Video Article

# A Versatile Method for Mounting *Arabidopsis* Leaves for Intravital Time-lapse Imaging

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## Abstract

The plant immune response associated with a genome-wide transcriptional reprogramming is initiated at the site of infection. Thus, the immune response is regulated spatially and temporally. The use of a fluorescent gene under the control of an immunity-related promoter in combination with an automated fluorescence microscopy is a simple way to understand spatiotemporal regulation of plant immunity. In contrast to the root tissues that have been used for a number of various intravital fluorescent imaging experiments, there exist few fluorescent live-imaging examples for the leaf tissues that encounter an array of airborne microbial infections. Therefore, we developed a simple method to mount leaves of *Arabidopsis thaliana* plants for live-cell imaging over an extended period of time. We used transgenic *Arabidopsis* plants expressing the *yellow fluorescent protein* (YFP) genefused to the nuclear localization signal (NLS) under the control of the promoter of a defense-related marker gene, *Pathogenesis-Related 1* (*PR1*). We infiltrated a transgenic leaf with *Pseudomonas syringae* pv. *tomato* DC3000 (*avrRpt2*) strain (*Pst_a2*) and performed in vivo time-lapse imaging of the YFP signal for a total of 40 h using an automated fluorescence stereomicroscope. This method can be utilized not only for studies on plant immune responses but also for analyses of various developmental events and environmental responses occurring in leaf tissues.

## Video Link

The video component of this article can be found at https://www.jove.com/video/59147/

## Introduction

Plant immune response involves a dynamic transcriptional reprogramming regulated by multiple transcription factors as well as phytohormones[1]. The accumulation of transcriptome data provides opportunities for collecting information on the plant immune system: for example, the network structure of signaling cascades[2]. However, our knowledge of the spatial and temporal dynamism of plant immunity still remains limited[3,4,5].

In previous studies, spatiotemporal regulation of defense-related gene expression has been mostly analyzed using in situ hybridization and a β-glucuronidase (GUS) reporter assay[6,7,8]. These methods enable us to visualize the transcriptional activation of various genes of interest in situ. However, these procedures require chemical fixation of specimens, and thus result in the loss of all temporal information. Biological events, such as immunity, progress over time. The use of luciferase as a reporter has enabled the capture of temporal dynamics of the promoter of interest[3]. However, luciferase-based assay requires an expensive substrate and highly sensitive detectors. To increase our understanding of the spatiotemporal aspects of plant immune response using a simple procedure, we generated transgenic *Arabidopsis thaliana* plants expressing the *yellow fluorescent protein* (*YFP*) gene fused to the nuclear localization signal (YFP-NLS) under the control of the promoter of the defense-related marker gene, *Pathogenesis-Related 1* (*PR1*)[9]. We used chlorophyll autofluorescence, a marker of living cells, to capture the process of programmed cell death (PCD), which often occurs during effector-triggered immunity (ETI), a form of plant immunity induced by specific pathogen infection[9,10]. Monitoring the temporal dynamics of fluorescence signal intensity in freely moving objects, such as living *Arabidopsis* leaves, requires a complex image processing software either built in-house or available commercially. Alternatively, preventing specimens from moving is a simple method to solve the issue. Here, we developed a versatile and simple method for mounting living leaves of a transgenic *Arabidopsis* plant for long-term observation under an automated fluorescence stereomicroscope. The method allows us to capture the promoter dynamics within soil-grown intact plant leaves over a few days.

## Protocol

NOTE: It is important to prevent sleep movement of living leaf samples during time-lapse imaging. To minimize mechanical stress on leaves, gentle fixation of leaves is necessary. Only adequately prepared leaf samples produce time-lapse images suitable for various image analyses. A

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protocol using transgenic *Arabidopsis* plants expressing *YFP-NLS* fusion under the control of *PR1* gene promoter (pPR1-YFP-NLS plants) and *Pseudomonas syringae* pv. *tomato* DC3000 (*avrRpt2*) strain (*Pst_a2*) is described below as an example.

## 1. Preparation of plants and pathogens

1. Fill a plastic cell plug tray (**Table of Materials**) with autoclaved soil.
2. Sow one transgenic *Arabidopsis* seed per cell (**Figure 1A**).
3. Transfer the tray to a growth room maintained at 23 °C and grow the plants under continuous white light for 2-3 weeks.
4. Two days prior to pathogen inoculation, streak *Pseudomonas syringae* pv. *tomato* DC3000 *avrRpt2* strain (*Pst_a2*) from a glycerol stock onto NYG medium (5 g/L peptone, 3 g/L yeast extract, 20 mL/L glycerol, 15 g/L bacteriological agar, pH 7.0) containing 100 mg/L rifampicin and 50 mg/L kanamycin and incubate at 28 °C for 48 h.
5. Harvest the bacterial cells that appear on the surface of the medium using plastic tips, transfer them to a plastic tube containing 10 mM $MgCl_2$, and resuspend. Measure the optical density (OD) of the solution at 600 nm ($OD_{600}$). Adjust the final cell concentration of bacterial cells to $10^8$ colony formation units (CFU)/mL, which normally corresponds to $OD_{600}$ = 0.2[11].

## 2. Pathogen inoculation

1. Carefully cut out a cell plug containing a 2-3-week-old plant without damaging the plant. Set the cell in an empty cell plug tray (2 x 2 cells are sufficient) to maintain a good balance (**Figure 1B**).
2. Select a visibly healthy leaf for inoculation. Generally, the third, fourth, and fifth leaves (#3, #4, and #5, respectively, in **Figure 1C**) from the bottom of the plant are easy to handle. Use leaves at the same position in a set of experiments for better reproducibility. Water the soil holding the plant before inoculation for long-term time-lapse imaging.
3. Optionally, in the case of analyzing stress-responsive promoters such as *pPR1*, to ensure that plants are not naturally stressed, examine the leaves under a fluorescence stereomicroscope prior to pathogen inoculation to verify the absence of YFP signal. Exclude leaves showing YFP signal from the experiment.
4. Wear disposable latex gloves before infiltration to avoid direct contact with the pathogen. Using a 1 mL needleless plastic syringe, carefully infiltrate the abaxial side of the leaf with the bacterial suspension ($10^8$ CFU/mL)[11] (**Figure 1D**). Inoculation of a small portion on one-half of the leaf enables a good visualization of *pPR1* activity; the infiltrated area becomes visible as darker green in color compared with the remaining leaf. Be extremely careful not to cause any mechanical damage to the leaf during infiltration.
   NOTE: Make sure that all the intercellular spaces in the infiltrated area are completely (vertically) fulfilled with the pathogen suspension; otherwise, the PCD domain will be difficult to visualize under the fluorescent stereomicroscope. This can be simply confirmed by completion of dark greening in the infiltrated area.
5. Absorb the excess of bacterial suspension from the area surrounding the infiltrated section of the infiltrated leaf with a soft paper towel.

## 3. Mounting the inoculated leaf

1. Immediately after inoculation, fix a glass slide on the plastic tray using surgical tape (**Table of Materials**) such that the infiltrated leaf is located at the center of the glass slide. Ensure that the inoculated leaf blade is completely fitted within the glass slide (**Figure 2A**).
2. Prepare double-layered plastic tape (in the case of 0.2 mm thick tape, see **Table of Materials**) and cut it into two pieces (**Figure 2B**; pieces 1 and 2) to fit the spaces along the petiole of the infiltrated leaf. Arrange the length of these two pieces, indicated with a double-headed arrow in **Figure 2B**, to fit into the length of the double-headed arrow shown in **Figure 2A**.
   NOTE: Cutting out a corner from each of the two pieces helps avoid damage to the leaf blade (**Figure 2B**, arrowheads; also see step 3.4 below). Any kind of plastic tape with similar thickness is suitable for making a bridge over the petiole. The stiffness of plastic tape is important for easy handling during the procedure described below.
3. Using a pair of fine tweezers, stick tape pieces 1 and 2 on either side of the petiole such that the cut corners of each piece align with the base of the leaf blade (**Figure 2C**). Ensure that the tape pieces do not touch the petiole or leaf blade.
   NOTE: These double-layered plastic tape pieces at the base of the leaf blade act as spacers and prevent physical stress on the petiole during mounting.
4. Prepare an additional piece of double-layered plastic tape (**Figure 2B**, piece 3) to fit the size of the double-headed arrow in **Figure 2B** and stick it on top of the tape pieces 1 and 2 to form a bridge over the petiole (**Figure 2D,E**). Be extremely careful not to catch the petiole and leaf blade directly between the tape pieces at positions indicated with arrows in **Figure 2D,E**.
5. Gently stick a small piece of surgical tape (**Figure 2F**, piece 4) on the glass slide above the tip of the leaf blade so that the leaf blade is fixed very softly on the glass slide. **Only** firmly press down the part of surgical tape directly touching the glass slide (**Figure 2F**, area outlined with a dashed red line), **not** the other part overlying the leaf.
6. Gently stick another small piece of surgical tape (**Figure 2G**, piece 5) at the border of the petiole and plastic tape pieces (1, 2, and 3) so that the petiole is very softly fixed onto both the glass slide and plastic tape pieces. **Only** firmly attach the part of surgical tape directly touching the glass slide to the glass slide and plastic tape pieces (**Figure 2G**, area outlined with a dashed red line), **not** the other part overlying the petiole.
7. Prevent neighboring leaves from moving into the field of view of the microscope using 200 µL pipette tips (**Figure 2H**). Insert the pipette tips in the soil to gently hold the neighboring leaves away from the infiltrated leaf. Be careful not to insert the tips too deep in the soil to avoid possible root damage.
   NOTE: The prepared plant is now ready for fluorescence stereomicroscope imaging.

## 4. Microscopic time-lapse observation

1. Turn on the fluorescent stereomicroscope.

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NOTE: Here, an automated stereomicroscope equipped with a highly sensitive 1.4 megapixel monochrome digital camera in 12-bit mode is used (**Table of Materials**). The microscope should be placed in a dark room fitted with an air-conditioning unit or in a dark cabinet with adequate ventilation to maintain the room temperature at 23 °C during time-lapse imaging.

2. Set the plant in the space above under the objective lens of the stereomicroscope for imaging.
3. Set up the parameters for time-lapse imaging (see examples in **Table 1**). Make sure to program steps for light exposure during the interval period of time-lapse imaging since light has a major impact on plant immunity[12].
    1. Use the conventional YFP filter (excitation 500-520 nm; emission 540-580 nm) to visualize the YFP signal.
    2. Use the Texas Red (TXR) filter (excitation 540-580 nm; emission 610 nm long pass) to visualize chlorophyll autofluorescence so that the PCD domain is visible as a dark area (no autofluorescence) surrounded by YFP-positive cell layers[9] (**Figure 3A**).
    3. Use the conventional epi-bright field setup for additional light exposure steps.
    NOTE: Before the experiment, measure the power of epi-bright light source and adjust it to the level of own plant growth condition.
4. Run the time-lapse imaging program. For long-term observation over several days, consider watering the plant appropriately, for example, during the light exposure steps.
5. After image acquisition, omit extra channels used for light exposure in the intervals (corresponding to channels 3-8 in **Table 1**) from the data set. Analyze data with various methods, such as region-of-interest (ROI) analysis, using different image analysis software such as Fiji[9].

## Representative Results

Here, we used *Pst_a2*-induced ETI as an example for time-lapse imaging. Time-lapse data were obtained as a series of images, a few of which are shown in **Figure 3B**, and as a time-lapse movie (**Supplemental Movie 1**)[9]. In successful experiments using pPR1-YFP-NLS during *Pst_a2*-induced ETI, transient activation of *pPR1* was observed, as evident from YFP expressing foci, in several layers of cells surrounding the PCD domain (**Figure 3B**)[9]. The activation of *pPR1* in cells surrounding the PCD domain usually starts at approximately 5 hours post inoculation (hpi), peaks at approximately 12 hpi, and lasts up to 40 hpi (**Figure 3B**)[9]. Since images were acquired using an epi-fluorescent system, the YFP signals obtained here were generated from several adaxial cell layers, including epidermal as well as upper mesophyll cells.

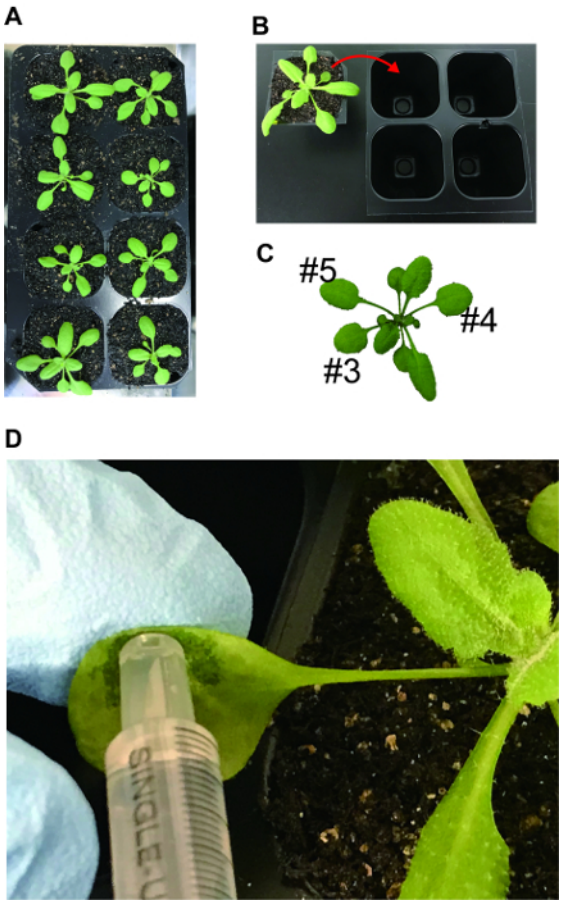



**Figure 1: Method used for infiltration of bacterial pathogen into the *Arabidopsis thaliana* leaf.** (**A**) Two- to three-week-old *Arabidopsis* plants were grown in a cell plug tray. (**B**) One-cell plug containing the selected plant to be used for inoculation and observation was cut out and placed into the empty cell plug tray to maintain balance. (**C**)The third, fourth, and fifth leaves (#3, #4, and #5, respectively) are suitable for image analysis. (**D**) Infiltration of the pathogen suspension into a selected leaf using a needleless syringe. The infiltrated area can be recognized based on its darker green color than the remaining leaf. Please click here to view a larger version of this figure.

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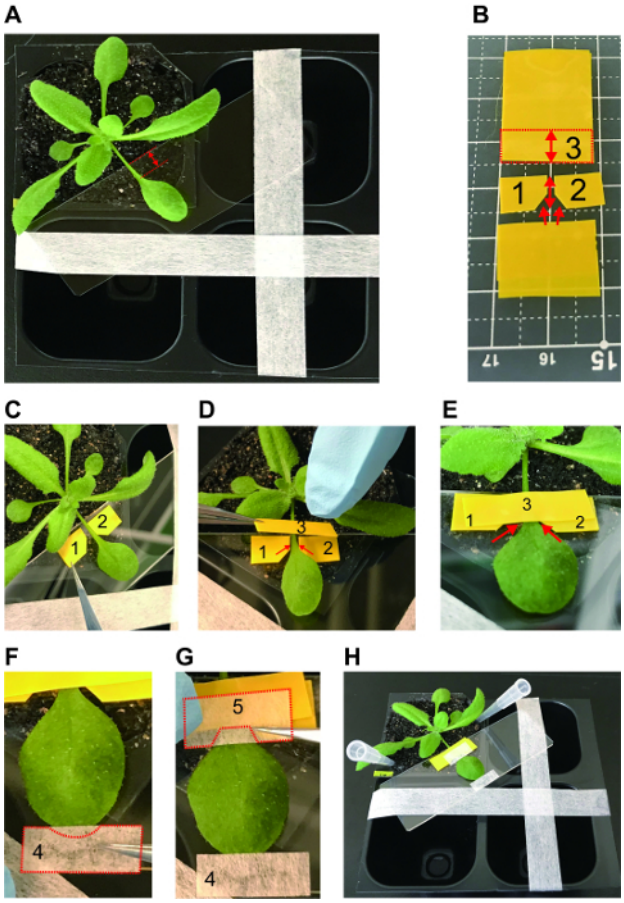



**Figure 2: Method used for mounting the infiltrated *Arabidopsis* leaf for time-lapse imaging.** (**A**) Photograph of the *Arabidopsis* plant with a glass slide fixed under the inoculated leaf. The infiltrated leaf was positioned at the center of the glass slide. The double-headed arrow indicates the length of the plastic tape spacers. (**B**) Preparation of plastic tape spacers and bridge. Double -layered plastic tape was cut into two pieces (1 and 2) for the spacers, and another piece (3, outlined with a dashed red line) was cut to prepare a bridge. The lengths of double-headed arrows are almost identical to the length of the double-headed arrow in (**A**). One of the four corners of pieces 1 and 2, indicated with arrows, were cut. (**C**) Two pieces of double-layered plastic tape (numbered as 1 and 2) were carefully pasted onto the slide glass along the petiole using a pair of fine tweezers, without making direct contact with the petiole and leaf blade. (**D**) An additional piece of double-layered plastic tape (numbered as 3), which was prepared from (**B**), was placed on both basal spacers (1 and 2) to form a bridge over the petiole, while ensuring not to catch the petiole between tapes 1 and 2 at positions indicated with arrows. (**E**) Photograph showing the taped petiole. It is important not to catch plant tissue directly between the plastic tape pieces at positions indicated with arrows. (**F**) A small piece of surgical tape (numbered as 4) was pasted gently on the glass slide around the tip of the leaf blade. Only the area outlined by the dashed red line was pressed firmly onto the glass slide. (**G**) Another small piece of surgical tape (numbered as 5) was gently pasted at the border of the petiole and plastic tape pieces. Only the area outlined by the dashed red line was pressed for fixing the petiole softly on the slide glass and plastic tape pieces. (**H**) Two disposable pipette tips were used to prevent neighboring leaves from moving into the field of view of the stereomicroscope. The pipette tips were inserted directly into the soil at appropriate positions. Please click here to view a larger version of this figure.

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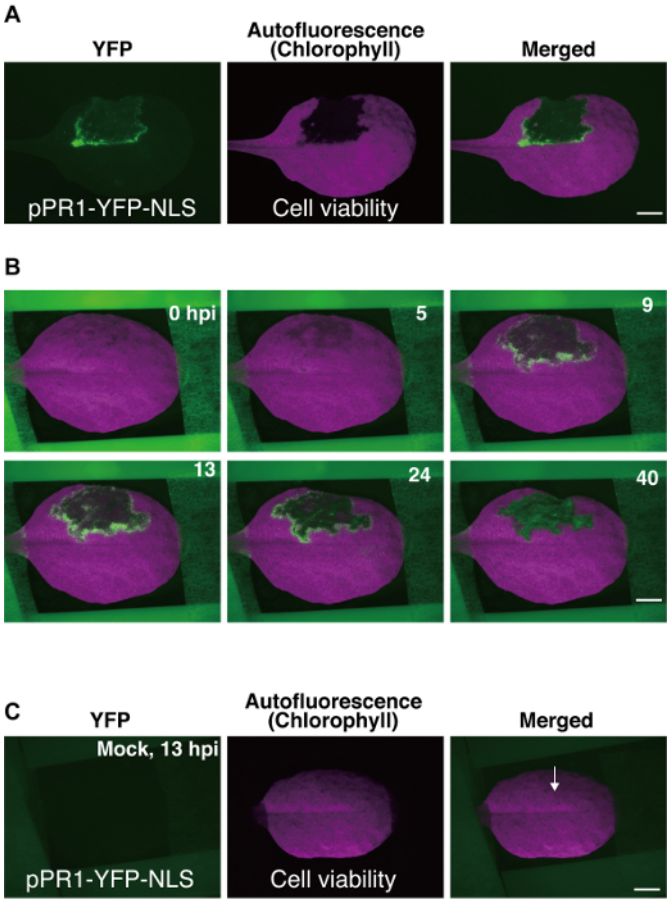


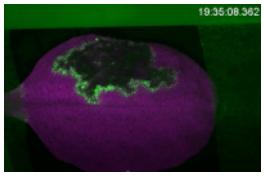

**Figure 3: Representative results using the *Arabidopsis* leaf mounting method.** (**A**) Fluorescent stereomicroscopic images of a transgenic *Arabidopsis* leaf (pPR1-YFP-NLS plant). A portion of the leaf was infiltrated with *Pst_a2* ($10^8$ CFU/mL) on its abaxial surface, and images were captured at 22 hours post inoculation (hpi). Nuclei in which the *pPR1* promoter was activated were detected using the YFP filter, and programmed cell death (PCD) was detected based on the loss of chlorophyll autofluorescence using the Texas Red (TXR) filter. Scale bar = 2.5 mm. (**B**) A few time-lapse images selected from a collection of 800 images obtained using the protocol described here. These data provide an *in vivo* overview of the spatiotemporal dynamics of *pPR1* activity for 40 hpi. Merged images of YFP and TXR images are shown. Scale bar = 2.5 mm. (**C**) Mock infiltration of a transgenic leaf (pPR1-YFP-NLS plant). In mock infiltration, 10 mM $MgCl_2$ was infiltrated into the abaxial side of the leaf, followed by time-lapse imaging. Mock treatment did not cause ectopic *pPR1* activity and did not interfere with chlorophyll autofluorescence at 13 hpi. An arrow indicates the position of mock infiltration. Scale bar = 2.5 mm**.** These images were modified from a previous study[9]. Please click here to view a larger version of this figure.

| Channel ID | Filter set | Duration of exposure | Usage |
|---|---|---|---|
| Channnel 1 | YFP | 5.0 s | YFP images |
| Channnel 2 | TXR | 10.0 ms | Chlorophyll autofluorescence images |
| Channnel 3 | Bright Field | 30.40 s | Interval light exposure |
| Channnel 4 | Bright Field | 30.40 s | Interval light exposure |
| Channnel 5 | Bright Field | 30.40 s | Interval light exposure |
| Channnel 6 | Bright Field | 30.40 s | Interval light exposure |
| Channnel 7 | Bright Field | 30.40 s | Interval light exposure |
| Channnel 8 | Bright Field | 30.0 ms | Bright field images |

Note: A maximum number of eight channels available in the system were used for running the time-lapse imaging program. The program was run at 3 min intervals for a total of 40 h (800 cycles). Maximum intensity of the mercuy lamp (**Table of Materials**) was used to capture live-cell images with YFP and TXR filters.

**Table 1:Time-lapse imaging program used in this study.**

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**Supplemental Movie 1: Time-lapse movie showing the spatiotemporal dynamics of *pPR1* activation in a transgenic *Arabidopsis* leaf following effector-triggered immunity (ETI) induced by *Pst_a2* inoculation.** A portion of the abaxial side of a transgenic leaf carrying the *pPR1-YFP-NLS* construct was infiltrated with *Pst_a2* ($10^8$ CFU/mL). Images were acquired at 3-min intervals for a total of 40 h. The time stamp shown is in the format dd:hh:mm:ss.sss. This movie was modified from a previous study[9]. Please click here to view this video. (Right-click to download.)

## Discussion

Here, we report a simple method to mount a living *Arabidopsis* leaf expressing a fluorescent reporter gene under the control of a promoter of interest for long-term observation using an automated fluorescent stereomicroscope. Time-lapse imaging of a fluorescent reporter has been frequently performed in root tissues; however, only a few similar studies have been conducted in leaf tissues. This is most likely because leaves are able to freely move in space, whereas roots are often buried and fixed in solid agar medium.

In this report, we focused on the spatiotemporal dynamics of *pPR1* activity during ETI induced by *Pst_a2*. In addition to the gentle fixation of the leaf detailed above, it is important to clearly visualize the spatiotemporal dynamics of cellular events such as promoter activation and PCD. If the distinction between cells showing pPR1 activity and PCD is not sharp, make sure that all of the intercellular spaces in the infiltrated area are completely filled with the pathogen suspension (see step 2.4). This is critical when using wide-field fluorescence stereomicroscopes since these microscopes capture all detectable signals along the same vertical position of the specimen. Chlorophyll autofluorescence from surviving cells above or beneath the cells in the PCD domain easily masks the dead cells exhibiting no autofluorescence. This is also true for YFP signal.

Conditions for time-lapse imaging need to be established carefully through several preliminary experiments under different experimental conditions. Parameters for time-lapse imaging depend on several factors such as the microscopic system, transgenic plants, and pathogens. To obtain these parameters, we first analyzed various exposure times for YFP signal intensity in the infiltrated leaf at 7 hpi, which almost coincides with the initial activation of *pPR1*. A 5 s exposure was determined as appropriate for capturing YFP signal with the stereomicroscope used in this study. A similar test was performed for imaging chlorophyll autofluorescence. Exposure of the specimen to light between 3 min intervals was programmed into the time-lapse imaging program as normal bright field imaging with maximum exposure time. Our system (**Table of Materials**) allowed us to have 2.5 min in addition to YFP, TXR, and bright field imaging. This constraint was the primary reason for choosing a 3 min interval. Next, we confirmed that this time-lapse condition caused no apparent damage to the plant samples, and did not induce ectopic light stress-related activation of *pPR1* (**Figure 3B,C**). This led to the development of the program used in this study. Thus, 3-min intervals of fluorescence imaging were deemed sufficient for capturing *pPR1* dynamics during *Pst_a2*-mediated ETI[9].

Promoter-reporter constructs, especially with the fluorescent reporter fused to the NLS, have been utilized by many groups, and are easily available from the research community; we used the construct published by Kubo *et al.*[13]. Thus, the protocol described here can be used in any plant biology study examining leaf tissues, if appropriate transgenic plants are available. Our simple and easy protocol provides a great opportunity for researchers who are keen to analyze the spatiotemporal dynamics of any biological event occurring in leaves, such as immune response. It is plausible that our method using tape pieces induces a slight physical stress on the specimens. However, this issue can be controlled by including appropriate positive and negative controls, such as mock treatments, in the experiments (**Figure 3C**). The experimental conditions can be further modified and optimized by analyzing these controls under different conditions.

In recent years, rapid development of imaging instruments and techniques has stimulated the interest of researchers in the complex spatiotemporal aspects of biological events. In any imaging analysis, appropriate mounting and fixing of specimens are among the most important issues. The simple and versatile method of mounting living *Arabidopsis* leaves developed in this study can be applied and optimized for various imaging experiments.

## Disclosures

The authors have nothing to disclose.

## Acknowledgments

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