Video Article

Assay for Pathogen-Associated Molecular Pattern (PAMP)-Triggered Immunity (PTI) in Plants

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Abstract

To perceive potential pathogens in their environment, plants use pattern recognition receptors (PRRs) present on their plasma membranes. PRRs recognize conserved microbial features called pathogen-associated molecular patterns (PAMPs) and this detection leads to PAMP-triggered immunity (PTI), which effectively prevents colonization of plant tissues by non-pathogens[1,2]. The most well studied system in PTI is the *FLS2*-dependent pathway[3]. FLS2 recognizes the PAMP flg22 that is a component of bacterial flagellin.

Successful pathogens possess virulence factors or effectors that can suppress PTI and allow the pathogen to cause disease[1]. Some plants in turn possess resistance genes that detect effectors or their activity, which leads to effector-triggered immunity (ETI)[2].

We describe a cell death-based assay for PTI modified from Oh and Collmer[4]. The assay was standardized in *N. benthamiana*, which is being used increasingly as a model system for the study of plant-pathogen interactions[5]. PTI is induced by infiltration of a non-pathogenic bacterial strain into leaves. Seven hours later, a bacterial strain that either causes disease or which activates ETI is infiltrated into an area overlapping the original infiltration zone. PTI induced by the first infiltration is able to delay or prevent the appearance of cell death due to the second challenge infiltration. Conversely, the appearance of cell death in the overlapping area of inoculation indicates a breakdown of PTI.

Four different combinations of inducers of PTI and challenge inoculations were standardized (Table 1). The assay was tested on non-silenced *N. benthamiana* plants that served as the control and plants silenced for *FLS2* that were predicted to be compromised in their ability to develop PTI.

Video Link

The video component of this article can be found at https://www.jove.com/video/1442/

Protocol

Part 1: Plant growth and maintenance

Nicotiana benthamiana plants used in the assay should be about 7 weeks old. They should be trimmed at least 4-5 days prior to the assay to remove all axillary branches and flowers. It is a good idea to remove axillary branches soon after they emerge in order to make the plants more manageable.

Part 2: Bacterial culture

DAY 1:

1. Plates or cultures for all the strains used in the assay are initiated at the same time. For the *Pseudomonas* spp. which include 2 inducers and 3 challenge strains, streak bacteria onto KBM plates containing the appropriate antibiotics from frozen glycerol stocks (Table 2). A small amount of bacteria should be spread on the center of the plate. Incubate the plate at 30°C for about 18-24 hours. For *Agrobacterium*, start a liquid culture in 2 ml LB from a fresh plate and grow overnight at 250 r.p.m., 30°C.

DAY 2:

2. For *Pseudomonas*, add 150-200 µl of liquid KBM to the bacteria and spread them over the entire plate using a sterile glass spreader. Put the plate back in the incubator and let it grow for another 20-24 hours. There should be a bacterial lawn on the plate the next day, indicating good

growth. For *Agrobacterium*, start a secondary culture in about 5-10 ml LB with 20 µM acetosyringone and let it grow overnight to 20 hours at 250 r.p.m., 30°C.

Part 3: Preparing plants for the assay

DAY 2:

1. One day before the assay the plants should be moved to a room at 22-24°C, ~35-40% RH and constant light.
2. Mark circles on leaves that are at least 1.5 cm in diameter using a thick black marker. Two to four circles can be marked per leaf. The circles should be well spaced and preferably not cross a large vein. Choose well-expanded leaves. Avoid older leaves or leaves that are tough or thick to the touch and avoid marking circles on the lower portions of the leaf.

Marking circles a day prior to the experiment is not essential to the protocol. However, it saves time if there are large numbers of plants being assayed, and makes it easier to achieve precise timing between the induction of PTI and challenge inoculations.

Part 4: Induction of PTI

DAY 3:

1. Harvest the cells from the plate in 10 mM $MgCl_2$ for *P. fluorescens* and *P. putida*. For *Agrobacterium*, centrifuge the culture to collect cells and resuspend in 10mM $MgCl_2$ + 10 mM MES pH 5.6. Repeat the centrifugation and resuspend in the $MgCl_2$ -MES solution. Measure the optical density (O.D.) of the cells at 600 nM. Adjust the O.D.s to the final required values as described in Table 2. *Pseudomonas* should be finally resuspended in 10 mM $MgCl_2$ and *Agrobacterium* in the $MgCl_2$ –MES solution. A total volume of 25 ml is sufficient to inoculate about 40-50 spots.
2. Infiltrate the PTI inducers into the pre-marked circles on the leaves using a 1 ml needleless syringe. Write down the order in which the plants were infiltrated and the exact time when the infiltration was begun.

Part 5: Challenge inoculation

1. Prepare *P. syringae* pv. *tomato* DC3000, *P.s.t.* DC3000 Δ*hopQ1-1* and *P.s.* pv. *tabaci* for the challenge as described in part 4.1 and Table 2.
2. Seven hours after the inducer infiltration, perform the challenge infiltration in the same order of plants as the induction was done. Generally, a point on the periphery of the first inoculation circle can be used as the center of the second inoculation circle. If there are multiple spots on a leaf then make sure the infiltrations do not overlap.
3. Plate serial dilutions of all the cultures used in the assay on KBM or LB plates containing the appropriate antibiotics to determine the exact C.F.U. number.

Part 6: Scoring for breakdown of PTI

Day 5:

1. Look for the appearance of cell death due to ETI in the spots that were challenged with *P.s.t.* DC3000. Cell death inside the overlapping area of infiltration indicates a breakdown of PTI and should be scored as a positive phenotype.

Day 7:

2. Look for the appearance of cell death due to disease in the spots that were challenged with *P.s.t.* DC3000 Δ*hopQ1-1* or *P.s.* pv. *tabaci*. Score for a positive phenotype in the same manner as described in part 6.1.

When the control plants (that should not be compromised for PTI) begin to show cell death in the overlapping area of infiltration, stop making any further observations.

Part 7: Representative results

Figure 1 shows the outcome of an assay when *P. fluorescens* was used as the inducer and *P.s.t.* DC3000 as the challenge.

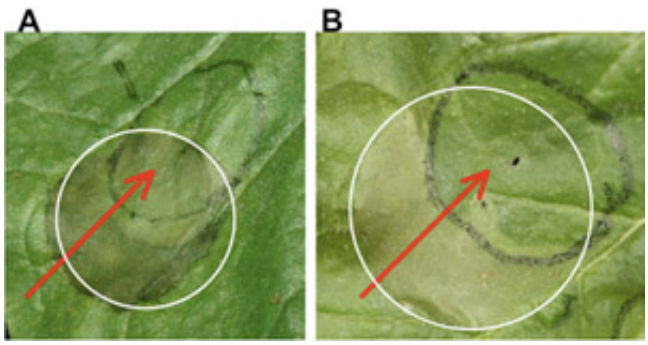

Figure 1. *P. fluorescens* was infiltrated onto *N. benthamiana* leaves (black circle) to induce PTI and 7 hours later, the spot was challenged with *P.s.t* DC3000 (white circle). Plants silenced for *FLS2* showed a breakdown of PTI in the region where *P. fluorescens* was infiltrated (A), as seen by cell death. Control plants that were not silenced showed no cell death in the overlapping area due to induction of PTI (B). Red arrows indicate lack of or presence of cell death in the overlapping area of infiltration. Photographs were taken 2 days after the infiltrations. Please click here to see a larger version of figure 1.

PTI inducer	Cell death-eliciting challenge	Nature of cell death	Code
Pseudomonas fluorescens 55	*Pseudomonas syringae* pv *tomato* (*P.s.t.*) DC3000[6]	ETI	Pf/DC
P. putida KT2440	*P.s.t.* DC3000	ETI	Pp/DC
P. fluorescens 55	*P.s.t.* DC3000 Δ*hopQ1-1*[6]	Disease	Pf/Q1-1
Agrobacterium tumefaciens GV2260	*P. syringae* pv. tabaci 11528R	Disease	Agro/Ptab

Table 1: Combinations of PTI inducing and cell death-eliciting microbes used in the cell death assay for PTI.

Bacterial strain	Selection medium	Final O.D. used in experiment	Corresponding C.F.U./ml
*P. fluorescens*55	KBM Ampicillin (100 ug/ml)	0.5	1×10^9
P. putida KT2440	KBM Ampicillin (100 ug/ml)	0.5	1×10^8
A. tumefaciens GV2260	LB Rifampicin (100 ug/ml)	0.5	5×10^8
P.s.t. DC3000	KBM Rifampicin (100 ug/ml)	0.02	2×10^7
P.s.t. DC3000 Δ*hopQ1-1*	KBM Rifampicin (100 ug/ml)	100 fold dilution of 0.1	1×10^6
P. syringae pv. tabaci 11528R	KBM Rifampicin (100 ug/ml)	100 fold dilution of 0.1	1×10^6

Table 2: Culture conditions and inoculum levels used in the assay. The O.D. and corresponding C.F.U. levels may vary between different brand of spectrophotometers.

Discussion

The cell death-based assay can be used to determine the involvement of a gene in PTI. For instance, loss-of-function mutants for a gene of interest can be tested using this assay. Out of the 4 combinations of inducer and challenge inoculations given in Table 1, it is possible that only one combination may result in a phenotype in your plant background. We have observed that plants silenced for *FLS2* show a breakdown of PTI in the Pf/DC and Pf/Q1-1 combinations of inducer and challenge inoculations (Table 1).

It is essential that the environmental conditions for the assay are as close as possible to those described in Part 3.1. Low humidity causes cell death to proceed too quickly, making the assay difficult to score. If the conditions described in our protocol do not work in your lab, then try altering the level of inducer or challenge inoculation. Another parameter that can be modified is the time gap between induction and challenge, although we have found that shorter time gaps caused the assay to break down too quickly in control plants.

We recommend that at least 4-5 plants be tested in an experiment and a positive phenotype should be repeated in at least 3-4 independent experiments.

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