Chapter 10

Illuminating *Phytophthora* Biology with Fluorescent Protein Tags

Audrey M. V. Ah-Fong, Meenakshi Kagda, and Howard S. Judelson

Abstract

Phytophthora species cause diseases that threaten agricultural, ornamental, and forest plants worldwide. Explorations of the biology of these pathogens have been aided by the availability of genome sequences, but much work remains to decipher the roles of their proteins. Insight into protein function can be obtained by visualizing them within cells, which has been facilitated by recent improvements in fluorescent protein and microscope technologies. Here, we describe strategies to permit investigators to generate strains of *Phytophthora* that express fluorescently tagged proteins and study their localization during growth in artificial media and during plant infection.

Key words Fluorescent protein, Oomycete, Transformation, Cell biology

1 Introduction

The genus *Phytophthora* includes some of the world's most destructive pathogens of crops and forest trees [1]. The last decade has witnessed a revolution in *Phytophthora* genomics, with genome assemblies and predicted protein sequences available for several species [2]. A major challenge now is ascribing functions to those proteins. Determining the subcellular location of proteins provides clues to their roles and modes of regulation. The advent of genetically encoded fluorescent tags has made it possible to visualize proteins with minimal perturbation to the cell, in contrast to traditional immunolocalization methods which require not only highly specific antibodies but also cell fixation and permeabilization [3]. Efforts to engineer fluorescent proteins have resulted in variants that span the visible spectrum, allowing multiple proteins to be monitored simultaneously [4]. Combined with advances in confocal microscopy, these tags provide powerful tools to study protein interactions, interrogate biological processes, and monitor the abundance, localization, and trafficking of proteins at high spatial

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and temporal resolution. In *Phytophthora*, fluorescent tagging has helped visualize organelles [5], track proteins involved in development [6, 7], and study the targeting of proteins involved in plant interactions [8–11].

Although the expression of *Phytophthora* proteins with fluorescent tags (FFP, fluorescent fusion proteins) is now common in several laboratories, it is not without challenges. These include the formation of insoluble aggregates, mislocalization, and instability of transgene expression. Here, we provide protocols for labeling *Phytophthora* proteins that take these issues into consideration. Information is provided about designing constructs, generating transgenic strains, purifying heterokaryons, choosing the appropriate transformants, fixing tissues, and imaging FFPs. Along with continuing advances in fluorescent tags and new genome editing techniques, this will enhance our understanding of *Phytophthora* biology.

2 Materials

2.1 Making Fluorescent Fusion Protein Constructs

1. Transformation vectors containing selectable (drug resistance) markers.
2. Genes encoding fluorescent proteins. GFP, YFP, CFP, mCherry, tdTomato, and mRFP have already been incorporated into vectors adapted to *Phytophthora* [5, 8].

2.2 Electroporation of Zoospores

1. Glass spreader, sterile beaker, 15 μm nylon mesh, hemocytometer.
2. Disposables: sterile 50 and 15 mL polypropylene tubes, 4-mm electroporation cuvettes, 150 mm petri plates, disposable pipettes.
3. Equipment: refrigerated centrifuge with swinging bucket rotor, electroporator, light microscope for counting spores.
4. Regeneration media: For *P. infestans,* this is Rye A (rye-sucrose) broth [12] clarified by centrifugation plus 100 mM mannitol, 1 mM KCl, and 2.5 mM $CaCl_2$. Some species may prefer other media as the base [1].
5. Cold 5 M LiCl and cold modified Petri's solution: 0.25 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 0.8 mM KCl.
6. Selective media: 1.5% agar media with antibiotics, usually G418 or hygromycin. For *P. infestans,* this is Rye A media [12].

2.3 Identifying Transformants Expressing FFPs

1. Microscope slides and coverslips.
2. Water, paper towels.
3. Optical fluorescence or confocal microscope.

2.4 Single-Nucleus Purification

1. Glass spreader, 60 mm petri dish, 15 μm nylon mesh, hemocytometer.
2. Sterile water, beaker.
3. Selective media with 1.5% agar.

2.5 Fixing Tissues

1. Single and double-strength (2×) fixative; single-strength is 4% formaldehyde, 50 mM Pipes pH 6.8.
2. Washing solution: 50 mM Pipes pH 6.8.

2.6 Confocal Imaging

1. Glass slides.
2. Coverslips and/or glass-bottom culture dishes designed for confocal microcopy; these are typically described as being made from "precision glass". Brands of coverslips include Marienfeld, Warner and World Precision Instruments (WPI). Glass-bottom dishes are available from MatTek, Nunc, Warner, and WPI.
3. Clear nail polish.

3 Methods

3.1 Design of Fluorescent Fusion Protein (FFP) Construct

1. Select the appropriate vector system. Used most are plasmids bearing *nptII* or *hpt*, which confer resistance to G418 or hygromycin, respectively.
2. Select the desired fluorescent protein. We advise using the brightest proteins in order to increase the signal-to-noise ratio (*see* **Note 1**).
3. Choose between a constitutive, native, or inducible promoter. A strong promoter will maximize expression but may introduce artifacts (*see* **Note 2**).
4. Design a cloning strategy to place the tag at the amino or carboxyl terminus of the protein of interest, or within the protein (*see* **Note 3**). Information about the optimal strategy may be obtained by searching the literature for studies of related proteins. If the protein contains a functional or targeting domain, place the tag at a site that will not interfere with the function of those domains.
5. If the goal is to coexpress two proteins, express both from the same plasmid or introduce them on separate plasmids by cotransformation and/or using different selectable markers (*see* **Note 4**).

3.2 Phytophthora Transformation

Several methods have been developed for transforming *Phytophthora*. Those used most commonly involve treating protoplasts with DNA or electroporating zoospores [5, 13].

Agrobacterium-mediated transformation has also been described [14, 15]. These protocols need to be tailored to the species and sometimes isolate. Presented below is an electroporation method that works well in *P. infestans*.

1. Inoculate five 15-cm rye-sucrose plates by spreading ~1 × 10^4 sporangia on each with a sterile glass rod. Grow for 8–9 days in the dark at 18 °C (*see* **Note 5**).
2. Pour 20 mL of cold Petri's solution on each plate. Use a glass spreader to rub off the sporangia, decanting what should be a milky solution into a 150 mm plate, i.e., one plate per 20 mL of sporangia suspension.
3. Induce zoosporogenesis by incubation at 10 °C for about 2 h (*see* **Note 5**).
4. Harvest the zoospores by pouring the suspension through 15 μm nylon mesh into a 400-mL beaker. This and the following steps should be done on ice. A convenient holder for nylon mesh is sold by Biodesign of New York.
5. Divide the zoospores into two 50 mL tubes, add one-fiftieth the volume of 5 M LiCl to each, and mix by gentle inversion. Remove an aliquot and count the zoospore concentration using a hemocytometer.
6. Spin at 400 × *g* for 5 min at 4 °C in a swinging bucket rotor.
7. Decant most of the supernatant and resuspend the pellet by gently pipetting up and down. Add enough Petri's solution to set the concentration between 6 × 10^6 and 2.5 × 10^7 zoospores per mL.
8. Gently mix 800 μL of zoospores with 30 μg of DNA in a prechilled tube. If coexpressing two plasmids, use 15 μg of each.
9. Pipette the zoospores into a precooled 4 mm-gap cuvette. Cap the cuvette, wipe-dry with a tissue, and electroporate at 550 V, 50 μF, and 1575 Ω. The time constant is usually 1.8–2.6 ms.
10. Rapidly place the cuvette on ice, add 800 μL of regeneration media, and pipette into a 15 mL tube containing an additional 9 mL of regeneration media (*see* **Note 6**). Lay the tube on its side and incubate at 18 °C for 20 h. Longer incubation is not advised since the hyphae will mat together and be difficult to spread on selection plates.
11. After 20 h, count the concentration of germinated cysts. Multiply by the volume, and divide by the original number of zoospores to determine the regeneration rate. This is normally between 5 and 40%.
12. Concentrate the regenerated material by spinning the cultures at 1000 × *g* for 5 min at room temperature. Decant all but 1.6 mL of the liquid.

13. Gently resuspend the zoospores in the residual liquid by pipetting up and down, and spread 0.2 mL on each of eight rye-sucrose agar plates containing the appropriate antibiotics (*see* **Note 7**). Incubate at 18 °C. Colonies will appear after 8–12 days.

3.3 Identifying Transformants Expressing the FFP

1. Screen for expression using a fluorescence microscope (*see* **Note 8**). If the construct is expressed constitutively, it is usually sufficient to place a tuft of mycelia in a drop of water on a slide under a cover glass. Other life stages (zoospores, plant infection, etc.) will need to be examined if expression is not expected to occur in hyphae.
2. To avoid artifacts, verify that the localization is the same in multiple transformants and those with high and low levels of expression (*see* **Note 9**). Also check for the formation of protein aggregates (*see* **Note 10**).
3. Confirm that the FFP is of the expected size by immunoblotting (*see* **Note 10**).
4. For colocalization studies, it is best to choose transformants in which expression of the FFPs is well balanced.

3.4 Zoospore (Single-Nucleus) Purification

Expression within a culture is often not uniform, because some nuclei may not be transformed or may be epigenetically silenced. Single-nuclear purification is recommended in such cases. A procedure for *P. infestans* is described below. We have also purified transformants using a cell sorter with zoospore cysts.

1. Isolate zoospores as in Subheading 3.2. After passing the zoospores through 15 μm mesh, determine their concentration.
2. Spread about 25 zoospores on a 100 mm plate containing the appropriate selective media (*see* **Note 11**).
3. Incubate at 18 °C, select single colonies, and recheck for expression.

3.5 Fixing Phytophthora Tissues

Subcellular structures are often unstable during prolonged incubation on a slide, or under the heat of the microscope (*see* **Note 12**). This instability can be reduced by using the following protocols for fixing tissues, adapted from reference [16]. Fixation is less necessary for visualizing *Phytophthora* in plants, as it may increase autofluorescence of the host tissue.

1. To fix hyphae, place a tuft of mycelia in single-strength fixative solution for 1 h at room temperature. Remove the fixative with a pipette, and rinse three times in 50 mM Pipes buffer, 5 min each.
2. To fix sporangia, zoospores, or cysts, add an equal volume of double-strength fixative to the spore suspension. Mix by gently

inverting the tube, and incubate for 30 min at room temperature. Wash three times. For each wash, centrifuge at 1500 × *g* for 3 min, remove supernatant, resuspend the pellet in 3 mL of 50 mM Pipes buffer, and wait for 5 min.

3.6 Mounting Fixed and Live Specimens for Confocal Imaging

1. Place a drop of spore suspension, or a tuft of hyphae in a drop of liquid, on a microscope slide. Use a fine needle to tease apart the hyphae.
2. Add a #1.5 coverslip, taking care to avoid trapping air bubbles.
3. Blot the excess mounting liquid, and seal the coverslip on the slide by placing a minimal amount of clear nail polish around all edges. Although we have not experienced problems, there have been reports that the solvent in nail polish may reduce fluorescence [27].

3.7 Confocal Imaging of Phytophthora in Plants

The following methods have proved useful for imaging *P. infestans* in tomato and potato leaves, or in tubers. Normally, plant tissues are infected with zoospores or hyphae. Because infection protocols are often specialized to the pathogen, host, or host organ, their description is beyond the scope of this chapter.

1. Obtain infected tissues in which actively growing hyphae are abundant. Older hyphae are vacuolated and yield lower signals (*see* **Note 13**).
2. The mounting of plant tissue depends on the sample and microscope. On an upright microscope, a leaf (or portion) can be viewed by laying it on a slide and placing a water drop between the leaf and a water-dipping objective. If an inverted microscope is used, or only an oil immersion objective is available, place the leaf between a coverslip and slide, seal three sides of the coverslip with clear tape, fill the space between the slide and coverslip with water, and seal the fourth side with tape (*see* **Note 14**).
3. Flat, thicker samples such as potato tuber slices are more easily visualized by placing the tissue on a glass bottom culture dish, using an inverted microscope. One or two coverslips can help hold down the plant tissue.
4. Since plant tissues are thicker than hyphae, *z*-stack imaging can be useful.

3.8 General Guidelines for Confocal Imaging

The reader is advised to consult the manuals for their particular instrument as well as other resources [17] on confocal microscopy. Some additional tips from our experience with *Phytophthora* are as follows:

1. FFPs can be imaged in live or fixed samples. Unfixed tissues in water should be viewed immediately, while hyphae left in media can be viewed within a few hours. Fixed specimens should be viewed within 24 h.
2. The accurate detection of multiple FFPs can be challenging, and microscopes vary in their abilities to separate wavelengths. To avoid cross-talk, use the appropriate band-pass emission filters, test the settings using transformants expressing only one tag, and consider sequential scanning if possible.
3. Consider using additional methods to validate the FFP results, including placing tags at both the N and C-termini (*see* **Note 15**).

4 Notes

1. A list of fluorescent proteins and their spectral properties are available at Rodriguez et al. [4]. We have not observed superior expression of GFP or tdTomato optimized to *Phytophthora* codon usage compared to those optimized for plants or animals, but this might not be true for all tags. Some tags such as tdTomato and DsRed are popular due to their brightness; however, they should be used with caution as they form oligomers, which may cause artifacts in targeting or protein–protein interactions.
2. The protein should be expressed at a level that enables reliable imaging with minimal interference with cellular functions. However, some native promoters may not yield enough protein. Strong oomycete promoters include *ham34* or ribosomal protein promoters [5, 18, 19].
3. One widely used system allows the construction of N-terminal, C-terminal, or internal fusions with GFP, CFP, YPF, or mCherry in backbones containing *hpt* or *nptII* [5]. We have also expressed tdTomato and mScarlet in that system. The simplest cloning method is to use polymerase chain reaction to amplify the gene of interest with primers containing restriction sites compatible with the vector. Make sure that the protein is inserted in-frame, contains Kozak initiation sites, and no stop codon is between the gene of interest and tag.
4. Similar numbers of transformants are obtained when one plasmid is introduced compared to when two plasmids, each bearing a separate FPP, are cotransformed. Cotransformation is relatively efficient [20], and can be done with plasmids using the same or different selectable markers. Expressing two FFPs from one vector should minimize the unbalanced expression of the tags, which may complicate microscopic analysis. Examples

of transformants expressing GFP and tdTomato FFPs introduced by cotransformation are shown in Fig. 1a, b.

5. Incubation times for growing hyphae and releasing zoospores are species- and strain-specific. Most *P. infestans* should not be grown for more than 10 days, otherwise zoospore release may be slower and less synchronous. Most zoospores are usually released by 2 h, and longer incubations may reduce transformation efficiency.
6. It is often prudent to include antibacterials (e.g., 25 μg/mL penicillin G, or 50 μg/mL ampicillin plus 25 μg/mL vancomycin) and antifungals (e.g., 40 units/mL nystatin) in the media to avoid contamination.
7. For most *P. infestans* strains, we use 9 μg/mL G418 or 45 μg/mL hygromycin. The concentrations should be determined empirically for each strain.
8. A conventional fluorescence microscope is usually sufficient for the initial screening, although higher sensitivity might be achieved with a confocal. It is best to use young cultures because older ones tend to autofluoresce, and to visualize freshly mounted tissue. If using a digital (or confocal) microscope, avoid false positives by ensuring that the signal is present without having to turn the gain to high; viewing samples through an eyepiece may provide more reliable results.
9. Due to position and copy number effects, expression levels of the transgene will vary between transformants [21, 22]. Highly expressed FFPs may exhibit false localization. The transgene may also interfere with normal cell functions due to overexpression or silencing of the native gene. Therefore, the fitness of the strain (growth rate, sporulation rate, etc.) should be noted as this may influence interpretations of results.
10. Some FFPs may misfold or form aggregates; punctate signals may indicate the latter and should be interpreted cautiously. In addition, some FFPs are prone to proteolytic cleavage at the linker between the tag and protein of interest. While linkers containing small or polar amino acids (e.g., glycine or serine) may add flexibility and solubility to the FFP, other compositions may increase the stability of the FFP [23].
11. On average, plating 25 zoospores should result in several drug-resistant colonies. Because the efficiency of colony establishment from zoospores can vary depending on the species and strain, a greater number of zoospores may be required.
12. Live cell imaging is not always practical. Fixation will reduce stress responses including those resulting from desiccation and heating, is the only option for viewing zoospores, and is needed to accurately assess FFP localization in sporangia undergoing zoosporogenesis (Fig. 1c). Fixing plant tissue can be problem-

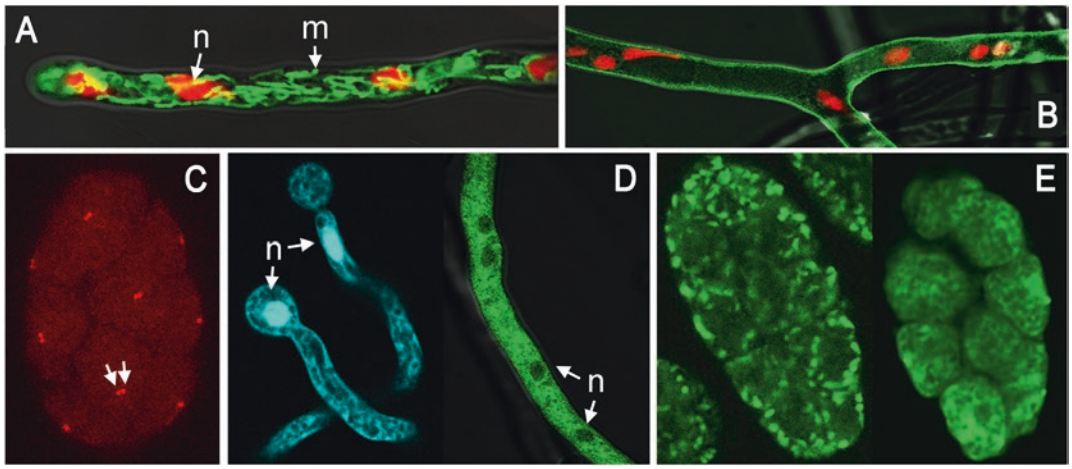

Fig. 1 Examples of FFPs in ***P. infestans*** transformants. (**a**) Colocalization in hyphae of GFP-tagged mitochondrial and tdTomato-tagged nuclear FFPs. Representative nuclei and mitochondria are denoted by n and m, respectively. (**b**) Cell wall and nuclear-localized FFPs in hyphae, expressing GFP and tdTomato, respectively. (**c**) tdTomato fused to basal body protein in sporangium cleaving into zoospores. Arrows point to a basal body doublet containing the FFP, and a weak signal is also in cytoplasm. With low signals, it is important to consider whether they are authentic or due to background noise. Here, examinations of multiple transformants and other FFPs that are targeted exclusively to basal bodies supported the veracity of the cytoplasmic signal. (**d**) The left image shows CFP in germinating cysts, with signals in cytoplasm (but not cytoplasmic vesicles) and nuclei. CFP, GFP, and YFP lack nuclear localization signals yet tend to accumulate in nuclei, so one must guard against drawing incorrect conclusions about protein targeting when expressing these small (27 kDa) markers alone or fused to small (<40 kDa) proteins. This is not an issue with larger FFPs, as illustrated in the right image where GFP was fused to a 68 kDa cytoplasmic protein; in this case, nuclei appear as nonfluorescing dark zones. Nuclear accumulation can also be reduced by using dimers or trimers of fluorescent protein such as GFP [5]. (**e**) Case where the location of the tag affects targeting. Both sporangia are undergoing zoosporogenesis and expressing the same ***P. infestans*** protein. The sporangium on the left is expressing the protein with N-terminal GFP, and resides in vesicles on the periphery of each developing zoospore. The right sporangium has a C-terminal GFP tag and is largely cytoplasmic. Subsequent experiments performed with other fluorescent tags indicated that the cytoplasmic location is more likely

atic as this usually damages its structure, making it harder to hold in place, and may increase autofluorescence.

13. Visualizing *Phytophthora* within plants can be challenging. It is helpful to scan at low magnification using eyepieces, and then switch to higher magnification and camera-assisted viewing. Autofluorescence can also be problematic. This derives mainly from chlorophyll (red), lignin (green), and compounds generated during wounding. It is thus preferable to minimize the number of cuts made in plant tissue, and use autofluorescence-blocking filters. Autofluorescence is greatest using blue or ultraviolet excitation wavelengths.
14. We recommend using inverted microscopes to visualize plant tissues. The objective on an upright instrument may damage the sample, and it is usually necessary to affix the sample in position using tape or a glue.

15. It is prudent to interpret with caution the results of experiments using FFPs. Confidence in subcellular localization data can be raised by testing both N and C-terminal tags, or different tags, since the site of fusion can affect targeting as shown in Fig. 1e. Members of the GFP family also show a propensity to translocate into nuclei on their own, unless they are part of a much larger protein (Fig. 1d). To help confirm the nature of an organelle, FFPs have been developed for *Phytophthora* basal bodies, cytoplasm, ER, Golgi, mitochondria, nuclei, peroxisomes, haustoria, and the cytoskeleton [5, 6, 8, 24]. Some organelles can also be labeled with fluorescent dyes, some of which are suited to imaging fixed or live cells. Dyes used for live-cell imaging are listed in Hickey et al. [25], but note that many of these have not been tested in *Phytophthora*. The use of FFPs to study dynamic cellular processes may also lead to erroneous conclusions since tags may affect a protein's half-life or its ability to interact with other proteins [26]. Therefore, researchers should consider supplementing studies using FFPs with other methods. For example, immunohistochemical analysis using an antibody against the native protein can confirm subcellular localization results from confocal microscopy.

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