

Workflow for a Functional Assay of Candidate Effectors From Phytopathogens Using a TMV-GFP-based System

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Abstract

The ability to efficiently screen plant pathogen effectors is crucial for understanding plant–pathogen interactions and developing disease-resistant crops. Traditional methods are often labor-intensive and time-consuming. Here, we present a robust, high-throughput screening assay using the tobacco mosaic virus–green fluorescent protein (TMV-GFP) vector system. The screening system combines the TMV-GFP vector and *Agrobacterium*-mediated transient expression in the model plant *Nicotiana benthamiana*. This system enables the rapid identification of effectors that interfere with plant immunity (both activation and suppression). The biological function of these effectors can be easily evaluated within six days by observing the GFP fluorescence signal using a UV lamp. This protocol significantly reduces the time required for screening and increases the throughput, making it suitable for large-scale studies. The method is versatile, cost-effective, and can be adapted to effectors with immune interference activity from various pathogens.

Key features

- A robust, cost-effective, and high-throughput functional screening system for plant pathogen effectors.
- Utilizes the TMV-GFP vector for rapid monitoring of effector activity.
- Evaluates the function of effectors within a few days using just a UV lamp.
- Adaptable to both apoplastic and cytoplasmic effectors from various phytopathogens.

Keywords: Effectors, TMV-GFP vector, Virulence, Avirulence, Functional screening

This protocol is used in: Plant J (2024), DOI: 10.1111/tpj.16774

Background

Understanding the molecular mechanisms of plant–pathogen interactions is essential for developing disease-resistant crops [1–3]. Traditional screening methods for plant pathogen effectors are often laborious and time-consuming, limiting their application in large-scale studies [4–8]. The tobacco mosaic virus (TMV) is a well-known positive-sense single-stranded RNA virus that can infect numerous plant species. Researchers have developed a TMV-based vector that expresses green fluorescent protein (GFP) to visualize viral movement. Under UV light, GFP fluorescence allows for easy tracking of the virus's spread [9]. When the TMV RNA is transcribed from the T-DNA, the vector begins to self-replicate and produces GFP. Consequently, the GFP fluorescence intensity provides a dependable measure of the TMV population size. The TMV-GFP vector system offers a robust approach, allowing for high-throughput screening of pathogen effectors. In this study, we took advantage of the TMV-GFP vector, established a TMV-GFP system, and demonstrated that it can be used to identify both functional cytoplasmic and apoplastic effectors from different plant pathogens based on TMV multiplication [9–11]. In our system, it is unnecessary to insert the effector gene into the TMV-GFP vector, overcoming size constraints about inserting foreign fragments when using a virus-based expression vector. *Agrobacteria* harboring the effector binary vector and the TMV-GFP vector were solely mixed before infiltration, and TMV infection was easily evaluated by observing the GFP fluorescence signal using a UV lamp within only a few days [12]. As such, as long as you already have an effector's binary vector library, this functional screening assay can be easily initiated. The protocol described here is optimized for *Nicotiana benthamiana* but can be adapted to other plant species and pathogens. This method streamlines the screening process, significantly reducing the time and labor required, and provides a versatile tool for plant pathology research.

Materials and reagents

Biological materials

1. *Nicotiana benthamiana* seeds
2. *Agrobacterium tumefaciens* strain GV3101 (pMP90)
3. *Escherichia coli* strain DH5a

Reagents

1. Sterile deionized water
2. Sodium chloride (NaCl) (Sangon Biotech, catalog number: A501218-0001)
3. Tryptone (OXOID, catalog number: LP0042)
4. Yeast extract (Sangon Biotech, catalog number: A515245-0500)
5. Agar (Solarbio, catalog number: A8190)

6. MES free acid monohydrate (MES) (Solarbio, catalog number: M8010)
7. Magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) (Sangon Biotech, catalog number: A601336-0500)
8. 3',5'-Dimethoxy-4'-hydroxyacetophenone (acetosyringone; AS) (Sigma-Aldrich, catalog number: 2478-38-8)
9. Rifampicin (Sangon Biotech, catalog number: A600812-0025)
10. Gentamycin (Sangon Biotech, catalog number: A620217-0005)
11. Spectinomycin dihydrochloride pentahydrate (Sangon Biotech, catalog number: A600901-0005)
12. Kanamycin sulfate (Sangon Biotech, catalog number: A600286-0005)
13. Dimethyl sulfoxide (DMSO) (Solarbio, catalog number: D8370)
14. Potassium hydroxide (KOH) (Sangon Biotech, catalog number: A610441-0500)

Solutions

1. Antibiotic solution (see Recipes)
2. LB medium (see Recipes)
3. *Agrobacterium* infiltration buffer (see Recipes)

Recipes

1. Antibiotic solution

Prepare a stock solution of gentamycin (50 mg/mL), spectinomycin dihydrochloride pentahydrate (50 mg/mL), and kanamycin sulfate (50 mg/mL) in sterile distilled water and rifampicin (25 mg/mL) in DMSO. Store at -20°C . Dilute $1,000\times$ and add to the medium.

Note: Depends on the construct and Agrobacterium strain. In this experiment, the GV3101 (pMP90) strain was used, and the nuclear gene contained the screening tag, rifampicin resistance gene, and the Ti plasmid pMP90, which conferred gentamycin resistance to the GV3101 strain. Also, different constructs have different screening tags; for example, the main construct used in this experiment is pGWB505, which has a spectinomycin dihydrochloride pentahydrate resistance. Therefore, media containing different antibiotics was used to grow the Agrobacterium needed for the experiment.

2. LB medium

Tryptone 10 g

NaCl 5 g

Yeast extract 5 g

Adjust pH to 7.5 with KOH and add sterile deionized water to 1 L. To prepare solid medium, add 15 g of agar to 1 L of medium and autoclave.

3. *Agrobacterium* infiltration buffer

1 M MES 100 μL

1 M MgCl_2 100 μL

150 mM AS 10 μL

Add 9.790 mL of sterile deionized water to reach 10 mL.

Adjust MES to pH 5.7 with KOH.

Note: Dissolve MES and MgCl_2 in sterile water and filter the solutions with a $0.22\ \mu\text{m}$ syringe filter to avoid contamination. Store the solution at 4°C . Dissolve AS in DMSO and store the stock solution at -20°C .

Laboratory supplies

1. Soil mix or substrate (potting soil and vermiculite)
2. Plastic cups (8 cm diameter and 10 cm height)
3. Plant trays
4. Syringes (1 mL)
5. Tissue paper
6. Petri dishes (60 mm diameter)
7. 1.5 and 2.0 mL tubes (BBI, catalog number: F600620-0001)
8. Pipette tips (AIBIO, catalog number: T1040000)
9. Medical rubber gloves
10. Metal beads (3 mm)

Equipment

1. Plant growth chamber at 24 °C under a light intensity of 150 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and a 16/8 h light/dark photoperiod
2. Spectrophotometer capable of OD₆₀₀ measurements (e.g., Shimadzu, catalog number: 206-67001-34)
3. Petri dish incubator at 28°C (Panasonic, model: MIR-262-PC)
4. Handheld UV lamp (BLAK-RAY B-100AP LAMP, UVP, USA)
5. Digital camera (Canon, model: EOS 200D II)

Software and datasets

1. ImageJ (<https://fiji.sc/>)
2. GraphPad Prism 8 (<http://www.graphpad.com>)

Procedure

A. Plant growth conditions (Figure 1)

1. Sow *N. benthamiana* seeds in a 2:5 mix of potting soil and vermiculite and place them in a growth chamber at 24 °C under a 16/8 h light/dark photoperiod. Water plants every 3–4 days (2 L per tray with 18 cups).
2. After 7 days, keep a well-growing seedling in each plastic cup and allow it to grow under the same growth conditions until it is 4–5 weeks old.

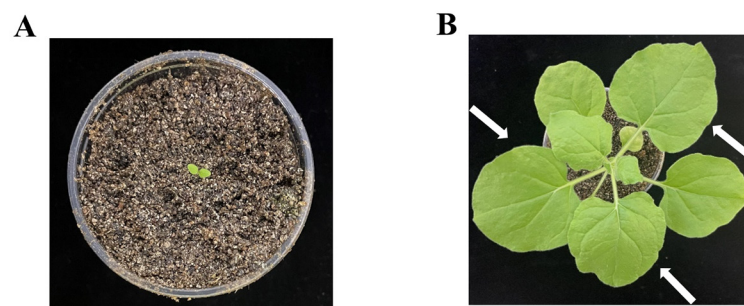


Figure 1. Growth of *N. benthamiana* seedlings in soil. (A) Germination of *N. benthamiana* seeds in a 2:5 mixture of standard potting soil and vermiculite. (B) Four-week-old *N. benthamiana* plant is ready for assay. White arrows indicate leaves suitable for infiltration.

B. Preparation of *Agrobacterium* cultures (Figure 2)

1. Separately culture the *A. tumefaciens* GV3101 (pMP90) carrying the TMV-GFP empty vector, the effectors to be tested (we use an effector that activates plant immunity from Cao et al. [12] as an example), or the mock (LTI6b, a non-immune-related control protein as negative control) on the plates containing the corresponding antibiotics at 28 °C.
2. Two days later, scrape the fresh *Agrobacterium* cells from the plates (or use a singly colony liquid culture), suspend, and adjust to specific concentrations ($OD_{600} = 0.4$ for TMV-GFP and $OD_{600} = 0.8$ for LTI6b or effectors).
3. Mix the TMV-GFP suspension with LTI6b or effector suspension in equal volumes and incubate them in a 28 °C incubator for 2–4 h.

Note: The total volume of the mix suspension is determined by the needs of the experiment; just pay attention to the accuracy of OD_{600} before mixing.

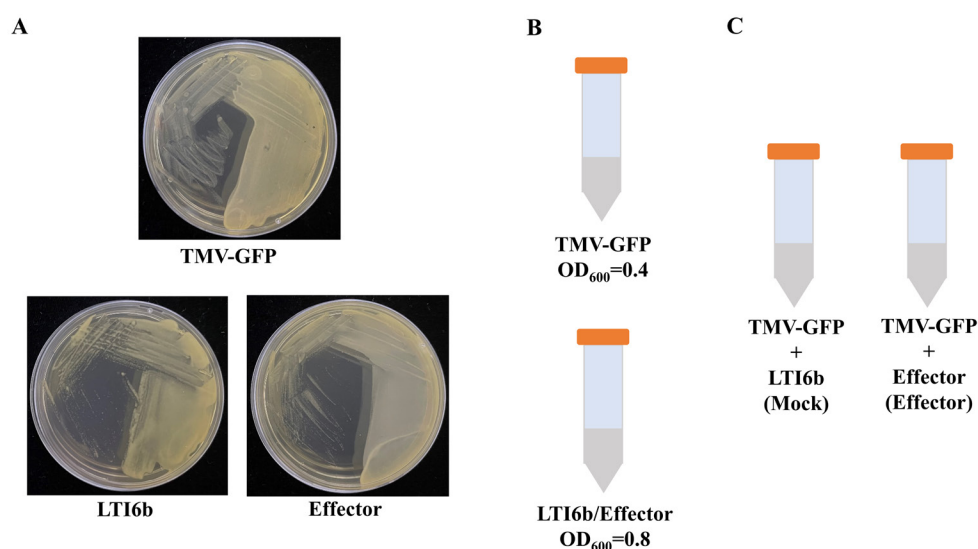


Figure 2. *Agrobacterium* culture and collection. (A) *Agrobacterium* growing on solid LB medium. (B) *Agrobacterium* suspension in infiltration buffer. (C) Mix the final *Agrobacterium* suspension to $OD_{600} = 0.2$ for TMV-GFP and $OD_{600} = 0.4$ for LTI6b or effectors.

C. Infiltration of *N. benthamiana* leaves (Figure 3)

1. Using a sterilized needle, gently score a minuscule wound on either side (left and right) of the leaf's lower epidermis without penetrating through the entire leaf.
2. On the upper surface of the leaf, label "Mock" on the left side and "Effector" on the right side using a non-toxic, water-resistant marker.
3. Prepare syringes without needles for infiltrating the *Agrobacterium* suspension.
4. Inject 100 μ L of the respective *Agrobacterium* suspensions—Mock and Effector—into the corresponding micro-wounds of the marked areas from the lower leaf surface.
5. Allow the plants to grow under the same growth conditions for 72 h.

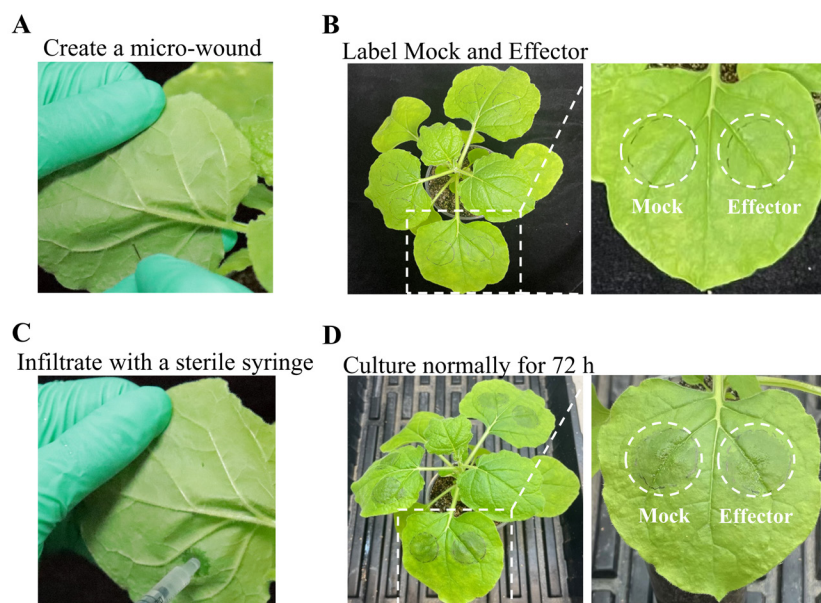


Figure 3. Illustration of the infiltration step. (A) On the abaxial side of the leaf, prick softly with a needle. (B) Using a marker pen, draw a circle on each half of the leaf to control the area for infiltration. (C) Infiltrate the "Mock" and the "Effector" *Agrobacterium* suspension into the marked area correspondingly. (D) Allow the plants to grow for 72 h.

D. Image capture (Figure 4)

1. Warm up the handheld UV lamp for 2 min to reach its peak luminosity.
2. Adjust the camera settings (Canon EOS 200D II) to optimize the photographic capture.
 - a. Set the focal length to 25 mm with an aperture range of F1.6 to F4.0.
 - b. Set the shutter speed to 1/15 s and adjust the ISO sensitivity between 3,200 and 6,400 to accommodate low-light conditions.

Note: These settings were carefully chosen to maximize light capture in the dark room while balancing exposure and maintaining image sharpness.

3. Excise the leaf sample approximately 1–2 cm from the petiole, gently tap it to remove any debris, and then lay it flat on a desk with a black background cloth.
4. Position the UV light source 10 cm above the leaf at a 45° angle laterally oblique to the leaf, ensuring that

the light is precisely focused on the center of the leaf area.

5. Align the camera lens perpendicularly to the leaf, maintaining a distance of 10 cm, and take the photo.

Note: Minor adjustments to the parameters mentioned above should be made as necessary to achieve a clear and detailed image of the leaf.

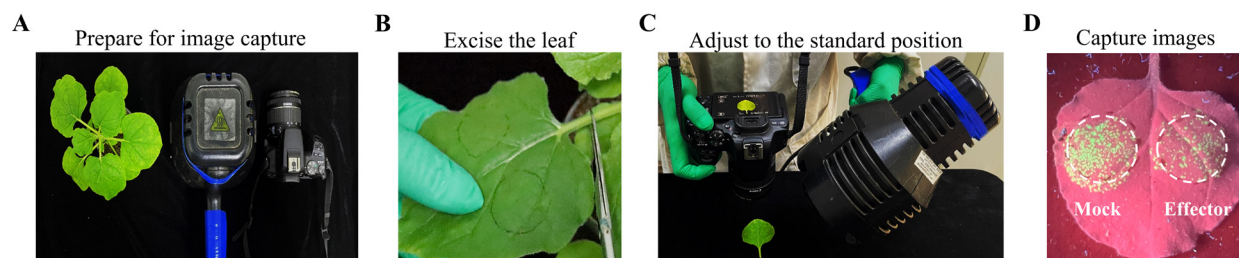


Figure 4. Images are captured after 72 h under a UV lamp in a dark room to monitor GFP expression. (A) Prepare to capture images after 72 h. (B) Excise the leaf sample from the petiole. (C) Move the camera or UV lamp to a position suitable for image capture. (D) Capture images under UV light. All of the above steps are performed in a dark room.

Data analysis

Proceed with the following steps for quantification of GFP intensity in images obtained from the above experiments.

1. Open the image in ImageJ, navigate to *Image*, and then *Color*. Select *Channels Tool* and check the *Channel 1* checkbox.
 2. Click on *Image*, then *Color*, and select *Split Channels* to separate the image into red, green, and blue channels. Choose the green-channel image for GFP intensity quantification.
 3. Click on *Image*, *Adjust*, then *Threshold*. Select *Default* and *B&W* settings and ensure the *Dark background* checkbox is checked.
 4. Adjust the slide bars to optimize image brightness and eliminate background noise.
- Note: Precise adjustments are essential to accurately distinguish fluorescence intensity differences between the infected areas on the left (Mock) and right (Effector) sides of the leaf.*
5. Proceed to *Analysis*, *Set Measurement*, and check the boxes for *Area*, *Mean grey value*, *Integrated density*, and *Limit to threshold*.
 6. Click on *Analyze*, then *Tools*, followed by *ROI Manager* to open the ROI Manager.
 7. Select *Oval* shape and draw around the infected area on the left side of the leaf to encompass all signals as the region of interest (ROI). Add this ROI by clicking *Add* in the ROI Manager, then click *Rename* to label it as *Mock*, and finally click *OK*.
 8. Repeat step 7 to set the ROI on the right side of the leaf, using identical settings including the size of the circle. Rename this ROI as *Effector*.
 9. In the ROI manager, select both *Mock* and *Effector*, then click *Measure*.
 10. Repeat steps 1–9 for the remaining replicates and export the collected data.
 11. Normalize the data with Mock set to 1.
 12. Analyze the compiled data using GraphPad Prism 8 software for further statistical analysis and interpretation. To compare results individually, perform a Student's t-test to indicate the statistical significance of the

differences (Figure 5). Differences between the GFP intensity of Mock and Effector reflect the influence of the effector on plant immunity.

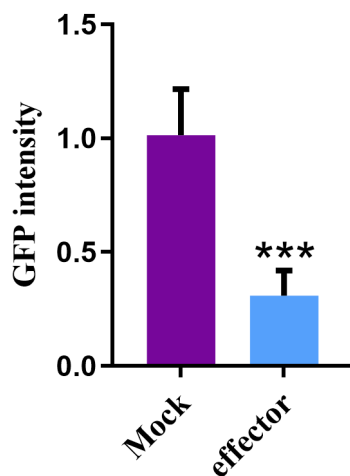


Figure 5. Effector function evaluation using the tobacco mosaic virus–green fluorescent protein (TMV-GFP) system. Values indicate the relative GFP intensity compared to the Mock. Data are represented as the mean ± SEM (n = 9, *** $P \leq 0.001$).

Validation of protocol

This protocol or its parts have been used in the following research articles:

- Cao et al. [12]. A robust high-throughput functional screening assay for plant pathogen effectors using the TMV-GFP vector. *The Plant Journal*. (Figures 2, 3, 4, 5, 6, and 7)

General notes and troubleshooting

1. Caution: To use plant material efficiently, inject 2–3 sets of mock/effectors on a leaf during screening.
2. Pause point: The protocol can be paused after *Agrobacterium* resuspension.
3. Critical step: Ensure *Agrobacterium* suspension is at the correct OD₆₀₀ for efficient infiltration.

Acknowledgments

This work was supported by the National Key Research and Development Program of China (2022YFD1602000) and the National Natural Science Foundation of China (32272641; 32372483; 32302296). We acknowledge the previous work described in [12], *The Plant Journal*.

Competing interests

The authors declare no competing interests.

Received: December 30, 2024; Accepted: March 18, 2025; Available online: April 1, 2025; Published: April 20, 2025

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