Roger de Pedro Jové[1,2$] Pau Sebastià[2$] and Marc Valls[1,2*]

[1] Department of Genetics, University of Barcelona, Barcelona, Catalonia, Spain

[2] Centre for Research in Agricultural Genomics (CSIC-IRTA-UAB-UB), Bellaterra, Catalonia, Spain

[$] This authors contributed equally to this work

* Corresponding author: marcvalls@ub.edu

Running head: Chemical inhibitors of bacterial type 3 secretion systems

IDENTIFICATION OF TYPE III SECRETION INHIBITORS FOR PLANT DISEASE MANAGEMENT

Abstract

Bacterial plant pathogens are amongst the most devastating threats to agriculture. To date, there are no effective means to control bacterial plant diseases due to the restrictions in the use of antibiotics in agriculture. A novel strategy under study is the use of chemical compounds that inhibit the expression of key bacterial virulence determinants. The type 3 secretion system is essential for virulence of many gram-negative bacteria, because it injects into the plant host cells bacterial proteins that interfere with their immune system. Here, we describe the methodology to identify bacterial type 3 secretion inhibitors including a series of protocols that combine *in planta* and *in vitro* experiments. We use *Ralstonia solanacearum* as a model because of the number of genetic tools available in this organism and because it causes bacterial wilt, one of the most threatening plant diseases worldwide. The procedures presented can be used to evaluate the effect of different chemical compounds on bacterial growth and virulence.

Key words: Bacterial plant pathogens, Type 3 Secretion System, *Ralstonia solanacearum*, Chemical inhibitors, Plants, Protocols, Immunodetection, *In vitro* inhibitory test.

1. Introduction

Bacteria can cause a range of diseases in economically important crops, leading to important losses. *Ralstonia solanacearum*, the causal agent of bacterial wilt, is one of the most devastating plant pathogens worldwide. The lack of effective means to control bacterial diseases and block the spread of these pathogens urge for new control strategies. The use of antibiotics and copper-based compounds is nowadays banned or tightly regulated in many countries [1, 2]. Using compounds that inhibit specific bacterial virulence factors are a promising and sustainable strategy.

The Type 3 Secretion System (T3SS) is one of the most distinctive hallmarks of gram-negative bacterial pathogens. These pathogens use the T3SS to inject small molecules called effectors inside the plant cell. Bacterial effectors hijack plant defence mechanisms and manipulate different metabolic pathways to successfully colonize the host [3]. Mutant bacteria devoid of the T3SS are totally non-pathogenic, so that a possible strategy to inhibit bacterial virulence is to use chemical compounds that block the expression of this secretion system and impede bacterial colonization throughout the plant [4–6].

In this protocol, we present a stepwise guide to assess the ability of different chemical compounds to transcriptionally down-regulate the expression of key T3SS genes and to test if they could be used as a means to decrease the virulence of the tested pathogens *in planta*.

2. Materials

2.1 Plant growth

1. *Nicotiana benthamiana; Nicotiana tabacum;* and *Solanum lycopersicum* cv. Marmande.
2. Soil mix: Peat soil substrate nº2 + vermiculite + perlite (*see* **Note 1**).
3. Plant growth chambers with temperature, humidity, and photoperiod control.

2.2 Bacterial strains and growth

1. *Ralstonia solanacearum* GMI1000 reporter strains for transcription of *hrpB* (*PhrpB*::*luxCDABE*), *psbA* (*PpsbA*::*luxCDABE*) and *hrpY* (*PhrpY*::*luxCDABE)*. *R. solanacearum* GMI1000 *PpsbA*::*avrA*-HA.
2. B medium: 10 g/L Bacteriological peptone, 1 g/L Yeast extract and 1 g/L Casamino Acids. Add 1.5% Agar for solid media before autoclaving. Before plating, add 0.5% Glucose and 0.005% Triphenyltetrazolium chloride (TTC). Supplement with the appropriate antibiotics (*see* **Note 2** and **Note 3**)**.**
3. Boucher's minimal medium [7]: To prepare 1L of 2X Boucher's medium, mix 100 mL of 5X M63 medium (10 g/L $(NH_4)_2SO_4$, 68 g/L KH_2PO_4 and 2.5 mg/L $FeSO_4 \cdot 7H_2O$, pH 7 with KOH) with 405 µL 1M $MgSO_4 \cdot 7H_2O$ and adjust to 1 L with sterile distilled water. Before use, dilute to 1X with sterile distilled water (or 2x agar on water for plates). Supplement with 20 mM glutamate and appropriate antibiotics.

2.3 T3SS inhibition test in vitro

1. Potential Type 3 Secretion inhibitory compound to test
2. DMSO
3. Incubator at 28ºC with rotor.
4. Luminometer
5. Spectrophotometer

2.4 Effect of the tested compound on bacterial T3E secretion

1. Sucrose

2. Congo red
3. 0.22 µM filter
4. 10 mL syringe
5. 25% Trichloroacetic acid
6. 90% Acetone
7. Phosphate-saline buffer (PBS) 1X: 8 g/L NaCl, 0.201 g/L KCl, 1.42 g/L Na_2HPO_4, 0.272 g/L KH_2PO_4.
8. 4X Laemmli buffer
9. Digital sonifier)
10. Primary anti-HA rat monoclonal antibody conjugated to horseradish peroxidase (HRP) in Tris-buffered saline (TBS) with 0.1% Tween-20 and 1% skimmed milk (see **note 4**).
11. Coomassie blue
12. LAS-4000 mini system.

2.5 *In planta* experiments

1. Blunt-end syringe
2. 100% ethanol
3. Leaf disk puncher
4. Potter S homogeniser

3. Methods

3.1 Plant and bacterial growth

N. benthamiana / N. tabacum

1. Sow *N. benthamiana* or *N. tabacum* seeds in a pot at 26ºC and 14 hours light/10 hours darkness.
2. After 10 days, transfer each seedling to individual pots.
3. After 10 days, transfer each individual plant to single big pots. These plants will be ready for assays after 3 weeks (*see* **Note 5** and **Note 6**).

Solanum lycopersicum cv. Marmande

1. Sterilise Marmande tomato seeds with a sterile solution containing 1:3.33 of commercial bleach (4.7% concentrated) and 0.05% triton. Keep the seeds in the solution for 10 minutes. Wash with sterile distilled water at least 5 times.
2. Sow the sterilised seeds and cover with plastic film.
3. Keep the plants in the growth chamber at 22ºC, 16 hours light and 8 hours darkness for one week, until tomato seedlings emerge and touch the plastic film on top.
4. Transfer each tomato seedling to individual soil pots with the soil mix and let them grow for 3 weeks in a chamber at 22ºC and 16 hours light and 8 hours darkness (*see* **Note 5**).

Ralstonia solanacearum

1. Streak the bacterial strain from a glycerol stock at -80 ºC on B medium supplemented with antibiotics for 2 days at 28 ºC.
2. Pick a single colony and incubate in liquid B or minimal media.

3.2 In vitro T3SS inhibitor screening in *Ralstonia solanacearum*

1. Grow an overnight pre-culture in liquid B media supplemented with antibiotics (*see* **Note 7** and **Note 8**).

2. Centrifuge the overnight pre-culture in 2 mL Eppendorf tubes at RT for 1 min at maximum speed, discard the supernatant and re-suspend the bacterial pellet in 1 mL of sterile distilled H_2O.
3. Measure the OD_{600} with the spectrophotometer (*see* **Note 9**).
4. Adjust to a final OD_{600} of 0.3 adding the right pre-culture volume to a culture tube containing 1.5 mL of fresh Boucher's minimal medium supplemented with 20mM glutamate, antibiotic and 100mM of the inhibitory test compound/DMSO (*see* **Note 10**).
5. Mix by vortexing for few seconds and incubate in a shaker.
6. Measure luminescence at times 0, 4, 6, 8 and 24 hours transferring 200 µL from each tube into a 1.5 mL Eppendorf and quantifying light emission from the reporter in the luminometer. For each time point, measure as well OD_{600} in a spectrophotometer by transferring the 200 µL into a cuvette containing 800 µL of distilled water (*see* **Notes 11**, **12** and **13**).

3.3 Effect of the tested compound on bacterial T3E secretion

1. From an overnight culture of liquid B medium supplemented with antibiotics, adjust to a final OD of 0.2 ($2x10^8$ CFUs/mL) in a final volume of 10 mL of minimal medium supplemented with antibiotics, 10mM glutamate, 10 mM sucrose, 100 µg/mL congo red (*see* **Note 14**) and 100 µg/mL of the test inhibitor compound (or 10 µL DMSO as a control).
2. Incubate at RT for 12-14h (or until OD_{600} reaches 1).
3. Transfer the culture to a 50 mL falcon tube and centrifuge 4000 g, 10 min.
4. Filter the supernatant through a 0.22 µM filter with a syringe in order to remove any bacteria. The bacterial pellet is also kept at -20 ºC for further analysis.
5. Add 10 mL of cold 25% TCA to the filtered supernatant and let it precipitate all night long at 4ºC.
6. Centrifuge 6000 g, 30 min at 4 ºC and discard the supernatant.
7. Wash the protein pellet (it will contain all secreted proteins in the medium) twice with cold 90% acetone and let it dry at RT.

8. Re-suspend the protein pellet in 100 µL of PBS 1X. Mix 15 µL of this solution with 15 µL Laemmli buffer.
9. Recover the frozen bacterial pellet, freeze-thaw 3-4 times (-80 ºC - RT cycles), re-suspend in 1 mL 1X PBS and sonicate the cells using a sonifier (see note 15). Mix 15 µL of the mixture with 15 µL of Laemmli buffer.
10. Boil the samples for 5 min and load it in a SDS-PAGE (it will be a 100X concentration from initial culture).
11. Presence of particular proteins in the extracts can be analysed by immunoblot using an antibody against the protein of interest. Coomassie-stained sodium dedecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) membranes can be visualised using LAS-4000 mini system (*see* Fig. 1).

3.4 *In vivo* T3E translocation test using hypersensitive response assays

1. To the overnight culture of the desired bacterial strain (e.g. *R. solanacearum* GMI1000*)* in Boucher's minimal medium supplemented with 20 mM glutamate and antibiotic, add the tested inhibitory compound at 100 µM (or with DMSO for the non-treated condition) and incubate for 8 hours.
2. Centrifuge at maximum speed for 8 min and discard the supernatant.
3. Re-suspend bacterial pellet with sterile distilled water and measure the OD. Make serial dilutions ranging from 10^7 to 10^5 CFUs/mL (*see* **Note 16**).
4. Leaf-infiltrate *N. benthamiana* and *N. tabacum* plants with a blunt-end syringe following a predesigned scheme (*see* **Note 17** and Fig. 2).
5. The first signs of hypersensitive response are visible 12 hours post-infiltration but they can be better appreciated when the dead tissue is totally dry, so the pictures are taken two days post-infiltration in *N. tabacum*, and 5 days post-infiltration in *N. benthamiana*, (*see* **Note 18**).

3.5 Compound effect on bacterial fitness *in planta*

1. Grow an overnight pre-culture in liquid B medium supplemented with antibiotics.

2. Measure the OD_{600} of the pre-culture and adjust a bacterial suspension to 10^5 CFU/mL (OD_{600}=0.0001) with autoclaved tap water supplemented with each test compound at 100 µM (or DMSO alone for control condition).
3. Hand-infiltrate 4 tomato leaves per tested compound with a blunt-end syringe (*see* **Note 19**).
4. Place the infiltrated plants in the growth chamber for 1 hour at 27ºC and 60% relative humidity.
5. At time 0 (just after infiltration) and at 4 days post-infiltration (d.p.i.), collect 2 leaf discs (5mm diameter) from the infiltrated area of six independent leaves. Combine in a 1.5 mL Eppendorf tube the disks from 2 leaves (4 disks total) to generate three biological replicates.
6. Homogenise the plant material with a Potter S homogeniser in 200 µL of sterile distilled water (*see* **Note 20**).
7. Add 800 µL of sterile distilled water to each Eppendorf tube.
8. Place the plants back in the growth chamber.
9. Prepare ten-fold dilutions from the leaf homogenates (*see* **Note 21**).
10. Plate 10 µL drops of the 4 dilutions on plates of B medium (containing TTC and glucose) supplemented with antibiotic and incubate at 28ºC for 1-2 days to count colonies (*see* **Note 22**).

3.6 Effect of the T3 Secretion inhibitor on bacterial virulence to plants

1. Grow an overnight pre-culture in liquid B medium supplemented with antibiotics.
2. For each treatment, wound the roots of 12 plants grown in independent pots with a 1-mL pipette tip by making 4 holes in the soil around the stem. Water each plant with 40 mL of a bacterial suspension containing 10^8 CFUs/mL supplemented with 100 µM of the compound to test or DMSO (*see* **Note 23**).
3. Record wilting symptoms during 9 days after infection for each plant using a semi-quantitative scale ranging from 0 (no wilting) to 4 (death) (*see* **Note 24**).

4. Notes

1. For 24 individual square pots mix: 7 L of peat soil, 0,2 L of vermiculite and 0,2 L of perlite.
2. For gentamicin and tetracycline, use half of the recommended concentration in liquid media (e.g. 10 µg/mL gentamicin in solid medium and 5 µg/mL in liquid medium).
3. Keep the TTC solution and tetracycline away from direct light contact. Glucose strongly enhances exopolysaccharide production and TTC turns red through bacterial metabolism, so that wild type *R. solanacearum* colonies appear red with a thick mucus halo in this medium. Spontaneous non-mucous mutants (usually rare) are non-pathogenic and can be discarded.
4. The anti-HA antibody (clone 3F10) from Roche, Switzerland works well for us at 1:4000 dilution. Anti-HA antibodies from our resources might work as well and we recommend to test for ideal dilutions before use.
5. To acclimate the plants, 2 days prior to bacterial inoculation, transfer them to the infection growth chamber (27ºC and 60% humidity).
6. For HR assays, plants should not be stressed. Clear signs of stress are chlorotic leaves and flowering. To avoid this, do not water in excess, always use high intensity light. Plants can be grown at 24-26ºC without any difference.
7. Minimal medium is appropriate when type 3 secretion gene expression has to be induced (e.g. *PhrpY::lux*). B medium is appropriate when high growth is desired, or expression of the type 3 secretion genes has to be repressed
8. Normally, 10 mL of overnight culture should be enough to prepare 20 tubes for the inhibition test.
9. We recommend measuring OD_{600} from 1/10 dilutions of overnight cultures to avoid saturation, as spectrophotometers usually measure linearly between 0.01 and 2.
10. To ease the experiment, prepare these minimal media culture tubes the day before and store at 4ºC. Pre-warm the media before use.
11. Use a cuvette with the same growth medium as blank to calibrate the spectrophotometer.

12. This protocol can be up-scaled to 96 well-plates in case a larger set of inhibitors has to be tested. For growth measurements a transparent bottom plate must be used. For luminescence measurements use white opaque plates, which help reflecting luminescence and amplify the signal. The 96 well plates can be measured using a Spectramax M3 from Molecular Devices.
13. Luminescence measurements allow quantification of the transcriptional output at different time points and OD_{600} measurements quantify bacterial growth to normalise luminescence per cell and rule out eventual inhibitory or bacteriostatic effects of the tested compounds.
14. Congo red enhances bacterial protein secretion through the type 3 secretion system [8].
15. We normally sonicate for 90s at 30% amplification and 10s ON/OFF intervals using a digital sonifier Model 250/450 from BRANSON, USA. The required sonication time and intervals can vary for different sonifiers.
16. In *R. solanacearum*, an $OD_{600} = 1$ usually corresponds to 10^9 CFUs/mL.
17. It is recommended to randomize the infiltration of the bacterial dilutions in different leaves in order to get rid of eventual position effects. Infiltrate in the inter-vein areas to avoid mixture of treatments.
18. For a better HR cell death visualization, the treated leaves can be bleached using 100% ethanol in a water bath at 60ºC for 20 min.
19. Tomato plants can be vacuum-infiltrated instead using silwett as an adjuvant to facilitate infiltration (80 µL/L). Usually 20-30 seconds of vacuum infiltration is enough per tomato plant but timings might change in other plant species depending on the hardness of their leaves. A change in the leaf colour to dark green indicates proper vacuum infiltration.
20. We use the mechanic drill with a plastic pestle, but a tissue lyser with beads or a classical mortar can also be used.
21. To ease manipulation, it is advisory to perform dilutions in 96-well plates using a multichannel pipette by transferring 10 µL into 90 µL of sterile distilled H_2O consecutively. Make sure to mix well each dilution.

22. For colony count, make sure that colonies are well separated. Bacterial growth is calculated as recovered CFU/cm^2 (area depends on the size of the leaf disk puncher).
23. In order to facilitate plant infection, it is better to stop watering them 2 days prior to inoculation.
24. Wilting symptoms are recorded based on a scale from 0 to 4. 0= no wilting, 1 = 25% of the leaves wilted, 2= 50% of the leaves wilted, 3= 75% of the leaves wilted, 4= 100% of the leaves wilted. It is recommended that the same person carries out the whole symptom recording to avoid biases.

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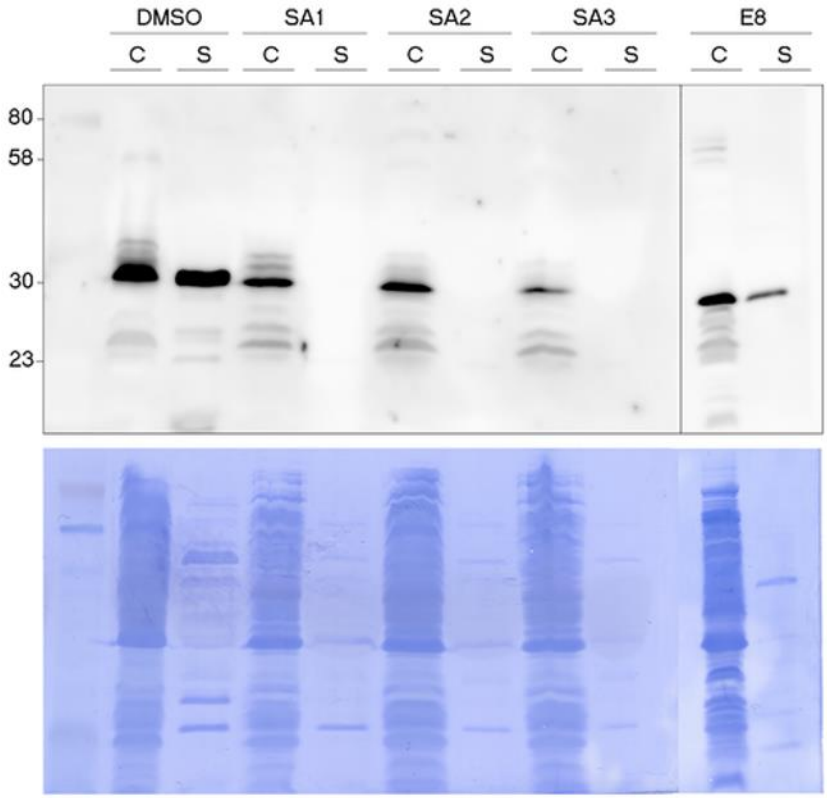

Fig 1. Immunoblot of the secreted T3 effector (in this case AvrA-HA) after treatment with four different inhibitory compound (SA1-3 and E8) or the control (DMSO). The cytosolic (C) and secreted (S) fractions were separated by centrifugation. The protein of interest was detected with anti-HA antibody. Coomassie blue-stained membranes (below) used in the western blotting are also shown. Reproduced from [9] with permission of John Wiley and Sons.

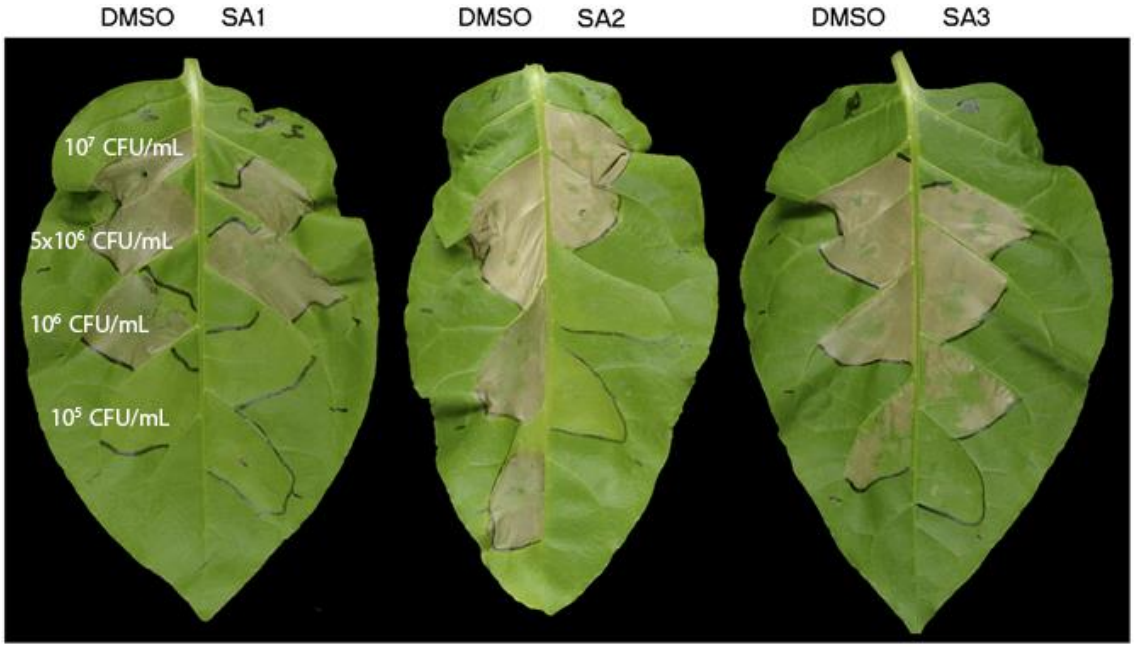

Fig 2. *N. benthamiana* leaves infiltrated with serial dilutions of *R. solanacearum* preincubated with inhibitory compounds (in this case SA1, SA2, SA3) or with a control solution (DMSO). Leaves were photographed 2 days post-infiltration Reproduced from [9] with permission of John Wiley and Sons.