Chapter 1

Promoter Analysis and Dissection Using Reporter Genes, Comparative Genomics, and Gel Shift Assays in *Phytophthora*

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Abstract

Transcriptional regulation allows cells to execute developmental programs, maintain homeostasis, and respond to intra- and extracellular signals. Central to these processes are promoters, which in eukaryotes are sequences upstream of genes that bind transcription factors (TFs) and which recruit RNA polymerase to initiate mRNA synthesis. Valuable tools for studying promoters include reporter genes, which can be used to indicate when and where genes are activated. Moreover, functional regions within promoters (typically TF binding sites) can be identified by integrating reporter assays with promoter mutagenesis. These sites may also be revealed through comparative genomics, or by the DNA-protein binding procedure known as a gel shift or electrophoretic mobility shift assay (EMSA). The latter can also be used to test if a specific TF binds a DNA target or assess the binding kinetics or affinity of the complex. In this chapter, we describe procedures for expressing reporter genes in *Phytophthora*, assaying reporter activity, identifying functional sites within promoters, and testing purified TFs or proteins within nuclear extracts for DNA binding.

Key words Reporter gene, Oomycete, Transformation, Transcription factor, DNA-protein binding assay, Comparative genomics, Phylogenetic footprinting

1 Introduction

The genus *Phytophthora* is infamous for causing major losses on food and fiber crops, ornamentals, and plants in natural environments [1]. Critical to the growth and development of *Phytophthora* is transcriptional regulation, which largely involves the binding of transcription factors (TFs) to promoters, which are sequences upstream of the coding sequences of genes. Several TFs controlling metabolism or developmental processes such as sporulation have been identified in *Phytophthora* [2–8]. Learning more about TFs, their targets in promoters, and environmental cues that regulate promoters has the potential to lead to strategies for controlling oomycete diseases.

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Reporter genes are useful tools for monitoring in vivo the temporal and spatial patterns of gene expression and identifying the signaling events that drive cellular and developmental processes. A diversity of reporters useful in oomycetes have been developed, including fluorescent proteins such as green fluorescent protein from *Aequorea victoria* (GFP; [9]) and β-glucuronidase from the *uidA* gene of *E. coli* (GUS; [10]). A prerequisite for using these is a system for inserting DNA into the genome, i.e., transformation. When fused to a constitutive promoter, reporters have been shown to be useful for tracing the growth of *Phytophthora* within a plant host [9]. Insight into the biology of plant pathogens may also be revealed when the reporter is fused to a promoter sensitive to signals such as the plant cell wall, redox state, or stimuli regulating spore germination or effector secretion [11–13].

In combination with promoter mutagenesis, reporters can also be used to define regulatory sites within promoters. Past studies of *Phytophthora* promoters have indicated that TF binding sites typically reside within a few hundred bases of the transcription start site, which is consistent with the relatively small (400–500 nt) intergenic regions found within oomycete genomes [12, 14, 15]. Proteins that bind such sites can be investigated using gel shift assays, also known as electrophoretic mobility shift assays (EMSA; [16]). Such assays are often used to confirm the binding of a TF to a DNA site, but can also be employed to measure binding affinities and stoichiometries within the DNA-protein complex [17], or to delineate the sequences required for TF binding which are often semi-degenerate [18].

A common strategy for dissecting the structure of promoters begins by fusing sequences upstream of the translation start site with a reporter, which is then tested in vivo. *Cis*-regulatory elements are then identified by testing the effect of base changes or deletions. The function of many motifs can also be measured by fusing their sequences to a minimal promoter. The latter, also known as a core promoter, is a short sequence that allows the initiation complex to form but has been stripped of upstream regulatory sites [19]. Whether regions within a promoter bind a transcription factor (or other protein) can also be assessed in vitro through gel shift assays using crude nuclear extracts or purified TFs. Clues can also be obtained through phylogenetic footprinting [20]. This usually involves identifying conserved blocks within alignments of orthologous promoters, but it is also possible to search for over-represented motifs within unaligned regions. The latter approach can be extended beyond analyses of orthologs to studies of co-expressed genes mined from whole-genome transcriptome data [15, 21].

In this chapter, we describe approaches that have proved to be successful for studying *Phytophthora* promoters using reporter genes and gel shift assays. While our protocols focus on the use of

the GUS gene and the potato pathogen *Phytophthora infestans,* the methods can be adapted to other reporters or species. We also discuss considerations that can be used when choosing a reporter, strategies for promoter mutagenesis, and ways in which comparative genomics and other bioinformatic tools can aid the analysis.

2 Materials

2.1 Constructing Reporter Plasmids

1. 37 °C incubators.
2. Microcentrifuge.
3. Transformation vector containing a selectable marker for drug resistance and a reporter gene containing a cloning site at its 5′ end. For GUS, a convenient vector is pNP-GUS [15], which contains the *nptII* marker (Fig. 1; Subheading 3, **step 1**). Promoter motifs can be tested for function using pNIFS-GUS, which contains a minimal promoter fused to GUS (Subheading 3, **step 6**).
4. PCR-amplified or synthesized promoter sequences.
5. Restriction enzymes for inserting promoter sequences into the reporter plasmids, such as *Xba*I and B*am*HI.
6. T4 DNA ligase.
7. Competent *E. coli* cells (e.g., DH5α).
8. Lysogeny Broth (LB): 1.0% NaCl, 1.0% tryptone, 0.5% yeast extract, with and without 1.5% agar.
9. TE buffer: 10 mM Tris pH 8.0, 1 mM EDTA.
10. Ampicillin.

2.2 Phytophthora Electroporation

1. Electroporator.
2. Electroporation cuvettes with a 4 mM gap.
3. Refrigerated centrifuge with swinging bucket rotor.
4. Light microscope.
5. Hemocytometer.
6. 15 μm nylon mesh.
7. Bent glass rod or disposable plastic spreader.
8. Regeneration media: Rye A (rye-sucrose) broth [22] is clarified by centrifugation at 5000× *g*, and then mannitol is added to 100 mM, $CaCl_2$ to 2.5 mM, and KCl to 1 mM. Other media may be preferred for species other than *P. infestans.*
9. 5 M LiCl, kept at 4 °C.
10. Modified Petri's solution (0.8 mM KCl, 0.25 mM $CaCl_2$, 1 mM KH_2PO_4, 1 mM $MgSO_4$), kept at 4 °C.

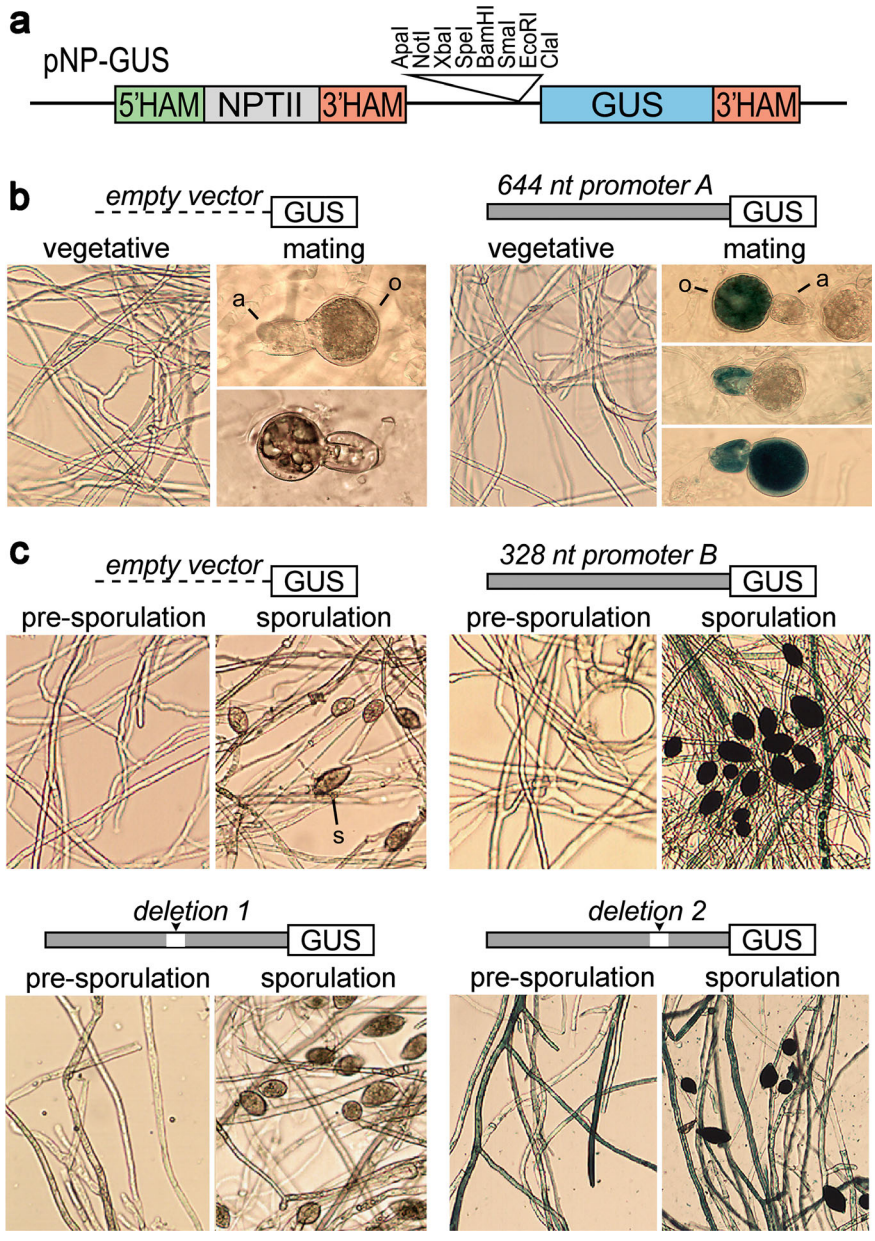

Fig. 1 Testing promoters using GUS. (**a**) Linearized schematic of pNP-GUS, which contains a promoterless GUS gene and *nptII* for resistance to G-418. (**b**) GUS driven by a 406-nt mating-induced promoter and detected by histochemical staining (blue color). No signal was detected using empty vector controls in vegetative hyphae, or in mating structures obtained by co-culture with an untransformed strain of the opposite mating type (oogonia, o, and antheridia, a). The oospore in the lower empty vector panel displays the darkening typical of maturing oospores, not the blue color from GUS. In contrast, the promoter-GUS fusion caused staining only in the sexual structures. Illustrated are expression in an antheridium (top right panel) or oogonium (middle right) in outcrossed pairings, and in both during selfing (lower right). (**c**) Analysis of a promoter induced during asexual sporulation. The intact 328-nt promoter is shown driving expression in sporangia (s) and nearby hyphae, but not in pre-sporulation cultures. The 328-nt region had been identified as sufficient for expression based on the analysis of sequential 5′ deletions starting from an 800-nt promoter. Erasure of a predicted motif (deletion 1) eliminated transcription, indicating that the block binds an activator. Loss of a second motif (deletion 2) resulted in constitutive expression, suggesting that region binds a repressor

11. Selective media: Rye A media with 1.5% agar and a drug for selecting transformants. For plasmids mentioned in this chapter, this is normally 8 μg/mL G-418.
12. Optional antibiotics to guard against contamination: Nystatin and/or Penicillin G.

2.3 Histochemical Staining for GUS

1. Equipment: Light microscope with slides and coverslips.
2. 37 °C incubator.
3. Staining solution: 50 mM $NaPO_4$, pH 7.0, 0.1% Triton X-100, 0.1% bromochloroindoyl-β-D-glucuronide (X-Gluc), 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$. X-Gluc is added from a 10% stock in dimethyl formamide. These should be stored in the dark at −20 °C.

2.4 Quantitative Assay for GUS

1. Equipment: Fluorometer, preferably one capable of handling 96-well plates.
2. Homogenization equipment: We normally use a mortar and pestle with liquid nitrogen. Hyphae or plant tissue may also be disrupted using a mechanical homogenizer such as a Polytron (Kinemetica).
3. Extraction buffer: 0.25 M sodium phosphate, pH 7.0, 10 mM ethylenediaminetetraacetic acid (EDTA), 50 mM β-mercaptoethanol, 0.5% sodium N-lauroylsarcosine, 0.5% Triton X-100.
4. Assay buffer: Extraction buffer plus 1 mM 4-methylumbelliferyl-β-D-glucuronide (4-MUG).
5. Stop buffer: 0.2 M sodium carbonate.
6. 4-Methylumbelliferone (4-MU) for making standard curve.

2.5 Nuclear Protein Extracts

1. Equipment: Bead-Beater (Biospec Products).
2. Refrigerated centrifuge.
3. Nuclear Buffer 1: 10 mM piperazine-N,N′-bis(2-ethanesulfonic acid) pH 7.0, 10 mM $MgCl_2$, 25% (vol/vol) glycerol, 10 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF); add the PMSF just before use from a 200 mM stock solution in isopropanol. Cocktails of protease inhibitors may be substituted for the PMSF.
4. Nuclear Buffer 2: 10% glycerol, 15 mM HEPES, pH 7.9, 0.5 M KCl, 5 mM MgCl2, 0.5 mM EDTA, 1 mM dithiothreitol (DTT), 1% Triton X-100.
5. Nuclear Buffer 3: 15 mM HEPES pH 7.9, 100 mM KCl, 1 mM EDTA, 2 mM DTT, 15% glycerol, and 0.1 mM PMSF (added just before use).

6. 10% Triton X-100.
7. 3 M KCl.

2.6 Gel Shift Assay (EMSA)

1. Vertical electrophoresis unit including power supply and low fluorescence gel plates.
2. Instrument for scanning the gel for fluorescence (e.g., GE Typhoon or Azure Sapphire).
3. Oligonucleotides containing the target site. This would include a Cy5-labeled oligonucleotide containing the target (stored in the dark), and its unlabeled complementary strand.
4. Unlabeled oligonucleotides for use as a specific competitor (same as above but without the Cy5 label).
5. Unlabeled oligonucleotides for use as nonspecific competitor. This could be an unrelated sequence or the same as above but with the putative target altered, e.g., changing A to C, G to T, C to A, and T to G.
6. TE buffer for resuspending the oligos (10 mM Tris pH 8.0, 1 mM EDTA).
7. 50 mM KCl.
8. 100 mM $MgCl_2$.
9. 1 M Tris pH 7.9.
10. 10× Tris-glycine buffer (0.25 M Tris pH 8.3, 1.92 M glycine).
11. Gel running buffer: 100 mL 10× Tris-Glycine buffer, 62.5 mL 80% glycerol, dH_2O to 1 L.
12. Gels containing 5% acrylamide (29:1 acrylamide:bis), 5% glycerol, 10% (v/v) 10× Tris-glycine buffer. These are made by standard protocols using 10% ammonium persulfate (APS), and *N, N, N′, N′*-tetramethylethylenediamine (TEMED) for polymerization.
13. Binding Buffer A for DNA dilution: 20 mM HEPES pH 7.9, 1 mM DTT, 8 mM $MgCl_2$, 0.1 mM $ZnCl_2$. When using nuclear extracts, add 1 μg poly(dI-dC) per 15 μL.
14. Binding Buffer B for protein dilution: 186 μL dH_2O, 75 μL 80% glycerol, 3 μL 2 M Tris pH 7.9, 30 μL 1 M KCl, 3 μL 0.1 M DTT, 3 μL 5 mg/mL bovine serum albumin.
15. Orange Loading Dye: 10 mM Tris pH 7.9, 15% Orange G, 60% glycerol.

3 Methods

3.1 Design of Promoter Fused Reporter Gene Constructs

1. Select a suitable vector system. We typically use pNP-GUS, which contains multiple cloning sites upstream of a promoterless β-glucuronidase (GUS) gene, along with the *nptII* marker which confers resistance to G-418. Other vectors can also be used, such as pGFPN [23] after deleting the *ham34* promoter (*see* **Note 1**).
2. Select a promoter region to fuse upstream of the reporter gene (*see* **Note 2**). This can be amplified by PCR using oligonucleotides containing restriction sites compatible with the vector. We normally add unique sites to the 5′ and 3′ ends of the promoter region to force directional cloning; check to ensure that those sites are not in the promoter region. An alternative is to have the sequence of interest synthesized; this is useful when mutagenized versions of the promoter are to be tested.
3. Digest the plasmid (e.g., pNP-GUS) and promoter insert with the appropriate restriction enzymes, and then purify the two DNAs using a commercial kit.
4. Mix the plasmid and insert in a 1:3 molar ratio and ligate. We normally perform ligations in a microcentrifuge tube containing 1 μL of 10× T4 DNA ligase buffer containing ATP, 1 μL (400 units) of T4 DNA ligase, 100 ng total of plasmid and insert DNA, plus water to 10 μL. Incubate overnight at 12 °C.
5. Mix 1–10 μL of the ligation reaction with 50 μL competent *E. coli* cells (e.g., DH5α), incubate on ice for 30 min, and heat at 42 °C for 90 s. Then, add 0.8 mL of LB broth and place in a shaking incubator at 37 °C for 1 h. Plate aliquots on LB agar containing 50–100 μg/mL ampicillin and incubate at 37 °C until colonies are observed, about 14–18 h.
6. Identify clones containing the desired insert by performing a small-scale plasmid prep followed by restriction digestion analysis and/or Sanger sequencing. Alternatively, identify clones with inserts on the original plates by PCR.
7. Prepare DNA for *Phytophthora* transformation using a scaled-up plasmid prep. In our hands, about 50 to 100 μg of DNA is typically adequate for obtaining enough transformants.

3.2 Phytophthora Transformation

Several strategies are available for transforming *Phytophthora* spp. and related oomycetes. With *P. infestans,* we have had the best success with protoplast-based and zoospore electroporation methods [23, 24]. While both appear equally satisfactory for promoter analyses, here we present an electroporation protocol.

1. Inoculate seven 15-cm rye-sucrose agar plates with ~10^4 sporangia. These are harvested from an 8- to 10-day-old 100-mm

culture plate by scraping with a sterile bent glass rod in 10 mL of sterile H_2O or Petri's solution. After counting with a hemocytometer, spread ~10^4 sporangia on each of the 15-cm plates with a sterile glass rod. Incubate in the dark at 18 °C (*see* **Note 3**).

2. After 8–9 days of growth, sporulation should have occurred. Harvest the sporangia by pouring 20 mL of ice-cold Petri's solution on each plate, followed by rubbing off the sporangia with the glass rod, and decanting the resulting milky solution into an empty 15-cm plate.
3. Induce zoosporogenesis by incubation at 10 °C for about 2 h (*see* **Note 3**).
4. To separate the zoospores from ungerminated or empty sporangia, filter through 15 μm nylon mesh into a 400-mL beaker. This and the following steps should be done on ice to retard zoospore encystment. Holders for nylon mesh are sold by Biodesign of New York. Alternatively, cell strainers that fit into 50 mL conical tubes can be used.
5. Transfer the zoospores into 50 mL conical tubes, add one-fiftieth of the volume of cold 5 M LiCl, and mix gently by inversion. Count the zoospore concentration using a hemocytometer.
6. Spin at 400× *g* for 5 min at 4 °C in a swinging bucket rotor.
7. Discard the liquid and resuspend the pellet by adding enough cold Petri's solution containing 0.1 M LiCl to set the concentration between 6×10^6 and 2.5×10^7 per mL.
8. For each reaction, gently mix 800 μL of zoospores with 30 μg DNA in a pre-chilled tube.
9. Pipette 800 μL of the zoospore-DNA mixture into a precooled 4 mm-gap cuvette. Close the cuvette, wipe the contacts dry with a tissue, and electroporate at 550 V, 1575 Ω, and 50 μF (*see* **Note 4**). A typical time constant is around 1.8 to 2.6 ms. If several cuvettes will be used with the same DNA, step 8 can be scaled up.
10. Immediately place the cuvette on ice and add 800 μL of regeneration media. Then, pipette the mixture into a 15 mL tube containing an additional 9 mL of regeneration media (*see* **Note 5**). Incubate the tube, lying on its side, at 18 °C for 20 h. Longer incubations may cause the resulting germinating cysts to stick together, making them difficult to spread on plates.
11. After the regeneration period, count the concentration of germinated cysts using a hemocytometer. Calculate the regeneration rate (germlings divided by the original number of zoospores per reaction); this normally ranges between 5 and 40%.

12. Spin the regenerated liquid cultures at 1000 × *g* for 5 min at room temperature. Decant the supernatant, retaining 1.6 mL of the liquid in the tube.
13. Resuspend the zoospores by gently pipetting, then spread 0.2 mL on each of eight 100-mm rye-sucrose agar plates containing 8 μg/mL G-418 (*see* **Note 6**). Incubate at 18 °C.
14. Colonies typically appear around day 8, and should be transferred to fresh plates containing G-418. It is prudent to check early so that colonies can be transferred before they overlap.

3.3 Identifying Transformants Expressing GUS by Histochemical Staining

1. Select several transformants for analysis. We typically aim to identify at least three GUS-expressing strains with a consistent expression pattern (*see* **Note 7**).
2. In a microcentrifuge tube, combine the GUS staining solution with the relevant *Phytophthora* sample which may include hyphae, sporangia, germinated cysts, oospores, plant tissue, etc. For spores, it may be useful to concentrate the tissue by gentle centrifugation. For hyphae, a small tuft of tissue scraped from the surface of a plant is usually sufficient. We normally perform the reaction in a volume of 50 μL, but add more staining solution as needed to keep the tissue submerged.
3. Incubate for 10 min to overnight; strong promoters may yield a useful signal (a blue color) after a few minutes while several hours may be needed for weak promoters. The signal will appear faster if incubation is performed at 37 °C, but good results can also be obtained at room temperature.
4. Visualize by light microscopy.

3.4 Quantitative GUS Assay

Precise measurements of GUS expression may sometimes be required. For example, promoter mutagenesis studies (Subheading 3, **step 5**) may result in a quantitative increase or decrease in expression not detected easily by histochemical staining, which is only semi-quantitative. Options for accurate measurements of the GUS reporter include enzymatic assays with colorimetric or fluorescent substrates, immunoblots, or RNA analysis. Here, we describe a fluorescent assay using 4-MUG as a substrate.

1. For studying spore stages, hyphae, or infected plant tissues, grind under liquid nitrogen using a mortar and pestle (*see* **Note 8**). Then, mix ~100 μl of frozen ground tissue with an equal volume of extraction buffer (100 μl buffer for every 100 μl of packed tissue) and vortex vigorously for 30 s.
2. For hyphae or plant tissues only, lysis may alternatively be achieved by homogenization in a mechanical tissue disrupter. We have had good success by adding a fivefold volume of cold extraction buffer to ~1 mL of loosely packed hyphae in a 15-mL polypropylene tube, following by 30 s at speed 7 in a Polytron (Kinematica) with a 5-mm diameter probe.

3. Spin the lysates from steps 1 or 2 at 12000× *g* for 10 min at 4 °C, and transfer the supernatant to a new tube.
4. Measure the protein concentrations in each extract, and then normalize all samples to the same concentration by adding extraction buffer. We normally use the Bradford or BCA methods for quantification [25].
5. For the fluorescence assay, add to a microcentrifuge 190 μL of extraction buffer containing 4-MUG and then 1 to 2 μg of the protein in 10 μL. Incubate at 37 °C for 1 h, and then add 1 mL of stop buffer and mix. For a time zero control, set up reactions in a similar manner but add the stop buffer immediately after the protein.
6. Make a standard curve by adding 200 μL of extraction buffer containing 0, 1, 4, 16, and 32 μM 4-MU to a microcentrifuge tube. Add 1 mL stop buffer and mix.
7. Measure the fluorescence at 365 mm excitation, 455 nm emission. We normally do this in a microplate reader by transferring 200 μl of each reaction into 96-well black plates.
8. Use the standard curve to calculate the specific activity of GUS enzyme (nmol MU/μg protein/min).

3.5 Promoter Mutagenesis

This is performed to identify functional regions, which are usually TF binding sites. Base changes or deletions at important locations should alter the expression pattern of the reporter gene unless redundant sites are present. As changes may be quantitative, the use of a reporter that is measured easily should be considered. These studies are often iterative, as one series of mutations may lead to testing of additional changes. These mutagenesis studies can be integrated with the gel shift experiments described in Subheading 3, **step 7**, which tests DNA for protein binding in vitro.

1. When genome data are available for related species, as is the case for *Phytophthora*, we recommend starting by aligning promoters from orthologs, i.e., phylogenetic footprinting. The logic is that functional regions should evolve more slowly than others, thus conserved regions would be candidates for mutagenesis (Fig. 2). Typically, we perform alignments of sequences from three to five species using MUSCLE or another alignment program [26]. Simultaneously, we recommend checking for over-represented motifs in co-expressed promoters using a program such as STREME [27]. The latter may be effective for cases where a TF-binding site occurs in each promoter but at unaligned sites (*see* **Note 9**).
2. Consider searching databases of known or predicted transcription factor binding sites. While this may yield false hits, the results may guide the mutagenesis strategy (*see* **Note 10**).

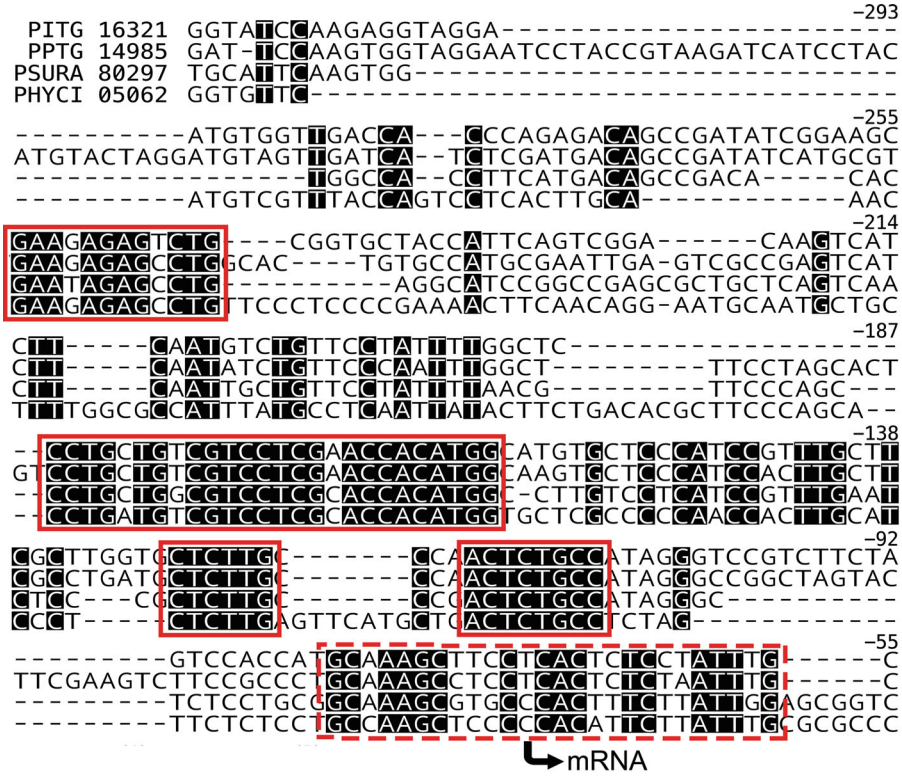

Fig. 2 Detecting conserved blocks within promoters. MUSCLE was used to align 500 nt of DNA upstream of the start codon of *P. infestans* gene PITG_16321 with its orthologs from *P. parasitica* (PPTG), *P. ramorum* (PSURA), and *P. cinnamomi* (PHYCI). The numbers correspond to the distance from the start codon in *P. infestans.* Major conserved blocks are outlined in red. These generally reside within 250 nt of the start codon and include the transcription start site (bent arrow, dashed box) and two to five upstream sites. The ends of the alignment were trimmed to save space

3. Based on results from the proceeding steps, clone modified versions into pNP-GUS. When the goal is to test regions internal to the promoter, we find it convenient to order synthetic DNA for insertion into pNP-GUS. When the goal is to generate truncate promoters at their 5′ or 3′ ends, the altered promoters are usually generated by PCR.
4. Introduce the resulting plasmids into *Phytophthora* by transformation as described in Subheading 3, **step 2**.
5. Test transformants for expression, comparing the mutagenized version with wild type, and interpret the results (*see* **Note 11**).
6. Conduct additional rounds of mutagenesis as needed to narrow down the functional sites.

3.6 Testing Motifs by Fusion to a Minimal Promoter

Many eukaryotic TF binding sites will drive transcription when cloned upstream of a minimal promoter [28]. Described below is a strategy that we have applied successfully to several motifs from *P. infestans,* using pNIFS-GUS (Fig. 3; *see* **Note 12**). The plasmid contains a minimal promoter comprised of 55-nt of DNA 5′ and 152-nt of DNA 3′ of the major transcription start site of the *PiNIFS* gene (Genbank accession AY751569), plus the *nptII* marker for selecting transformants. GUS expression does not occur in the absence of additional upstream sequences, but usually results if a site binding a TF with an activation domain has been added.

1. Select the motif for testing. This might be identified by methods in this chapter such as phylogenetic footprinting, deletion analysis, or EMSA. Motifs might also be identified from approaches such as over-representation analysis or SELEX-seq [15, 29].
2. Design a double-stranded oligonucleotide containing the motif, including restriction sites compatible with the vector at each end (e.g., *Xba*I and *Bam*HI). We typically design a 35-nt fragment in which the motif of interest resides close to the 5′ end. The oligonucleotides should be staggered such that cohesive ends will form after the following annealing step.

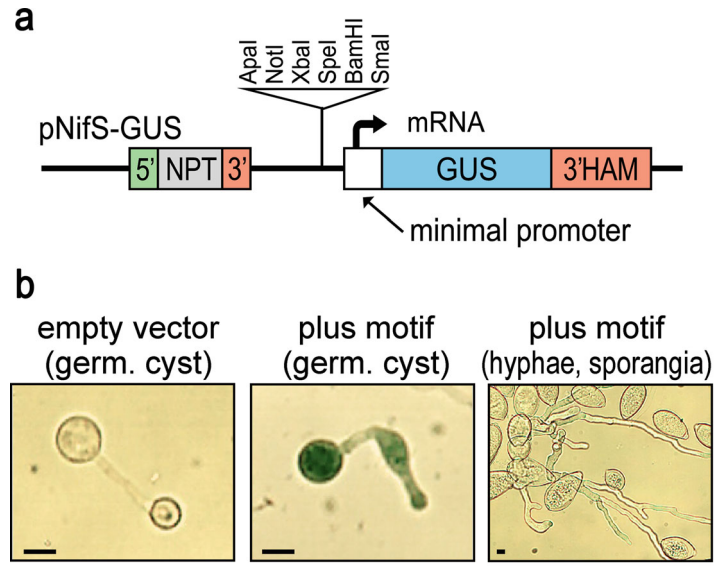

Fig. 3 Testing a motif with minimal promoter plasmid. (**a**) Linearized schematic map of pNIFS-GUS, which bears a minimal promoter from the *P. infestans* NIFS gene. (**b**) Testing the function of a 6-nt sequence previously associated with cyst germination based on motif over-representation analysis of co-expressed genes. When cloned into the *Xba*I and *Sma*I sites of the polylinker, GUS was detected in germinating cysts but not in sporangia or vegetative hyphae. Bars equal 5 μm

3. After obtaining the oligonucleotides, resuspend each in TE to a concentration of 100 μM.
4. Combine 10 μL of each strand in a single tube, and heat at 95 °C for 2 min. The mixture is then cooled to 4 °C over about 15 min. This can be done in a thermal cycler with a ramp program, or in steps by incubating the tube at 72 °C for 4 min, 37 °C for 4 min, 25 °C for 4 min, and then holding the tube at 4 °C.
5. Following the steps in Sect. 3.1.4, digest pNIFS-GUS with the appropriate restriction enzymes, combine with the annealed oligonucleotides, ligate, transform *E. coli,* and identify colonies containing the insert.
6. Transform the resulting plasmid to *Phytophthora* by transformation as described in Subheading 3, **step 2**.
7. Observe the pattern of GUS expression in the transformants and interpret the results (*see* **Note 13**).

3.7 Gel Shift Assays (EMSA)

This helps link TFs to their targets in the promoter, based on the principle that a protein-nucleic acid complex has less electrophoretic mobility compared to the free nucleic acid. The assay is straightforward although some TFs may exhibit weak binding and be challenging to study (*see* **Note 14**).

1. Identify the DNA to be tested. When testing a single motif, we normally use double-stranded DNA targets (dsDNAs) having a minimum size of about 35-nt, in which the site of interest (TF binding sites are typically 6–12 nt) is flanked by additional bases. These dsDNAs are typically made by annealing a Cy5-labeled oligonucleotide with a complementary unlabeled sequence. If greater sensitivity is desired, both strands may be labeled. Other fluorescent tags such as Cy3 or fluorescein, or a 5' ^{32}P label added using T4 kinase, may also be used. A dsDNA fragment lacking the target sequence should also be designed as a control, to be used as a nonspecific competitor in the binding reaction (*see* **Note 15**).
2. Obtain your protein sample, either a purified TF or a nuclear extract. For the latter, harvest and resuspend tissue in Nuclear Buffer 1. Homogenize on ice using 0.5-mm glass beads in a Bead-Beater, employing three strokes of 30 s, each separated by a 2 min cooling period. Subsequent steps should be at 4 °C. Add an equal volume of Nuclear Buffer 1 containing 1% Triton X-100 to the lysed tissues and incubate for 10 min. Pellet the nuclei at 8000 × *g* for 5 min and then wash twice with 25 mL Nuclear Buffer 1. Resuspend the pellet in 1.5 times the pellet volume of Nuclear Buffer 2 containing 0.5 mM PMSF. Next, add 4.75 volumes of water, followed by 1.25 volumes of 3 M KCl added dropwise. Stir gently for 30 min. Centrifuge for

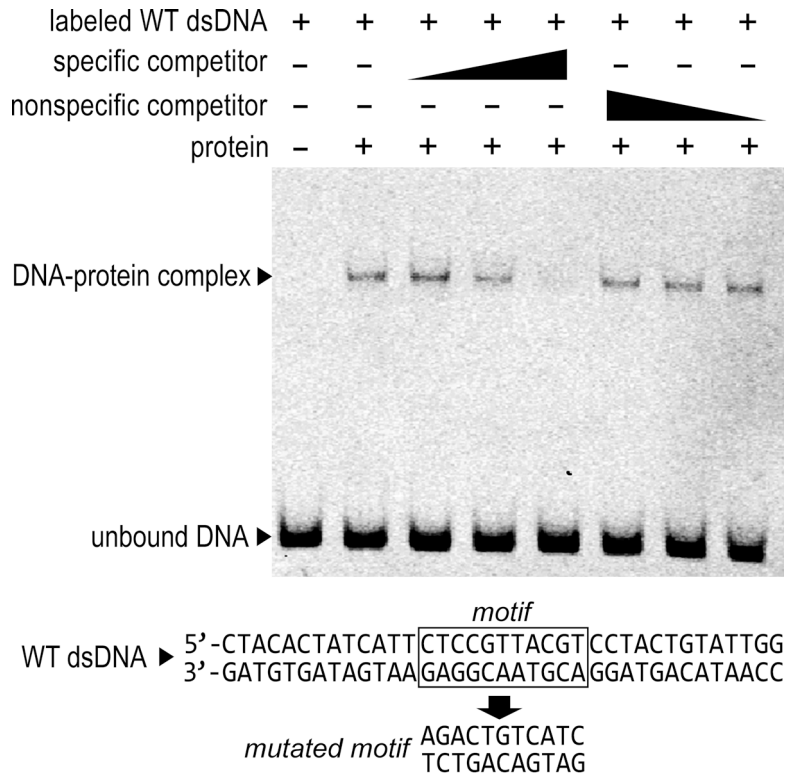

Fig. 4 Fluorescent EMSA. A TF was incubated with a 37-nt double-stranded Cy5-labeled oligonucleotide containing a candidate motif and subjected to electrophoresis. As indicated above the gel, some lanes included unlabeled 37-mer as a specific competitor or an unlabeled nonspecific competitor, which was the 37-mer with the motif mutated. The triangles represent competitors added at 1, 10, and 100-fold the concentration of the Cy5-labeled target. The sequences of the unmutated (WT) and mutated 37-mers are represented beneath the gel. Specific binding of the TF to the motif is indicated since only the specific competitor diminished the intensity of the retarded band, which represents the DNA-protein complex

30 min at 16,000× *g* to collect the supernatant, which should be dialyzed overnight against Nuclear Buffer 3. Finally, clarify by centrifugation at 16,000× *g* for 30 min. Store at −80 °C until ready to use.

3. Resuspend each oligonucleotide at 100 μM in TE buffer.
4. In separate tubes, anneal the three pairs of oligonucleotides. The first will be the Cy5-labeled target and its unlabeled complement; the second will be a specific competitor made from the same two sequences but without Cy5; and the third will be a nonspecific competitor control without Cy5. The latter could be random DNA of the same size, or the same sequence used for the Cy5 oligo but with the predicted target site altered, as

shown in Fig. 4. Set up the annealing reaction by combining 1 μL of each oligonucleotide, 1 μL of 1 M Tris pH 7.9, 2.5 μL of 50 mM KCl, 1 μL of 100 mM $MgCl_2$, and 43.5 μL of dH_2O. After heating for 3 min in a heat block at 95 °C, cool slowly by placing the block at room temperature (on the benchtop) for 1 h. Alternatively, the mixture can be cooled in steps in a thermal cycler or a series of heat blocks as in Sect. 3.6.4. Run a small amount of the reaction on a 5% polyacrylamide gel to confirm the generation of a band of the expected size.

5. Cast the 5% polyacrylamide gel. We normally use 7-cm tall gels with 1.5-mm spacers and wells 5-mm in width.
6. Prepare the DNA and protein samples, keeping each at 4 °C. The optimal concentrations of Cy5-labeled DNA and protein will vary with each transcription factor, so we advise that these be measured through titration experiments (*see* **Note 16**). A typical starting point would be a final concentration of 15 nM DNA and 150 nM purified protein (or 6 μg of nuclear extract protein) in a 20 μl reaction. When a nuclear extract is used, we include 1.3 μg of poly (dI-dC) to reduce non-specific binding.

   Once the optimal concentrations are established, the assays are assembled by adding the appropriate DNA to 10 μl of Binding Buffer A, the protein to 10 μl of Binding Buffer B, and then combining the two tubes. This should result in the following mixtures:

   (a) Cy5-labeled dsDNA alone.
   (b) Cy5-labeled dsDNA plus protein.
   (c) Cy5-labeled dsDNA with an equal amount of the same unlabeled DNA, plus protein.
   (d) Cy5-labeled dsDNA with a tenfold excess of the same unlabeled DNA, plus protein.
   (e) Cy5-labeled dsDNA with a 100-fold excess of the same unlabeled DNA, plus protein.
   (f) Cy5-labeled dsDNA with an equal amount of control DNA, plus protein.
   (g) Cy5-labeled dsDNA with a tenfold excess of control DNA, plus protein.
   (h) Cy5-labeled dsDNA with a 100-fold excess of control DNA, plus protein.

   The concentration of the Cy5-labeled dsDNA should remain the same in each of the eight reactions. Reactions c to e represent those with the specific competitor, while f to h represent those with the nonspecific competitor.

7. Incubate at room temperature for 15 min in the dark.
8. Add 2 μL of Orange Loading Dye to each reaction and load 19 μL on the gel. Run for 30 min at 15 V/cm (110 V for a 7-cm gel) at room temperature (*see* **Note 17**).
9. Detect the signal using a scanner. For Cy5, we use 633 nm excitation, 670 nm emission.
10. Interpret the results. Binding will be signaled by a retarded band (Fig. 4). Specific binding will be indicated if the signal drops off proportionately with the amount of unlabeled specific competitor. The nonspecific competitor should have less of an effect, although it might partially reduce the intensity of the retarded band since most TFs have some nonspecific DNA-binding ability. Depending on the application, the results may lead to an additional round of EMSA with altered dsDNAs to refine the definition of the TF binding site.

4 Notes

1. GUS should provide a stronger result than a fluorescent reporter since the former benefits from enzymatic amplification of its signal. Histochemical staining for GUS also does not require the instrumentation required for measuring fluorescence. However, the staining profiles may be deceptive since the assay is only semiquantitative. Also, weak signals may be detected with difficulty in small cells such as zoospores. One reason for this is that the initial reaction product, 5-bromo-4-chloro-indoxyl, must dimerize with its tautomer in an oxidation reaction that forms the blue precipitate, 5,5′-dibromo-4,4′-dichloro-indigo. The initial product may diffuse from the cell prior to dimerization if GUS levels are very low, although the ferro- and ferricyanates are included to accelerate dimerization by catalyzing the oxidation reaction [30]. Do not be tempted to leave these out of the reaction! This problem does not occur with fluorescent protein reporters, which unlike GUS are amenable to live imaging. One issue with both types of reporters is that they have long half-lives, with GUS appearing to be more stable than GFP at least in plants [31]. This helps to augment their signals but may complicate some temporal studies. Destabilized reporters have been developed for some taxa but not yet for oomycetes [32].
2. Most promoters of protein-coding genes in eukaryotes range in size from a few hundred bases to about 1 kb [33]. This does not include distal enhancer sites which in mammals may be tens of kb 5′ or 3′ of the coding sequences [34]; whether such sites exist in oomycetes is unknown. We generally start our

experiments by testing the 500 bases upstream of the translation start site or the intergenic region, whichever is smaller. Phylogenetic footprinting may also suggest the size of the functional promoter (Sec. 3.5.1), which can be tested by 5′ deletion analysis. Identifying the transcription start site is also recommended. While this can be defined by traditional techniques such as 5' RACE, it can be estimated by mapping RNA-seq reads, which are now widely available for many *Phytophthora* spp. including *P. infestans* [35].

3. These times may vary depending on the strain or species. While *P. infestans* normally sporulates as cultures age, other species may require treatments with light or starvation. The goal is to obtain young synchronous sporangia that will effectively release zoospores within a fairly narrow window of time (typically 1.5 to 2 h after chilling for *P. infestans*). A simple 10 °C chamber for chilling can be made by filling a plastic tub with ice, covering the ice with a 3 to 6-mm acrylic sheet upon which the plates of sporangia are positioned, and then covering the apparatus with an inverted tub.
4. These parameters work well for most strains of *P. infestans*. Changes in the electrical conditions may be needed for species making larger or smaller zoospores to achieve the optimal time constant and voltage drop across the cell.
5. To prevent contamination, we often add an antibacterial (e.g., 50 μg/mL penicillin G) and antifungal (e.g., 40 units/mL nystatin) to the media.
6. This concentration works well for most strains of *P. infestans*. Adjustments may be needed depending on the species, strain, or batch of chemical.
7. It is prudent to examine multiple transformants to avoid artifacts due to position effects or variation in copy number [36]. This will also reduce the possibility of drawing a false conclusion if the reporter has integrated adjacent to another promoter. Alternatively, use CRISPR [37] to insert the reporter into a safe harbor [38]. Promoter swaps using CRISPR have been reported in *Phytophthora sojae,* but note that these run the risk of being lethal if the targeted gene was essential [39].
8. Protein extracted from ~150 μg of tissue is usually sufficient. For hyphae, it is convenient to use either liquid cultures or grow the strain on top of a 0.4 μm pore size polycarbonate filter laid on top of agar media; the hyphae can be easily peeled off the membrane.
9. It is essential to use orthologs and not paralogs, since many genes belong to families in which members may show diverse patterns of transcription. Moreover, the promoters should be

suitably distant (typically <70% identical in alignments) to allow non-conserved and thus likely nonfunctional sites to be distinguished from conserved blocks. We usually start by aligning promoters from at least five species, and then eliminate species if needed to optimize the discrimination of the analysis. Alignments are usually effective at revealing conserved blocks as shown in Fig. 2, although modifying gap penalties may help to optimize the alignment. However, DNA rearrangements may have moved TF binding sites to locations that would remain unaligned. Such rearrangements are not uncommon, even within a species [40]. Programs such as STREME, which uses expectation maximization to identify over-represented words, may succeed in identifying conserved motifs even within a set of rearranged promoters [27]. This approach may also be useful for studying promoters where orthologs are unavailable, as is the case for some effectors. In such cases, over-represented motifs (putative TF binding sites) may be detected within promoters showing similar patterns of activity [15].

10. Several papers have reported predicted or experimentally proven regulatory sites within *Phytophthora* promoters [15, 41–43]. There may also be utility in searching the extensive databases that exist for TFs from model plants, animals, and fungi [44]. Our experimental analysis of the DNA binding sites of over 100 *P. infestans* TFs indicates that many resemble those of TFs in the same family from other kingdoms. However, be aware that many hits in databases will be false positives [45].
11. Possible results include a loss of stage or condition-specific transcription due to altering the binding of a stage-specific activator, expression in a new pattern due to eliminating a repressor site, or a quantitative reduction if a motif bound by a general transcription factor was deleted. No change means either that the mutated site was nonfunctional or redundant.
12. This was named p74NIF in our prior publications based on expressed sequence tag data that indicated that it included 74-nt of DNA 5′ of the transcription site [14]. More accurate techniques have shown that the start site is 27–29 nt further upstream.
13. There are limitations to this method. While sites that bind TFs with activation domains usually yield visible GUS expression, the signal is usually lower than that obtained with the complete promoter due to the absence of binding sites for general transcription factors. It is also possible that no reporter may be made if the TF binding the motif must operate in combination with other regulatory elements. In addition, TFs acting as repressors will not yield results in this assay.

14. Our protocol has succeeded with multiple TFs, but no single combination of protein extraction, binding, or electrophoresis conditions may work well for all [46]. For example, protein-nucleic acid interactions are sensitive to pH and the levels of mono- and divalent salts. While our protocol uses a Tris-HEPES buffer at an approximately physiological ionic strength and pH, and on ice, some TFs may bind better under alternative conditions. Our inclusion of bases flanking the putative TF binding site is meant to strengthen the DNA-protein interaction as the TF might "sit down" better on a longer target. However, the longer template may bear non-specific binding sites, which can be a drawback when binding the DNA to a complex nuclear extract. The longer targets also migrate more slowly on gels which provides more time for the desired complex to dissociate, although this could be mitigated by running the gel in the cold or for a shorter time. Another example of a challenge is when binding occurs only when the TF includes a post-translational modification such as phosphorylation, and thus it may be prudent to include a phosphatase inhibitor during extractions.
15. Poly (dI-dC) can also be used as a nonspecific competitor [47].
16. Titration can improve the results of EMSA, particularly when using a purified TF [46]. If the protein concentration is too low, a signal will not be detected while if too high, the DNA competitor may not have an effect. We typically perform titrations using DNA at about 25 nM in Binding Buffer A and proteins ranging from 0 to 300 nM in Binding Buffer B.
17. It may be useful to pre-run the gel until the current stabilizes (20–30 min) to remove persulfate ions which may weaken the protein-DNA complex. If a certain ion or other molecule was found to be important for binding, this could be included in the electrophoresis buffer to stabilize the complex. This is why glycerol is present both in the running buffer and gel, as this fortifies most protein-DNA interactions.

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