) 500 μg/mL ampicillin, and (3) a not supplemented (without antibiotics) control plate (*see* Note 11). Incubate the plates overnight at 28 ºC.

Check growth on the plates: double recombinant clones carrying the desired chromosomal fusion would grow in LB + kanamycin, would not grow in LB + ampicillin, and would grow on the control, not supplemented plate (without antibiotics) (*see* Note 12). Pick several clones showing the correct antibiotic resistance profile from the control plate and inoculate them into liquid LB medium supplemented with 15 μg/mL kanamycin. Incubate overnight at 28 ºC with shaking. Store these clones at −80 ºC in 25% glycerol. The correct chromosome insertion must be confirmed by Southern blot analysis (*see* Subheading 3.4).

3.3 Removing the Kanamycin Resistance Gene

3.3.1 P. syringae Transformation and Selection of the Clones

1. Prepare electrocompetent cells of the *P. syringae* strain to be used, as indicated in Subheading 3.2.1.
2. Add up to 2 μL of a pFLP2 vector preparation and transform as indicated in Subheading 3.2.2.
3. Plate the cells onto LB agar plates supplemented with ampicillin at 500 μg/mL. Incubate the plates at 28 ºC for 48 h.
4. Pick up to five colonies from the selection plate and inoculate them into liquid LB medium without any antibiotic. Incubate overnight at 28 ºC with aeration. Make serial dilutions into 10 mM $MgCl_2$ using the saturated cultures (*see* Note 13). Incubate at 28 ºC for 48 h.
5. Replicate up to 5 colonies from each plate in different LB agar supplemented with: (1) kanamycin 15 μg/mL, (2) ampicillin (500 μg/mL) and (3) not supplemented (without antibiotic). Incubate at 28 ºC for 48 h.

Check bacterial growth on the plates. The desired clones are those that do not grow in either kanamycin or ampicillin plates, but grow in the control LB plates without selection.

Pick one colony from each clone from the LB nonsupplemented control plate and use to inoculate LB liquid medium. Incubate overnight at 28 ºC with aeration. Store the clones at −80 ºC in 25% glycerol. The correct deletion of the antibiotic resistance gene must be confirmed by Southern blot analysis (*see* Subheading 3.4).

3.4 Confirming Allelic Exchange and/or Removal of the Kanamycin Resistance Gene by Southern Blot Analysis

1. Inoculate 5 mL of LB liquid medium with the strain to be tested. Incubate overnight at 28 ºC with aeration. Harvest the cells by centrifugation at 12,000 × *g* for 5 min and extract genomic DNA using the Jet Flex extraction kit or equivalent, following the instructions of the manufacturer. Measure DNA concentration.
2. Digest 2 μg of DNA with the appropriate enzyme to ensure transformants carry a single insertion on the correct position and/or have lost the *nptII* gene (*see* Note 7).
3. Load the restrictions into a 0.7% agarose gel and separate by electrophoresis for 1–2 hours at 100 V (*see* Note 14).
4. Depurinate DNA by submerging the gel into 0.25 N HCl for 15 min at room temperature with gentle shaking.
5. Wash the gel by submerging it into $_{d}H_2O$. Repeat three times.
6. Submerge the gel in denaturing solution and incubate for 30 min at room temperature with gentle shaking.
7. Remove denaturing solution. Add neutralizing solution and incubate for 30 min at room temperature with gentle shaking.
8. Transfer DNA onto a nylon membrane (*see* Note 15), and cross-link it by exposing the DNA-bound side of the membrane to UV light (0.120 J).
9. Carry out prehybridization and hybridization stages at 65 ºC, and a digoxigenin-labeled DNA fragment containing FRT-*nptII*-FRT as probe (*see* Note 16).
10. Develop the membrane. In our example, membrane was developed using anti-digoxigenin antibody and CSPD (Roche, Germany), following instructions of the manufacturers.

3.5 Analysis of the Strain

Once the strains carrying the chromosome-located transcriptional fusions are confirmed by Southern Blot analysis (Fig. 4a), they are ready for experimental work. Since our purpose was to use the generated strain to carry out confocal microscopy, we tested expression of the transcriptional fusion in bacteria extracted from the plant apoplast (*see* Note 17), where in planta activation of *hrpL* expression can be analyzed [10]. Confocal microscopy (*see* Note 18) on *Arabidopsis* apoplast-extracted bacteria carrying the *hrpL::mturquoise2* transcriptional fusion showed an activation of gene expression. In this particular case, this activation was characterized by a strong phenotypic heterogeneity, as previously reported for the same gene in the closely related pathogen *P. syringae* pv. *phaseolicola* 1448A in bean leaf apoplasts [10] (Fig. 4b).

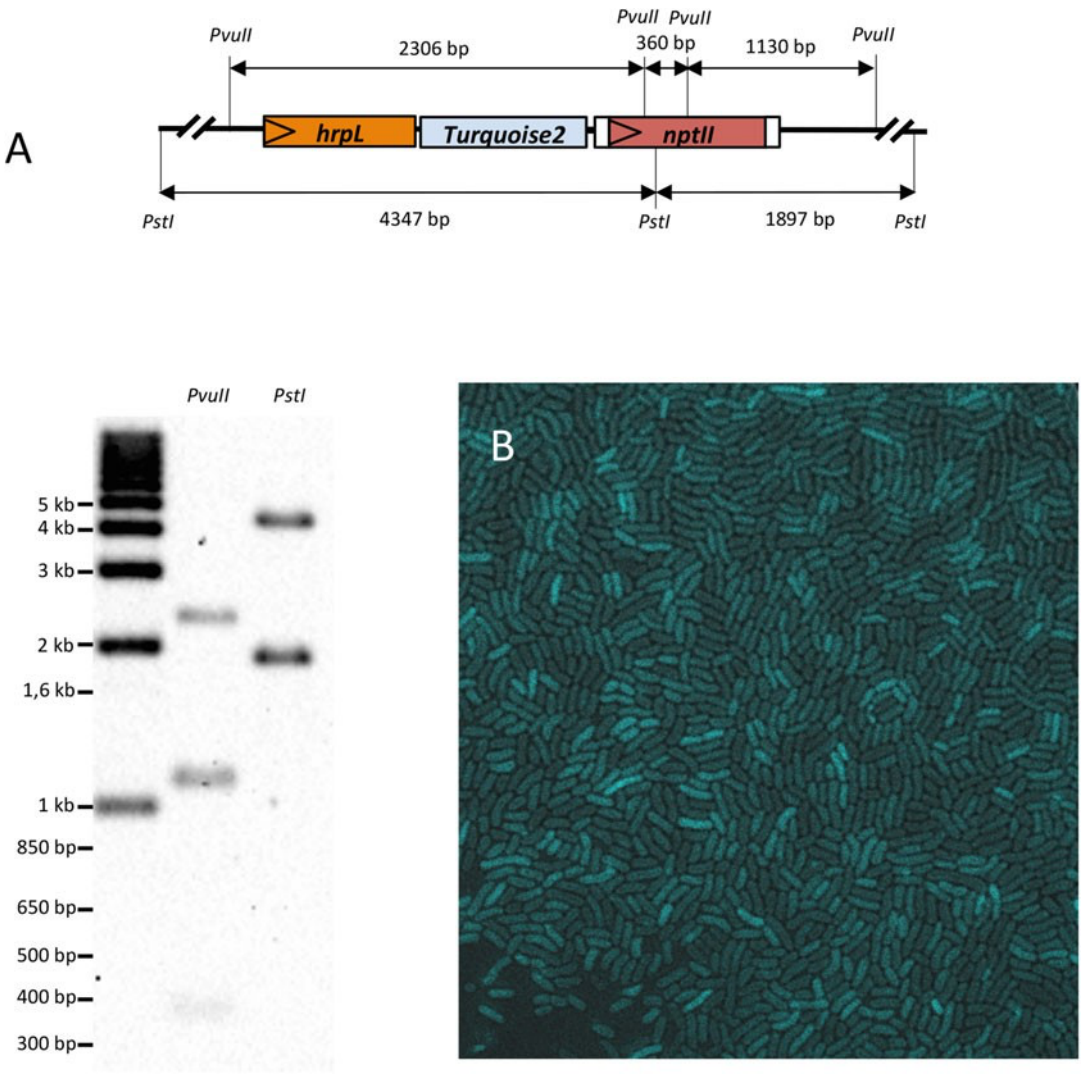

Fig. 4 Southern blot analysis and confocal microscopy analysis of *P. syringae* pv. tomato DC3000 carrying a *hrpL::mturquoise2*. (a) Southern blot analysis of genomic DNA from DC3000 derivative carrying the *hrpL::mturquoise2* transcriptional fusion. The diagram shows the organization and expected sizes of the construct upon digestion of genomic DNA using the indicated restriction enzymes. Below, blot of genomic DNA of the strain digested with either *Pvu*II or *Pst*I after probe hybridization, displaying bands with the expected sizes (b) Confocal microscopy image of *Arabidopsis* apoplast-extracted bacteria showing phenotypically heterogeneous activation of *hrpL::mturquoise2* expression

4 Notes

1. Optimize annealing temperature to the primers used.
2. For ligation of blunt-end fragments in pGEM-T, a regular A-tailing reaction must be performed, as follows: 1 μL of 10x standard buffer (with MgCl2), 2 μL of 1 mM dATP, 0.2 μL of Taq DNA Polymerase, and 6.8 μL of your purified PCR fragment. Run the reaction for 30 min at 72 ºC.

3. Any high-efficiency E. coli transformation protocol can be used. We obtained competent DH5a cells using a modification of the method described by Inoue and colleagues [23]. Briefly, a 1:1000 dilution of a saturated bacterial culture into 200 mL of SOB medium [19] was incubated with aeration at 22 ºC cells reached an OD_{600} of 0.5. After chilling the culture in ice for 10 min, cells were collected by centrifugation (2500 × *g* 4 ºC 10 min), suspended in 80 mL of ice-cold TB buffer and kept in ice for 10 min. The process was repeated twice with the cells suspended in 20 mL of TB after the second centrifugation step. After adding 1.5 mL of DMSO cells aliquots were kept at -80 ºC prior to use. Transformation was carried out by heat-shock [19].
4. DNA plasmid extractions were carried out using the method described by [24], using isopropanol to precipitate DNA.
5. We have used *Eco*RI but any other 6-cutter restriction enzyme not cutting within the sequences of the 1 Kb A + B fragment generated flanking the fluorophore-antibiotic resistance reporter cassette can be used.
6. The plasmids containing the fusions of the fluorescent proteins to Km were generated as follows: The ORF of each fluorescent protein (Turquoise2, eYFP, mPlum, mOrange2, mCherry) was amplified using the primers Prot Fluor F and Prot Fluor-Km R or GFP^+ F and GFP^+Km R, in the case of GFP+ (Table 1), and the corresponding plasmids (Table 2). The FRT-Km-FRT fragment was amplified using the primers Km-Prot Fluor F and P2. The PCR reactions were set up as in Subheading 3.1.2. Each fluorescent protein ORF was fused to Km by PCR using equal amount of each fragment (10–20 ng) and the primers Prot Fluor F and P2 (for Turquoise2-Km, eYFP-Km, mPlum-Km, mOrange2-Km and mCherry-Km fusions) or GFP^+ and P2 (for the GFP^+-Km fusion). The resulting fragments were cloned in pGemT (*see* Subheading 3.1.3) to generate plasmids pGT-Turquoise2, pGT-YFP, pGT-mPlum, pGT-mOrange2, pGT-mCherry, and pGT-GFP^+ (Table 2).
7. Percentage of agarose within the gel and electrophoresis conditions should be adjusted depending on the expected size of the fragments.
8. Antibiotic resistance genes different from *nptII* could be used and therefore the antibiotic for plate selection should be modified accordingly.
9. The ORF of the gene encoding the fluorophore must be in the same orientation of the gene to which is to be fused. The selection of the restriction enzymes to be used in the restriction analysis of the resulting plasmid must generate DNA fragments of sufficiently different sizes in each possible orientation as to allow unequivocally determination of the correct one.

10. Plates should be fresh as storage at 4 ºC can reduce the efficiency of transformation.
11. Storage of *P. syringae* competent cells at −80 ºC drastically reduces transformation efficiency.
12. An LB plate is used in order to make completely sure that the selected clone is not ampicillin resistant. An LB plate supplemented with kanamycin could be used as well.
13. Dilutions 10^{-4} and 10^{-5} should be sufficient to ensure isolated colonies that have lost the *nptII* gene are obtained.
14. Digestions should be designed using two enzymes: one that cuts (e.g., *Pvu*II) and another that does not cut within the probe, respectively, as long as the fragments generated are not larger than 5 Kb.
15. DNA transfer from the agarose gel onto the membrane was carried out using upward capillarity transfer, but other means could be used as well.
16. A probe containing the *nptII* gene flanked by two FRT sites was generated by PCR using chemiluminescent digoxigenin-dNTPs and DIG Labelling Mix (Roche, Germany), primers P1 and P2, and pKD4 as the DNA template.
17. *Arabidopsis* leaves were pressure infiltrated using a needleless syringe and approximately 100 μL of a 5×10^5 cfu/mL bacterial suspension in 10 mM $MgCl_2$. Three days post inoculation (dpi) bacteria were recovered from the plant by an apoplastic fluid extraction. The apoplastic fluid extraction was carried out by pressure infiltrating a whole leaf with 3 mL of a 10 mM $MgCl_2$ solution inside a 10 mL syringe. Following 5 cycles of pressure application, the flow-through was removed and placed in two fresh 1,5 mL tubes and centrifuged for 1 min at max speed. Pellets were resuspended into 50 μL of $MgCl_2$ and analyzed by confocal microscopy.
18. Images of apoplast-extracted bacteria were taken using the Leica SP5 II confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany). Variable AOTF filters were used for the visualization of Turquoise2 (excitation 405 nm/emission 425 to 500 nm). Image processing was performed using Leica LAS AF (Leica Microsystems).

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