Quantifying the Contribution to Virulence of Phytophthora infestans Effectors in Potato

Solanum tuberosum

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Chapter 14

Quantifying the Contribution to Virulence of *Phytophthora infestans* Effectors in Potato

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Abstract

Late blight in potato, caused by the oomycete *Phytophthora infestans*, is a devastating disease that significantly impacts potato production. For a proper understanding of disease development, it is important to understand the interaction between plant and pathogen at a molecular level. Like other pathogens, *P. infestans* secretes effector molecules, which can be recognized by receptors in the plant and trigger immunity. In addition, effectors from *P. infestans* have been identified to enhance disease development. Here, we describe an assay to investigate the role of effectors in virulence of *P. infestans* on potato. We combine agroinfiltration to transiently express effectors in potato with detached leaf assays to monitor disease development. This protocol makes it possible to conveniently quantify the effect of individual effectors on virulence of *P. infestans*. The identification of effectors with an important role in late blight development can help to design better strategies to control the disease.

Key words *Phytophthora infestans*, Late blight, Effectors, *Avr* genes, Agroinfiltration, Disease test, Detached leaf assay

1 Introduction

Late blight, caused by the oomycete *Phytophthora infestans*, is a devastating disease of potato. The disease led to the great famine in Ireland from 1845 to 1849, causing a significant decline in the Irish population due to starvation and many Irish immigrating to other parts of the world. Late blight remains an important and remerging threat to global potato production today [1, 2].

A detailed understanding of the interaction between the pathogen and the host plant is important for breeding for resistance to the disease [3]. Host susceptibility and pathogen virulence can be studied using different methods. Susceptibility can, for example, be evaluated under natural conditions by performing field trials. This will make sure that resistance is studied in a relevant context. However, disease development may vary largely because of

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environmental conditions and field trials are costly and labor intensive [4, 5]. Moreover, tight regulations are in place when genetically modified organisms (GMOs) are involved in the experiment [6]. As an alternative, disease tests can be performed in greenhouses or climate chambers under much more stable conditions [7]. This will ensure reproducibility and allows for performing experiments with GMOs under contained conditions [6]; however, it does require relatively expensive greenhouse facilities. A widely used and more high-throughput method for testing large numbers of plants and pathogen isolates is the detached leaf assay [4, 8]. Using this somewhat artificial system compared to testing in the field or in the greenhouse, *P. infestans* infection can be studied under standardized laboratory conditions.

During infection, pathogens secrete effectors, molecules that manipulate host cell structure and function [9]. As effectors are encoded by a pathogen genome but exert their function in another organism, in this case the host plant, they are beautiful examples of genes with an extended phenotype [10]. On the one hand, effectors facilitate infection, for example by evading recognition by the plant immune system. However, effectors can have dual roles, as they can be recognized by immune receptors from host or nonhost plants. Such recognition usually leads to a strong defense reaction called hypersensitive response (HR). This strong cell death reaction is believed to restrict pathogen growth to the site of infection and can result in immunity of the plant. HR has been used as a readout in an approach called *effectoromics*, where germplasm collections are screened with effectors to detect genotypes with receptors that recognize the effector [11]. The effectoromics approach can help to accelerate the subsequent mapping and cloning of *R* genes and other types of immune receptors [3, 12].

Effector molecules are central to understanding the antagonistic interplay between pathogens and their host plants. Most known effectors of *P. infestans* have traditionally been named based on their avirulence (Avr) activity on host plants with matching resistance (R) genes [3, 13], but in more recent years, also studies for virulence activity are underway. High-throughput screens with transiently expressed GFP-tagged effectors followed by inoculation with *P. infestans* have been reported to lead to enhanced colonization in the model plant *Nicotiana benthamiana* [14]. For some of these effectors, their role in virulence has been characterized in great detail. For example, AVR2 is exploiting cross talk between brassinosteroid signaling and innate immunity. AVR2 interacts with the kelch-repeat-containing phosphatase, BSL1, which is involved in growth-promoting brassinosteroid pathway of potato. Transient expression of *Avr2* compromises immune responses to elicitins of *P. infestans* in *N. benthamiana* and stable overexpression of *Avr2* in transgenic potato leads to reduced infection rates [15–17]. AVR3a in turn is manipulating the host ubiquitin proteasome system, by

stabilizing the ubiquitin E3 ligase CMPG1. AVR3a also suppresses elicitin-mediated cell death responses and is believed to promote virulence during the early biotrophic infection phase [18–20]. *P. infestans* is a rapidly evolving pathogen and genes encoding *Avr* genes are typically quickly modified or lost to avoid detection by the plant immune system [21]. We anticipate that it is important to understand the role of effectors in pathogen virulence, and by targeting effectors with an important (or essential) role in virulence, it might be possible to obtain a more durable resistance [3, 13].

We have developed an assay to investigate and quantify the role of individual effectors in virulence of *P. infestans* on potato. In this protocol, effectors are transiently expressed in potato leaves through *Agrobacterium tumefaciens*-mediated transient transformation (ATTA) and *P. infestans* is inoculated on the leaf panels where the effectors are produced. By comparing the effect of different effectors with negative controls, the role of individual effectors on virulence of *P. infestans* can be assessed. A better understanding of the role of effectors of *P. infestans* in late blight development might enable us to design better strategies to control the disease.

2 Materials

All materials can be prepared and stored at room temperature, except when otherwise indicated. All solutions can be prepared with double-demineralized water, except when otherwise indicated. All antibiotics should be stored at −20 °C. The appropriate rules for handling GMOs and GMO waste should be followed.

2.1 Potato Propagation

1. MS20 medium: Dissolve 4.45 g *Murashige-Skoog* (MS) salts (including vitamins) and 20 g sucrose in 900 mL of double-demineralized water (ddH_2O). Then adjust the pH to 5.8 using KOH and complete the volume to 1 L. Add 8 g micro agar and autoclave for 15 min at 121 °C.
2. Greenhouse: 8 × 8 × 8 cm pots and sterilized soil.

2.2 Phytophthora infestans Spore Production

1. Rye agar medium: Soak 60 g rye grain for 36 h in distilled water. Separate the grains and supernatant, and keep both fractions. Macerate the swollen grain fraction, extract at 50 °C in 200 ML ddH_2O, and filter. Add the supernatant into the filtrate, and make up to a final volume of 1 L with ddH_2O. Add 15 g/L agar and 20 g/L sucrose and autoclave for 15 min at 121 °C [22]. Pour into Petri dishes for culturing *Phytophthora* isolates.
2. Ice-cold water.

3. 50 mL tubes.
4. Plate spreader.
5. Hemocytometer.

2.3 Agroinfiltration

1. *Agrobacterium tumefaciens* isolate AGL1 [23, 24].
2. LB (Luria Bertani) broth: 10 g tryptone, 5 g yeast extract, and 5 g NaCl dissolved in 1 L ddH_2O. Autoclave for 15 min at 121 °C.
3. 200 mM acetosyringone (4-hydroxy-3,5-dimethoxyacetophenone) stock solution: 0.393 g acetosyringone dissolved in 10 mL dimethyl sulfoxide (DMSO), filter-sterilized.
4. 1 M 2-(*N*-morpholino)-ethane sulfonic acid (MES) stock solution: 10.66 g MES monohydrate dissolved in 50 mL ddH_2O, set the pH to 5.6 using KOH and filter sterilize (*see* **Note 1**).
5. Yeast extract broth (YEB): 5 g beef extract, 5 g bacteriological peptone, 5 g/L sucrose, 1 g/L yeast extract, 0.48 g/L $MgSO_4 \cdot 7H_2O$ dissolved in 1 L ddH_2O. Autoclave for 15 min at 121 °C.
6. MMA (MS-MES-acetosyringone) buffer: 20 g sucrose, 5 g MS20 salts without vitamins, and 1.95 g MES dissolved in 900 mL of ddH_2O, set the pH to 5.6 using HCl or KOH and add ddH_2O to obtain a final volume of 1 L. Add 1 mL/L 200 mM acetosyringone (*see* **Note 2**).
7. Antibiotics stock solutions (1,000× concentrated): 50 mg/mL kanamycin, 50 mg/mL spectinomycin, and 50 mg/mL carbenicillin in ddH_2O and filter sterilize, 50 mg/mL chloramphenicol in ethanol.
8. Plant expression vector that bears the effectors, and the empty vector negative control [25] (*see* **Note 3**).

2.4 Detached Leaf Assay

1. Plastic trays.
2. Laboratory filter paper (0.13 mm × 47 cm × 57 cm).
3. Floral foam.
4. Toothpicks.
5. Clear plastic bags.
6. Laundry clips or tape.

3 Methods

Open all autoclaved materials and perform all methods inside a down-flow/cross-flow cabinet, unless indicated differently. Follow GMO regulations carefully when performing agroinfiltration. The overview of the protocol is shown in Fig. 1.

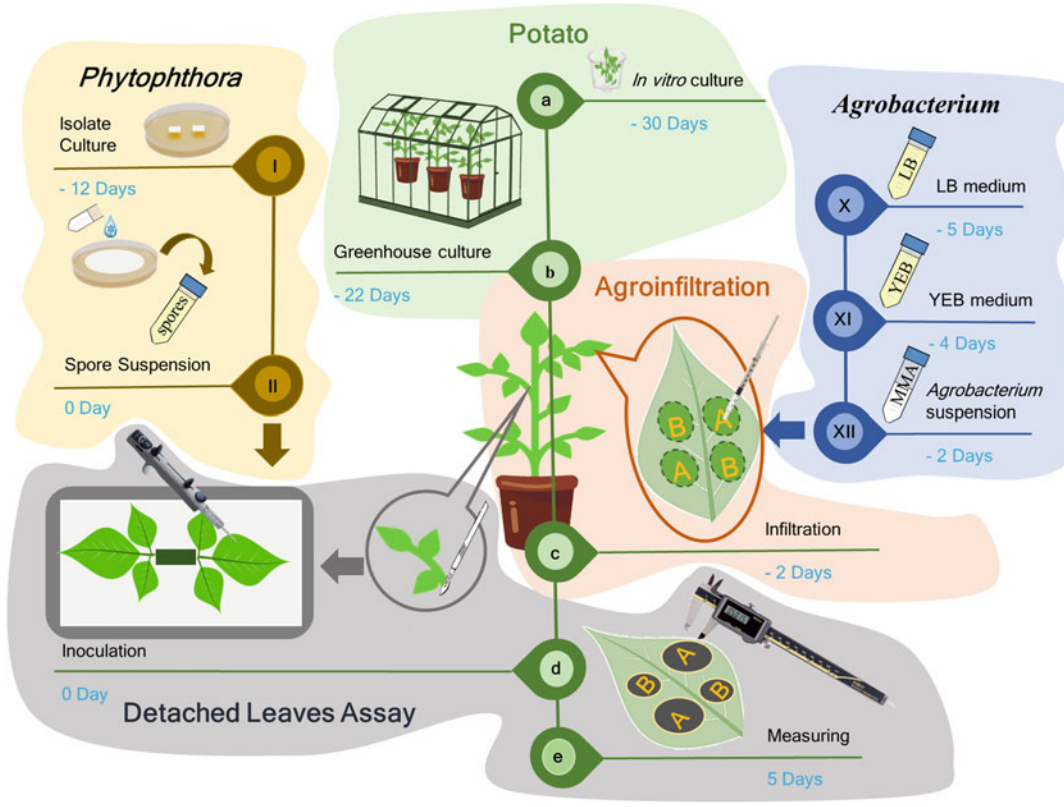

Fig. 1 Schematic overview of the protocol to investigate the effect of individual effector expression on virulence of *Phytophthora infestans* on potato. (**a**) Propagation of potato plants in vitro. (**b**) Growing plants in greenhouse. (**c**) Agroinfiltrated potato leaves. (**d**) Detached leaf assay. (**e**) Measuring lesion sizes of *P. infestans*. (**I**) Propagation of *P. infestans* on rye medium. (**II**) Preparation of spore suspension of *P. infestans*. (**X**) Growth of *Agrobacterium tumefaciens* containing the gene of interest construct in LB medium. (**XI**) Culturing *A. tumefaciens* in YEB medium. (**XII**) Preparation of *A. tumefaciens* suspension in MMA medium

3.1 Potato Propagation

1. Propagate shoots of in vitro plantlets on MS20 medium in sterile containers containing MS20 medium [26, 27].
2. Incubate shoots in a climate chamber at 18 °C with a 16 h/8 h day/night regime, for around 2 weeks until plantlets are well rooted.
3. Transplant the rooted in vitro plants into 8 × 8 × 8 cm pots containing sterile soil in a GMO-regulated greenhouse compartment for 3 weeks, at 18–22 °C with natural light complemented with artificial light under a 16 h/8 h day/night regime. Apply water and fertilizer according to normal practice and use biological pest control (*see* **Note 4**).

3.2 Phytophthora infestans Spore Production and Preparation of Spore Suspension

3.2.1 Spore Production

1. Culture the *P. infestans* isolates from glycerol stocks on fresh rye agar medium, and incubate at 18 °C in the dark for 14–21 days, as a starter plate for spore production.
2. Transfer agar plugs (~1 × 1 cm) with mycelium from the starter plate to a Petri dish containing fresh rye agar medium, and seal the lid with parafilm.
3. Incubate at 18 °C in the dark for 10–14 days prior to inoculation (*see* **Note 5**).

3.2.2 Preparation of Spore Suspension (the following steps do not need to be carried out under sterile conditions) (See **Note 6**)

1. Add 5 mL ice-cold water to a plate containing well-grown mycelium of *P. infestans* isolates (10–14 days old).
2. Rub the mycelium gently using a plate spreader.
3. Pour the sporangium suspension into a 50 mL tube and incubate at 4 °C for 1–3 h to release zoospores.
4. Adjust the zoospore concentration to 50,000 spores/mL using a hemocytometer. Zoospore counting method: pipet 20 μL zoospore suspension and apply to the hemocytometer. Count spores under a microscope using a 10 × 10 magnification and calculate the average number of spores (*see* **Note 7**).

3.3 Agroinfiltration

1. Prepare a tube with 5 mL LB medium supplemented with 5 μL of the appropriate antibiotics.
2. Inoculate LB with 20 μL glycerol stock (*see* **Note 8**) of the *Agrobacterium* strain containing the expression vector for the effector of interest and the empty vector (*see* **Note 9**).
3. Incubate the culture on a shaking incubator at 28 °C and 200 rpm for 1–2 days to grow a starter culture.
4. Prepare a 50 mL tube with 20 mL YEB medium complemented with 200 μL of 1 M MES buffer, 2 μL of 200 mM acetosyringone stock solution, and 20 μL of the appropriate antibiotics. Add 300 μL of the starter culture. Incubate the culture on a shaking incubator at 28 °C with 200 rpm for 24 h until the OD_{600} reaches about 1.0.
5. Collect the *Agrobacterium* cells by centrifuging the culture at 3,000 × *g* for 10 min.
6. Discard the supernatant, gently resuspend the pellet with MMA buffer (*see* **Note 2**), and adjust the OD_{600} to 0.2–0.3.
7. Incubate the *Agrobacterium* suspension at room temperature for 1 to maximum 6 h prior to infiltration.
8. Infiltrate potato leaves on the abaxial side (*see* **Note 10**) using a 1 mL syringe without needle.
9. Agroinfiltrate (*see* **Note 11**) two leaf panels with *Agrobacterium* containing the appropriate expression vector and two leaf panels with *Agrobacterium* containing the empty vector. To

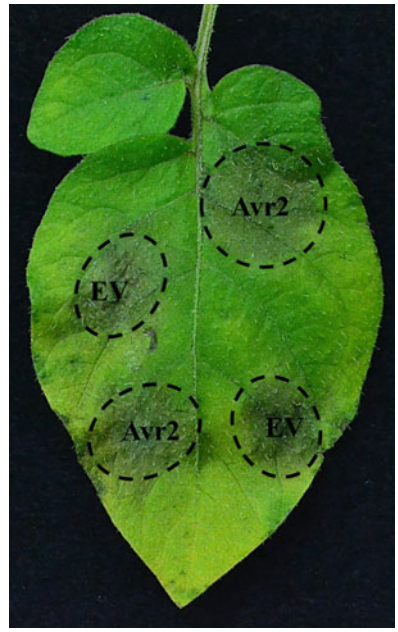

Fig. 2 Lesions from *Phytophthora infestans* isolate Dinteloord at 5 days postinoculation (dpi) on leaf panels treated with *Agrobacterium* expressing *Avr2* and EV (empty vector) in potato cultivar Bintje

exclude position effects, each treatment (effector and empty vector) should occur left and right from the vein as well as at top or bottom position (Figs. 1 and 2).

10. Use at least three fully expanded leaves from at least three plants of 2–3 weeks old (*see* **Note 12**).

3.4 Detached Leaf Assay

3.4.1 Preparation of Leaves

1. Collect three agroinfiltrated leaves at 48 h post-agroinfiltration, using a sharp knife or scissors. Keep the leaves moist by putting them on wet paper inside a plastic bag.
2. Soak floral foam in tap water for 15–20 min until well saturated. Cut into blocks of approximately 5 × 5 × 5 cm.
3. Place two layers of wet filter paper in plastic trays.
4. Cut the end of the petiole of the leaves using a sharp knife. In each block of floral foam, insert two leaves on opposite sides of the block, with the abaxial side facing upward. Place the floral foam containing the leaves into the tray. Each tray can usually accommodate three blocks of floral foam (*see* **Note 13** and Fig. 3).

3.4.2 Inoculation

1. Inoculate the abaxial side of the leaves at the center of each agroinfiltrated leaf panel with 10 μL suspension of *P. infestans* spores (*see* **Note 14**).

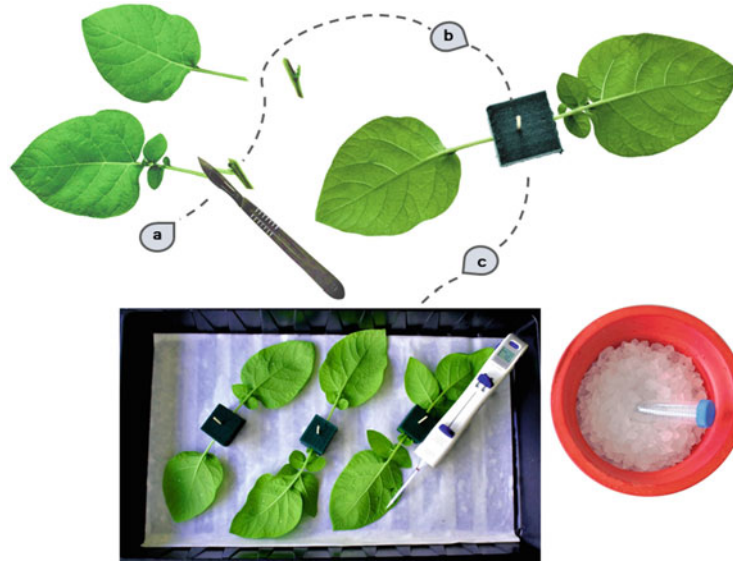

Fig. 3 Leaf preparation. (**a**) Potato leaves are collected, and petioles are cut with a sharp knife. (**b**) The leaves are inserted into blocks of wetted floral foam, and (**c**) placed in a tray with wetted filter paper. The leaves are now ready for inoculation with zoospore suspensions (kept on ice)

2. Put the trays in transparent plastic bags and close the bags using laundry clips or tape to keep relative humidity (RH) high.
3. Incubate the trays in a climate chamber at 18 °C and RH >95% under long-day conditions (16 h/8 h day/night regime) under fluorescent light tubes for 4–6 days (*see* **Note 15**).

3.4.3 Scoring

1. Determine the lesion sizes using a digital caliper (*see* **Note 16**).

4 Notes

1. To reach the final concentration in 50 mL, first add 10.66 g MES monohydrate to 25 mL of ddH_2O, add 5 mL KOH, and adjust pH to 5.6. Bring to a final volume of 50 mL with ddH_2O.
2. MMA buffer should be prepared fresh on the same day as the agroinfiltration. Add the acetosyringone after adjusting the pH.
3. We use pK7WG2 as plant expression vector; source and details can be found at https://gatewayvectors.vib.be/collection/pk7wg2 [28].
4. To avoid the risk that pesticides/fungicides can interfere with *P. infestans* infection, biological pest control is used. We use Thripex (contains predatory mites (*Neoseiulus cucumeris*)) to

control common greenhouse pests such as thrips and mites; source and details can be found at https://www.koppert.nl.

5. The duration of the incubation depends on the *P. infestans* isolate. For instance, *P. infestans* isolate Dinteloord will cover the entire Petri dish with white fluffy mycelium in about 10 days, whereas isolate Katshaar will need 14 days to cover the plate.
6. The zoospore suspension is freshly made on the same day as the detached leaf assay. Zoospores that are actively moving are preferred, as we found that these are more virulent.
7. Take sporangiospores into account when counting zoospores; they can be counted as zoospores.
8. The glycerol stocks are stored at −20 °C. Either defrost them on ice and take 20 μL or keep them in liquid nitrogen and take a small scoop from the frozen glycerol stock.
9. Always include a culture of *Agrobacterium* with empty vector as a negative control.
10. Choose young, healthy, and fully expanded leaves for agroinfiltration. These are usually leaves 2–4 (counted from the top) at the middle part of the plant. If the plants have more than one leaflet, choose the main leaflet.
11. Use eye protection during the agroinfiltration. To avoid cross contamination, always change gloves or sterilize using 70% ethanol when infiltrating different effector treatments.
12. Avoid watering the plants after agroinfiltration, especially using a water sprayer, to avoid cross contamination.
13. The number of floral foam blocks per tray depends on the size of the leaves. For medium-sized leaves, such as those from potato cultivar Bintje, two leaves fit per floral foam block, and six leaves fit in a tray.
14. One agroinfiltrated leaf panel can accommodate a 10 μL droplet of spore suspension from a single isolate (use different leaves for different isolates).
15. The length of the incubation is dependent on the aggressiveness of the inoculated *P. infestans* isolates. The more aggressive, the shorter the incubation time. For instance, the aggressive *P. infestans* isolate Dinteloord takes 5 days to cover the entire leaf of potato cultivar Bintje and the lesions from different inoculated spots will start to merge. In comparison, *P. infestans* isolate Katshaar will take 6 days on this cultivar.
16. The development of *P. infestans* is determined by measuring the lesion sizes before the lesions will start overlapping (*see* **Note 14**).

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