Chapter 9

Visualizing the Movement of *Magnaporthe oryzae* Effector Proteins in Rice Cells During Infection

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Abstract

During biotrophy, filamentous pathogens such as the rice blast fungus *Magnaporthe oryzae* deliver effector proteins into live host cells to facilitate colonization. We describe three complementary assays for visualizing *M. oryzae* effector translocation into the rice cytoplasm and cell-to-cell movement during infection. Our assays make use of live-cell confocal imaging of optically clear rice sheath cells infected with transgenic strains of *M. oryzae* that express the fluorescent protein-tagged effector known as PWL2. We highlight several important considerations for the analysis of effector translocation and movement dynamics during infection of host plants.

Key words Biotrophic interfacial complex, Confocal imaging, Effector proteins, Fluorescent proteins, Host translocation assay, Live-cell imaging, *Magnaporthe oryzae*, *Oryza sativa*, Plant–pathogen interactions, Rice blast

1 Introduction

During biotrophic invasion of plant cells, filamentous pathogens such as fungi and oomycetes secrete effector proteins to suppress host immunity and facilitate colonization. Effectors are increasingly shown to be translocated into host cells across the host-derived membrane encasing effector-secreting cells such as haustoria or invasive hyphae and then move cell-to-cell in the host [1–3]. The mechanisms of effector translocation and cell-to-cell movement remain largely unknown. The ability to directly visualize effector movement and subcellular localizations can facilitate the investigation of such mechanisms and effector function.

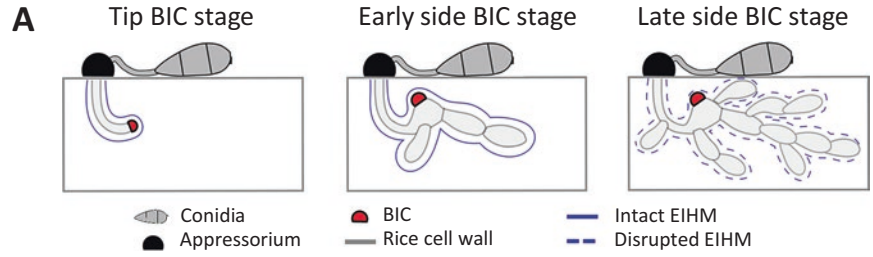
Magnaporthe oryzae is a hemibiotrophic filamentous fungus that causes the economically important blast disease on many crops such as rice and wheat. Using live-cell imaging of fluorescent protein (FP) reporters, subcellular localizations of several *M. oryzae* effectors with an N-terminal signal peptide have been

Wenbo Ma and Thomas Wolpert (eds.), *Plant Pathogenic Fungi and Oomycetes: Methods and Protocols*, Methods in Molecular Biology, vol. 1848, https://doi.org/10.1007/978-1-4939-8724-5_9,

visualized during fungal invasion of live rice cells [2, 4–8]. Apoplastic effectors such as BAS4 are localized in the *M. oryzae*-rice interfacial space enclosed by the tight-fitting plant-derived extra-invasive hyphal membrane (EIHM), whereas cytoplasmic effectors such as PWL2 enter the rice cytoplasm and move into adjacent cells. Cytoplasmic effectors preferentially accumulate at the biotrophic interfacial complex (BIC), a localized structure that has been hypothesized to be the site of effector translocation into host cells across the intact EIHM (Fig. 1a) [2, 4].

Here, we describe three complementary assays for visualizing *M. oryzae* effector movement during infection and discuss their advantages and potential limitations (Fig. 1b). All three assays make use of live-cell confocal imaging of optically clear rice sheath cells infected with transgenic strains of *M. oryzae* that express FP-tagged PWL2 proteins. In the first assay, mCherry-tagged PWL2 (PWL2:mCherry) is directly visualized in the host cytoplasm. In the second assay, the translocated PWL2:mCherry is more easily observed in the host cytoplasm after sucrose-induced plasmolysis. In the third assay, detection of translocated PWL2:mCherry is further improved by including a nuclear localization signal (NLS) to concentrate the proteins in the rice nucleus. Cell-to-cell movement of PWL2 can be detected in all three assays, particularly when NLS is included in the third assay.

There are a few points that are important to consider when studying translocation of FP-tagged effectors into host cells using live cell imaging. First, effector gene expression is coordinated with infection stages [1, 9], thus FP-tagged effectors should be expressed under the control of their native promoters. Second, to control for possible artifacts introduced by tagging, it is important to validate that FP-tagged effectors retain their biological function as shown for PWL2 and AVR-Pita1 [2]. For effectors whose functions are unknown, which is the case for most effectors, the localization of FP-tagged effectors must be interpreted with caution. Third, the EIHM loses integrity during infections, which results in leaking of EIHM matrix proteins into the host cytoplasm through the disrupted EIHM [2, 6, 10, 11]. Therefore, individual infection sites must be assessed for EIHM integrity when performing effector translocation studies. In our translocation assays, we introduced PWL2:mCherry or PWL2:mCherry:NLS constructs into an *M. oryzae* strain expressing the BAS4 signal peptide:EGFP fusion, which is secreted into the EIHM matrix, to monitor EIHM integrity. Exclusive localization of EGFP fluorescence around invasive hyphae indicates that the EIHM remains intact, whereas appearance of EGFP fluorescence in the host cytoplasm indicates that the EIHM has lost integrity [10].



B

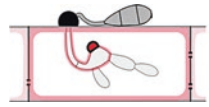
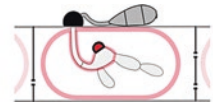
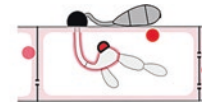
	Assay 1 	Assay 2 	Assay 3 
PWL2-associated mCherry localization	• Strongly at the BIC, weakly around the BIC-associated cell and absent from other invasive hyphae • In the cytoplasm of invaded and adjacent cells	• Strongly at the BIC, weakly around the BIC-associated cell and absent from other invasive hyphae • In the plasmolyzed cytoplasm of invaded and adjacent cells	• Strongly at the BIC, weakly around the BIC-associated cell and absent from other invasive hyphae • In nuclei of invaded and adjacent cells
Advantages	• Subcellular localization likely governed by the effector protein itself	• Easy to detect translocated effectors with host cytoplasm separated from cell walls • Plasmolyzed cells indicate live cells	• High sensitivity to detect effector translocation and cell-to-cell movement
Potential Limitations	• Not sensitive enough to detect effector translocation and cell-to-cell movement at low concentration • Possible confusion with cell wall autofluorescence	• Plasmolysis can cause structural alterations that may affect effector localization	• Endogenous effector localization not represented due to NLS-mediated nuclear targeting

Fig. 1 *M. oryzae* infection process and features of effector movement assays. (**a**) Schematic diagram showing hyphal growth, BIC development, and EIHM in the first-invaded rice cell. Tip BIC stage: A filamentous primary hypha with a tip BIC extends from the appressorium. The hypha is surrounded by an intact EIHM. Early side BIC stage: A few bulbous hyphal branches extend from the first bulbous invasive hyphal cell with a side BIC [2, 17]. Late side BIC stage: Invasive hyphae have branched to fill much of the space in the rice cell. The EIHM has disrupted, and the host cell may be dying or dead. (**b**) A table summarizing PWL2-associated mCherry localization as well as the advantages and potential limitations of each of the three effector movement assays

2 Materials

2.1 Cloning and Transformation

1. *Escherichia coli* competent cells and standard supplies for gene cloning.
2. *Agrobacterium tumefaciens* EHA105 competent cells.
3. *M. oryzae* strain CKF3816 (G418 resistance) expressing BAS4 signal peptide:EGFP fusion (secreted EGFP). CKF3816 is a transformant of *M. oryzae* wild-type strain O-137 [12].
4. Incubator(s) set at 24 °C and 37 °C.
5. Antibiotics (kanamycin for *E. coli* and *A. tumefaciens*; hygromycin for *M. oryzae*).
6. 24-well tissue culture plate containing V8 agar medium.

2.2 Plant and Fungal Materials and Inoculation

1. Rice (*Oryza sativa*) cultivar YT16: Grown in soil-filled pots in a growth chamber at 80% relative humidity with a 12/12 light/dark cycle at 28 °C during the day and 24 °C at night. Apply fertilizer (20-10-20 peatlite) once a week.
2. *M. oryzae* strain CKF3816: Grown on oatmeal agar (OMA [12]) in an incubator under constant light at 24 °C.
3. Adjustable pipettes and pipette tips (ranging 2–1000 μL).
4. Small scissors.
5. Miracloth.
6. Forceps.
7. Sterile distilled water.
8. 1.5 mL microcentrifuge tubes.
9. Hemacytometer.
10. Light microscope.
11. Humidity container (*see* **Note 1**).

2.3 Confocal Microscopy

1. Glass slides.
2. Coverslips 22 × 50 mm.
3. Forceps.
4. Razor blades (single edge).
5. Sterile distilled water.
6. 0.5 M sucrose.
7. Transfer pipettes.
8. Confocal microscope system. We use a Zeiss LSM 710 laser scanning confocal microscope and Zen software (Black edition) for image acquisition and analysis. However, the assay can be performed with most other confocal systems and epifluorescence microscopes (*see* **Note 2**).

3 Methods

3.1 Generate M. oryzae Transgenic Strains Expressing PWL2:mCherry or PWL2:mCherry:NLS

1. Generate PWL2:FP expression constructs by cloning the *PWL2* promoter and its entire 145-amino acid coding sequence fused to mCherry (*PWL2*: mCherry) or nuclear targeting mCherry (*PWL2*: mCherry:NLS) into the binary vector pBHt2 [13] (*see* **Note 3**).
2. Transform *M. oryzae* strain CKF3816 (G418 resistance) with each construct (hygromycin resistance) using *Agrobacterium*-mediated transformation [14]. CKF3816 expresses the BAS4 signal peptide:EGFP fusion (secreted EGFP) as a control for EIHM integrity as mentioned above.
3. Pick 10–12 transformants and grow them in a 24-well tissue culture plate with V8 agar.
4. Screen transformants after inoculating them onto rice sheaths (*see* Subheading 3.2 for inoculation and microscopy protocols)—check for morphology that is consistent with wildtype; typically growth on agar medium or in planta will not differ from wild-type. Confirm that all transformants for each construct show similar fluorescence patterns and then choose those with the brightest fluorescence to make filter disc stocks from monoconidia [12] and to use for further imaging.

3.2 Effector Localization Assay 1: Direct Visualization of Translocated PWL2:mCherry

3.2.1 Sheath Inoculation

1. Prepare rice sheaths (adapted from [15]): Choose 16–19 day old rice plants with their third leaf emerged about 4–10 cm. Excise the central sheath by cutting it just above where the tillers branch. Remove the outer and inner sheath layers carefully without bending the middle sheath layer. Cut the upper end of the middle sheath layer to produce a sheath segment ~8 cm in length. Make the cut at a 45° angle so that the midvein forms a point. Place the excised sheaths in a humidity container (*see* **Note 1**).
2. Prepare the inoculum: Harvest conidia from a one to 2 week old OMA culture of transgenic *M. oryzae* coexpressing PWL2:mCherry and secreted EGFP. Adjust the inoculum concentration to 5×10^4 spores/mL in water (*see* **Note 4**).
3. Inoculate excised sheaths (adapted from [15]): Homogenize the conidial suspension by vortexing and then pipette the suspension into the hollow space of an excised sheath from the opening at the end with the 45° cut. Completely fill each of the excised sheaths with inoculum from end-to-end. Each sheath typically requires about 200 μL of conidial suspension.
4. Incubate the inoculated sheaths in a humidity container in dark condition at 25 °C (*see* **Note 5**).

3.2.2 Live-Cell Confocal Imaging

1. Trim the inoculated rice sheath with a razor blade (*see* **Note 6**) at 26–28 h post inoculation (hpi) (*see* **Note 7**).
2. Immediately mount the trimmed sheath on a glass slide using sterile water. Ensure that the sheath is oriented correctly on the slide with the epidermal cells facing up (*see* **Note 8**). Use care when placing a coverslip over the trimmed sheath as not to introduce air bubbles or damage the sheath tissue (*see* **Note 9**).
3. Observe the trimmed sheath through the eyepiece using a 20× objective lens in bright-field for initial field selection (*see* **Note 10**). Dark pigmented appressoria can be easily recognized at this magnification. Be aware of the different types of epidermal cells (*see* **Note 11**).
4. Switch to a 40× objective lens and locate an infection site that is at an early growth stage with only the filamentous primary hypha (tip BIC stage) or up to a few bulbous IH branches (side BIC stage) (Fig. 1a).
5. After locating and focusing on the selected infection site in the bright-field, switch to image acquisition and use the appropriate laser lines for excitation along with the applicable emission spectra (e.g., 488 nm Argon laser line and 495–530 nm emission collection for EGFP and 543 nm Neon laser line and 560–615 nm emission collection for mCherry). Visualize the infection site using live scanning or an equivalent feature capable of acquiring fluorescence and bright-field channels at a fast scan speed to determine if it is an informative infection site (*see* **Note 12**).
6. After locating an informative infection site, set the pinhole size to 1 airy unit (AU). Adjust the pinhole to a higher AU if the fluorescence signal is too weak (*see* **Note 13**).
7. Set the zoom factor to a value of 1 for the 40× objective lens to capture the entire infected cell (*see* **Note 14**). To get optimal signal intensity and minimize background noise, adjust image acquisition settings (*see* **Note 15**). Choose a frame size (*see* **Note 16**).
8. Set the z-slice interval (*see* **Note 17**) and acquire a z-stack image of the informative infection site (*see* **Notes 12** and **18**).
9. Repeat **steps 3–8** to collect images of multiple informative infection sites in order to confirm the consistency of the protein localization patterns observed (*see* **Note 19**). When acquiring many images from the same trimmed sheath, check the slide about every 30 min to make sure the sheath does not dry out and add more water when necessary.
10. Use image analysis software such as Zen (Black edition) from Zeiss microscopy to confirm the EIHM integrity based on secreted EGFP distribution in the acquired images. Ensure

that fluorescence is associated with invasive hyphae and not located in the rice cytoplasm (*see* **Note 20**).

11. Use Zen to determine the PWL2:mCherry fluorescence localization in the acquired images. PWL2:mCherry fluorescence is strong at the BIC and relatively less intense in the cytoplasm of the infected cell (Fig. 2a) (*see* **Note 21**). Observing PWL2:mCherry fluorescence in the BIC and the infected cell's cytoplasm indicates effector secretion and translocation, respectively (*see* **Note 22**).

3.3 Effector Localization Assay 2: Visualizing Translocated PWL2:mCherry After Sucrose-Induced Plasmolysis

1. Prepare sheaths and inoculate them with the *M. oryzae* strain coexpressing PWL2:mCherry and secreted EGFP as described in Subheading 3.2. Follow all steps in Subheading 3.2.1.
2. Trim the inoculated sheaths by following **step 1** in Subheading 3.2.2.
3. Use 0.5 M sucrose as the slide mounting solution (*see* **Note 8**). Incubate the slide for 15 min to allow rice cell plasmolysis to occur (*see* **Note 23**).
4. Find suitable informative infection sites and optimize imaging settings by following **steps 3**–**7** in Subheading 3.2.2 (*see* **Note 24**).
5. Acquire z-stack images of informative infections by following **steps 8** and **9** in Subheading 3.2.2.
6. Follow **step 10** in Subheading 3.2.2 to confirm EIHM integrity by checking the localization of secreted EGFP.
7. To determine the PWL2:mCherry fluorescence localization in the acquired images, follow **step 11** in Subheading 3.2.2. The cytoplasm should be pulled away from the cell wall in plasmolyzed cells (Fig. 2b) (*see* **Note 25**).

3.4 Effector Localization Assay 3: Visualizing Host Nuclear-Targeted PWL2:mCherry:NLS

1. Prepare sheaths and inoculate them with *M. oryzae* coexpressing PWL2:mCherry:NLS and secreted EGFP by following all steps in Subheading 3.2.1.
2. Trim and mount the inoculated sheaths by following **steps 1** and **2** in Subheading 3.2.2.
3. Find infection sites and optimize imaging settings by following **steps 3**–**7** in Subheading 3.2.2 (*see* **Note 26**).
4. Acquire z-stack images of informative infections by following **steps 8** and **9** in Subheading 3.2.2.
5. To confirm that secreted EGFP fluorescence is associated with invasive hyphae and is not located in the rice cytoplasm in the acquired images, follow **step 10** in Subheading 3.2.2.
6. To determine the PWL2:mCherry:NLS fluorescence localization in the acquired images, follow **step 11** in Subheading

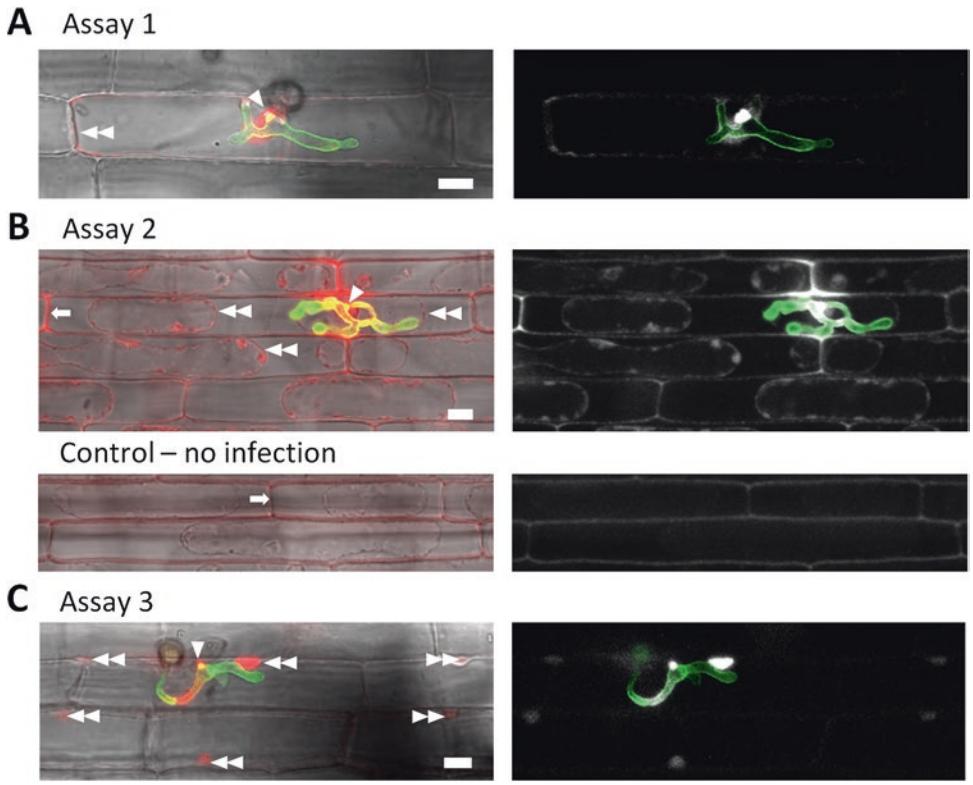


Fig. 2 Fluorescence-based assay to examine effector movement in plant cells. Three different assays for PWL2 movement during *M. oryzae* infection in rice. Shown are confocal images with merged fluorescence and bright-field (left) and merged fluorescence alone (right; red fluorescence pseudo-colored in white). Secreted EGFP (green) exclusively surrounds invasive hyphae in all images, indicating intact EIHM. Bars = 10 μm. (**a**) Direct visualization of PWL2:mCherry (red) preferentially accumulated at the BIC (arrowhead) and translocated into the rice cytoplasm (double arrowhead). (**b**) Plasmolysis-assisted detection of translocated PWL2:mCherry (red) accumulated at the BIC (arrowhead) and translocated into the rice cytoplasm of the infected cell and moved into surrounding cells (examples denoted by double arrowheads). The protoplast in this particular infected cell split into two upon plasmolysis. Note that some autofluorescence is associated with rice cell walls (example denoted by an arrow). In the control with no fungal infection shown below, plasmolyzed cells have the same cell wall autofluorescence (example denoted by an arrow) without cytoplasmic fluorescence. (**c**) PWL2:mCherry:NLS (red) accumulated at the BIC (arrowhead) as well as in the nuclei of the invaded cell and the surrounding cells (double arrowheads)

3.2.2. PWL2:mCherry:NLS fluorescence appears in BICs and also in rice nuclei for both the infected and adjacent uninfected rice cells (Fig. 2c).

4 Notes

1. Use any container that can maintain humidity and hold the excised sheaths upright. We found a convenient way to make a humidity container was to place a 10 cm filter paper at the

bottom of a 10 cm glass petri dish and add just enough water to wet the filter paper. To make a sheath holder, we cut the barrel of a plastic transfer pipette into ~3 cm sections and glued them together side-by-side.

2. Use a confocal microscope that is equipped with laser lines capable of 488 nm and 543 nm such as an Argon/2 laser (458, 488, 514 nm) and a HeNe1 laser (543/561 nm). Use the 488 nm laser for excitation of EGFP and collect emission between 495 and 530 nm (Ex/Em 489/508 nm) and a 543 nm laser for excitation of mCherry and collect emission between 560 and 615 nm (Ex/Em 587/610 nm).
3. We used a conventional cloning method to generate the constructs. Using PCR, we amplified the *PWL2* gene fragment, including the promoter (~1 kb) and the entire coding sequence (stop codon not included), from *M. oryzae* genomic DNA. The recognition sites for *Eco*RI or *Bam*HI were added to the 5′-end of the forward or reverse PCR primers, respectively. The *Bam*HI site serves to ligate *PWL2* and mCherry and also to encode a short linker peptide (Gly-Ser). Next, we cloned the *Eco*RI-*Bam*HI digested PCR product (1.4 kb *PWL2* gene fragment) together with mCherry:terminator (1 kb *Bam*HI-*Hind*III fragment isolated from pBV360; [2]) into *Eco*RI-*Hind*III sites of the binary vector pBHt2 to generate the *PWL2*:mCherry construct. Additionally, we cloned the same *PWL2* gene fragment together with mCherry:NLS:terminator (1.1 kb *Bam*HI-*Hind*III fragment isolated from pBV579; [2]) into *Eco*RI-*Hind*III sites of pBHt2 to generate the *PWL2*:mCherry:NLS construct.
4. To harvest conidia of *M. oryzae*, add 1 mL of sterile distilled water to the surface of the culture. Release conidia into the water by gently dislodging them from the outer edge of the mycelial colony with the tip of a sterile microcentrifuge tube. Filter the conidial suspension by pipetting it through miracloth into a new microcentrifuge tube. Vortex the spore suspension and load ~15 μL into a hemacytometer. Observe under a light microscope to determine the initial spore concentration. Conidial suspensions should be used within an hour after preparation.
5. Ensure that sheaths are positioned upright before leaving them for incubation so that conidia settle onto the epidermal cells along the midvein.
6. The ideal trimmed sheath should be (1) cut from the center of the inoculated sheath, (2) approximately 2 cm in length, (3) about 3–4 cell layers thick, (4) trimmed to a consistent thickness so that the coverslip lies flat over the top of it. To obtain ideal trimmed sheaths, cut each inoculated sheath in half and then excise an approximately 2 cm segment from the inner

portion of one of the pieces. A length of about 2 cm provides enough unperturbed infected cells between the cut ends of the sheath. Hold one end of the sheath gently with your hand and slice through the center of the hollow space from end-to-end with a single-edge razor blade. The remaining portion of the sheath should look U-shaped if viewed on end. Next, trim the sides of the sheath by slicing off the upright portions of the U-shape, leaving the mid vein. Then remove the lower cells of the midvein, leaving the inner epidermal layer and at least one intact mesophyll cell layer. Trimming the sheath too thin can damage the mesophyll layer directly beneath the epidermal layer, thus causing perturbation of the epidermal cells [16]. Trimming the sheath segment too thick reduces light transmission through the sample. Proper sheath trimming takes practice—we recommend practicing with noninoculated sheaths prior to performing the experiment.

7. We found that at 26–28 hpi infections of *M. oryzae* strain O-137 on the susceptible rice cultivar YT16 are typically at an early growth stage in the first-invaded rice cell (Fig. 1a Tip BIC stage and Early side BIC stage). That is, when there is just a filamentous primary hypha or up to a few bulbous IH branches. This is when EIHM is likely to be intact, and the translocation of PWL2:mCherry across the intact EIHM can be examined.
8. Maintain the orientation of the sheath (with the epidermal cells facing up) when mounting it on the slide. When observed on the microscope, the first layer of cells to come into focus should be the rectangular epidermal cells, in which *M. oryzae* initially colonizes. If large square cells (mesophyll cells) appear first, then the sheath was flipped over during mounting. To correct the orientation of the sheath, gently lift the coverslip and use forceps to reposition the sheath. Be sure to mount the trimmed sheath on a slide immediately after preparing it because it dries out quickly after trimmed.
9. Be aware that moving the coverslip can easily damage appressoria and cause the primary hypha to collapse when the infection is at the tip BIC stage or at the early side BIC stages before a septum is formed [17]. There is no need to seal the slide even if using an inverted microscope, provided the sheath was trimmed thin enough.
10. Initial field selection should be done using the bright-field rather than fluorescence to prevent unintentional photobleaching of the fluorescent proteins. We found that with a 20× objective lens (200× magnification), 20 or more rice cell rows are usually visible in the y-axis on the screen. The field of view is slightly larger when physically looking through the eyepiece. This magnification is good for finding infection sites but does

not reveal details of the invasive hyphae. With a 40× objective lens (400× magnification), less than 10 rows of rice cells are typically visible. However, the details of an infection site are much easier to view.

11. Typical trimmed rice sheaths contain more than 30 rows of cells with at least five alternating bands of grouped narrow cells and wide cells, though this can vary greatly based on the age of the plant and the location along the sheath, etc. These two different types of epidermal cells may show different degrees of effector cell-to-cell movement, for example, there is more movement of PWL2:FP in wider and shorter epidermal cells than in longer and thinner cells [2].
12. We found that the growth stage, the state of the EIHM, and the health of rice cells can vary between infection sites even at the same infection time point. These variations affect the interpretation of effector localization. Therefore, care must be taken to choose informative infection sites when assaying for effector translocation into host cells. Use the following criteria to choose informative infection sites: (1) Focus on infection sites at an early growth stage with just a filamentous primary hypha (tip BIC stage) or with up to a few bulbous IH branches (side BIC stage) (Fig. 1a) [10]. (2) Confirm that secreted EGFP fluorescence is localized exclusively around invasive hyphae. This indicates that the EIHM is intact. Do not use infection sites where EGFP fluorescence is dispersed in the host cytoplasm, which indicates that the EIHM has lost integrity [10]. (3) Choose rice cells that do not show excessive discoloration, granulation, or cytoplasmic aggregation. These are features of failed infections or resistance responses. (4) Avoid infection sites where the rice cell is damaged, for example at the cut end of the sheath. Also avoid rice cells with a high level of cell wall autofluorescence.
13. Lower pinhole settings allow for higher resolution in the z-axis and allow visualization of secreted EGFP fluorescence in an outline pattern around invasive hyphae, but this signal may be relatively weak. Higher pinhole settings allow for increased detection of fluorescent signals, although the outlining pattern around invasive hyphae will become indistinct, and secreted EGFP fluorescence will appear to be generally associated with invasive hyphae.
14. It is important to include at least the entire infected cell to determine whether secreted EGFP or PWL2:mCherry has entered the host cytoplasm because the cytoplasm is in close proximity to the cell wall. The 20× objective lens with a zoom factor of 2 or the 40× objective lens with a zoom factor of 1 can both be used to capture the entire infected cell together

with the surrounding uninfected cells, which is useful for determining effector cell-to-cell movement.

15. Optimize the signal-to-noise ratio to detect translocated PWL2:mCherry at low concentrations as well as instances of low intensity secreted EGFP fluorescence in the rice cytoplasm that occurs just after EIHM disruption. Be aware that with settings optimized for detection of low intensity signals, the BIC will likely be saturated because it has the brightest fluorescence intensity. To obtain the optimal settings, adjust the detector gain (maximum of 800), the digital offset, the digital gain, the scan speed, and the laser power. On confocal microscopes equipped with variable spectral filters, maximizing collection emission bandwidth for the fluorophore will also be helpful. Watch for potential autofluorescence associated with rice cell walls that could be mistaken for effector fluorescence.
16. We found that good results are obtained when starting with the 1024 × 1024 frame size. Reduce the scan field in the y-direction if possible because this reduces the image acquisition time. However, be sure to include some uninvaded cells above and below the infected cell to assay for cell-to-cell movement of effectors.
17. We found that a z-slice interval of 2 μm was sufficient for capturing all features of interest, including invasive hyphae, BICs, and rice nuclei. Make sure to set the z-stack parameters to capture the entire depth of the rice cell of about 10–15 μm, thus approximately 5–8 z-slices are needed.
18. Infection sites with a disrupted EIHM showing secreted EGFP spilled into the host cell can also be imaged if effector localization at the post-EIHM disruption stage and/or the state of the vacuole are of interest. The central vacuole has been shown to rupture after EIHM disruption during successful infections at the later stages of growth within the first-invaded cell [10]. This causes any translocated effectors or effectors spilled from the EIHM compartment to homogenize throughout the infected host cell.
19. The number of infection sites that need to be imaged is influenced by the level of variation in the fluorescence patterns. We suggest taking images from at least three different rice sheaths for a total of at least 10 infection sites. Observing comparable PWL2:mCherry fluorescence patterns across all infection sites constitutes strong evidence for effector translocation. The number of infection sites should be increased if major variation is observed across the infection sites. Some minor variations such as different fluorescence intensities or degrees of effector cell-to-cell movement may be observed and worth documenting.

20. To detect low intensity secreted EGFP fluorescence in the rice cytoplasm, lower the set white point in the green channel histogram. This results in an increase in the brightness and contrast, which allows low intensity signals to be more easily differentiated from background noise. Performing the white balance adjustment is important for identifying infections with a recently disrupted EIHM that have not accumulated high concentrations of spilled EGFP in the rice cytoplasm.
21. Some PWL2:mCherry fluorescence may be detected in the EIHM compartment along the primary hypha and the BIC-associated cell. However, PWL2:mCherry fluorescence is not found in the EIHM compartment surrounding other invasive hyphae. If testing a putative mCherry-tagged effector other than PWL2, observing exclusive colocalization of the effector with secreted EGFP in the EIHM compartment indicates that it is an apoplastic effector. However, the effector may still enter the cytoplasm together with secreted EGFP after the EIHM disrupts.
22. The degree of effector cell-to-cell movement can vary depending on the size of the protein and the size exclusion limit of the plasmodesmata [2].
23. We found that adding 0.5 M sucrose to a trimmed rice sheath was sufficient to induce convex plasmolysis, where the protoplast pulls away from the shorter traverse cell walls and forms convex ends [18]. For the purpose of this assay, convex plasmolysis is preferred over other forms of plasmolysis that can occur at higher concentrations of sucrose, such as concave, or sub-protoplasts [18]. It may be necessary to optimize plasmolysis if convex plasmolysis does not occur. This can be done by altering the concentration and/or incubation time to increase or decrease the intensity of plasmolysis. Step-wise plasmolysis, in which sucrose is added gradually at increasing concentrations, can lessen the stress of rapid plasmolysis and allow it to occur more gradually. This can be helpful in some instances when convex plasmolysis is difficult to produce with the addition of a single concentration of sucrose. Step-wise plasmolysis can be done by starting with 0.25 M sucrose and allowing 10 min for incubation before replacing with 0.5 M sucrose for another 10 min.
24. The optimal imaging settings should be the same for assays 1 and 2 because they use the same fungal strain.
25. Plasmolysis causes the plasma membrane and the enclosed protoplast of viable rice cells to pull away from the cell walls. The protoplast always shrinks around invasive hyphae rather than away from them [15], thus improving the distinction between

the cytoplasm and the apoplast as well as any autofluorescence associated with the cell wall.

26. The optimal imaging settings for the strain expressing PWL2:mCherry:NLS will be very similar to the optimal settings determined for the strain expressing PWL2:mCherry. Therefore, the settings for PWL2:mCherry can be used as a convenient starting point.

Acknowledgments

We acknowledge the assistance of the Biomedical Microscopy Core at the University of Georgia with imaging using a Zeiss LSM 710 confocal microscope. This work was supported by the Agriculture and Food Research Initiative competitive grants program, Award number 2014-67013-21717 from the USDA National Institute of Food and Agriculture.

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