Isolation of Powdery Mildew Haustoria from Infected Barley

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[Abstract] *Blumeria graminis* is a fungus that causes powdery mildews on grasses, such as barley. Investigations of this pathogen present many challenges due to its obligate biotrophic nature. This means that the fungus can only grow in the presence of a living host plant. *B. graminis* forms epiphytic mycelia on the plant surface and feeding organs (haustoria) inside the epidermal cells of the host plant. Therefore, it is difficult to separate the fungus from plant tissues. This protocol shows how to obtain different fungal structures from powdery mildew infected barley leaves. The epiphytic mycelia including conidia and conidiophores can be separated after immersing the infected leaves into 5% cellulose acetate dissolved in acetone, and peeling off the cellulose acetate membrane. Then, the haustoria are isolated from dissected epidermis after cellulase degradation of plant cell walls. The isolated haustoria remain intact with few plant impurities. The haustoria may be visualized by epifluorescence microscopy after staining with the chitin-specific dye WGA-Alexa Fluor 488. Finally, dissected material can be either processed immediately or kept at -80 °C for long-term storage for studies on gene expression and protein identification, for example by mass spectrometry.

Keywords: Powdery mildew, Barley, Epiphytic, Epidermis, Haustoria isolation, Cellulase, WGA-AF 488

[Background] Powdery mildew of cereals, caused by *Blumeria graminis*, leads to significant yield loss (Spanu *et al.,* 2010). The barley powdery mildew fungus *B. graminis* f. sp. *hordei*, is economically important, and one of the best studied powdery mildews (Both *et al.*, 2005a; Bindschedler *et al.*, 2009). This fungus is an obligate biotroph, which means it can only infect and propagate on living barley plants (Pedersen *et al.*, 2012). Its asexual life cycle involves an intimate relationship with the host plant. Airborne conidia germinate on the host producing first a primary and then a secondary germ tube. The secondary germ tube develops an appressorium, from which a hyphal peg penetrates into an epidermal cell. Inside the cell, a penetration peg enlarges and forms a multidigitate feeding structure: the haustorium. This is surrounded by a plant-derived extrahaustorial membrane (Both *et al.*, 2005b; Bindschedler *et al.*, 2016).

Several studies of *B. graminis* f. sp*. hordei* have characterized gene expression profiles and proteomics of this fungus (Bindschedler *et al.*, 2011; Pennington *et al.*, 2016). However, the obligate biotrophic nature of *B. graminis* poses exceptional challenges to the investigations of this fungus, especially the studies of haustoria. The intracellular haustorium and the extrahaustorial membrane constitute a special compartment, which is functionally essential for the interaction with the host (Wang *et al.*, 2009; Pliego *et al.*, 2013). Investigating haustoria is critical for further understanding the mechanism of nutrient uptake into the pathogen as well as plant-pathogen recognition. Therefore,

obtaining purified haustoria from infected plants has the potential of contributing significantly to these studies.

Previously, the procedure for isolating haustoria from obligate biotrophs employed affinity chromatography on rust fungi (Catanzariti *et al.*, 2006; Garnica *et al.*, 2014). During the 1990s, several centrifugation-based methods were introduced to isolate powdery mildew haustoria from pea plants (Mackie *et al.*, 1991; Testut *et al.*, 1999). This method was then applied to powdery mildews of other plants, including *Arabidopsis* (Wang *et al.*, 2009; Micali *et al.*, 2011). More recently, a filtration and gradient centrifugation-based method was described to isolate powdery mildew haustoria from barley (Godfrey *et al.*, 2009). The hallmark of these published methods is that they are relatively laborious and require several different kinds of isolation buffers, different sizes of steel meshes and multiple centrifugation steps. Besides, the isolation procedure was required to be performed on ice. In this protocol, we developed a simpler way to isolate *B. graminis* haustoria from infected barley leaves. Releasing of haustoria from plant cells is achieved by enzymatically degrading the epidermal cell walls, and the purification of haustoria by filtration through a nylon mesh. This method requires digestion of the host plant cell walls, a single filtration and one centrifugation step.

Materials and Reagents

1. Pipette tips
2. Dedicated enclosed transparent container
3. Razor blade
4. Parafilm
5. Pasteur pipettes
6. 15 ml/50 ml polypropylene tubes
7. Liquid nitrogen container
8. Microscope glass slide
9. Cover glass
10. Barley (*Hordeum vulgare*) cv. Golden Promise seedlings, barley seeds were sown in John Innes 1 compost mixed with vermiculite (4:1) in 12 x 12 cm pots
11. Liquid nitrogen
12. Cellulose acetate
13. Anhydrous acetone
14. MES hydrate powder
15. HCl
16. WGA, Alexa Fluor 488 conjugate (Thermo Fisher Scientific, catalog number: W11261)
    Note: This dye exhibits the bright, green fluorescence of the Alexa Fluor® 488 dye (excitation/emission maxima ~495/519 nm).
17. Cellulase Onozuka R-10 (Duchefa Biochemie, catalog number: C8001)

Note: "Cellulase Onozuka R-10" from Trichoderma viride.1 unit (U) of Cellulase will release 1.0 µmole of glucose from carboxymethyl cellulose.

18. NaCl
19. KCl
20. Na_2HPO_4
21. KH_2PO_4
22. PBS buffer (see Recipe 1)

Equipment

1. Forceps
2. Pipettes
3. Scissor
4. Tweezers
5. 70 µm nylon mesh sieve
6. Incubator
7. Centrifuge
8. GFP filter
9. Epifluorescence microscope
10. -80 °C freezer

Procedure

A. Dissection of infected barley leaves
1. Grow barley (*Hordeum vulgare*) cv. Golden Promise seedlings in a 16-h light/8-h dark cycle at 20 °C. It is essential to keep the plants well-watered, as drought stress prevents effective dissection of the epidermis.
2. Maintain the powdery mildew fungus (*B. graminis* f. sp. *hordei*) on living barley plants in a dedicated enclosed transparent container protected from air currents that may displace the conidia. Remove old conidia by shaking the inoculum pots one day before infecting healthy plants.
3. Inoculate seven-day barley seedlings. At this stage, the primary leaves are fully extended. The inoculation *B. graminis* is carried out by shaking heavily infected plants over the uninfected barley. The plants will be ready for dissection seven days after inoculation.
   Note: It is highly recommended that a tight-fitting face mask be used when carrying out this procedure to prevent conidia from entering the operator's airways.
4. Prepare scissor, tweezers, razor blade, Parafilm, Pasteur pipettes, 15 ml/50 ml polypropylene tubes and liquid nitrogen. Place two 50 ml tubes in a liquid nitrogen container and fill with liquid nitrogen.

5. Dissolve 5 g of cellulose acetate in 100 ml anhydrous acetone. Pour the solution into a 15 ml polypropylene tube.

6. Dissolve 0.2 g of MES hydrate powder in 100 ml H_2O to make 10 mM MES buffer. Adjust the pH to 5.3 with HCl. Pour 20 ml of MES buffer into a 50 ml tube at room temperature.

7. Cut one infected primary leaf and immerse it in 5% cellulose acetate acetone, holding it at the leaf base. Carefully take out the leaf and wait for a few seconds above the mouth of the tube to remove any cellulose acetate acetone droplets accumulating at the leaf tip. Then place the leaf onto two Pasteur pipettes.

8. Repeat the above step five times. Let the acetone evaporate from the leaf surface for 5-8 min.
   Note: This step is critical: the cellulose acetate membrane needs to be dry enough to allow peeling; however, if the leaves are left too long they dehydrate and wilt rendering the dissection of the epidermis impossible.

9. As the acetone evaporates, the epiphytic mycelia-containing cellulose acetate layer will harden and begin to peel away from the rest of the leaf. Now peel off the cellulose acetate membrane with the epiphytic fungal structures embedded in it. Keep the leaves stripped of epiphytic structures on water for 5 min to ensure full hydration.

10. Following this, turn the leaf so the adaxial surface faces upwards. Press a razor gently near the tip of the leaf, carefully rock the blade back and forward.
    Note: This step is challenging and requires some trial and error to gain sufficient experience to achieve correct dissection. The aim is to cut through the upper, adaxial epidermis and part of the mesophyll, while retaining the lower, abaxial epidermis intact.

11. Turn the leaf over to let the abaxial side facing upwards. While gripping the leaf at its tip (this forms a 'tab' which can be used to pull the epidermis) and holding the section of the leaf below the cut steady, pull back the abaxial epidermis which contains fungal haustoria.
    Note: Free the epidermal layer by pushing away other layers with forceps, if needed.

12. Once free, carefully peel the abaxial epidermis in a continuous smooth motion.

13. Once complete, take away the tip of the leaf (the 'tab') by cutting the epidermis and removing it with the tweezers.
    Note: To isolate haustoria from epidermis, skip to Step B1.

14. Snap freeze peeled epiphytic and epidermal materials separately in two 50 ml polypropylene tubes in liquid nitrogen for future use.

15. Store at -80 °C till needed.

B. Haustoria isolation

1. Float peeled epidermis in 15 ml 10 mM MES hydrate buffer (pH 5.3) in a 50 ml tube.

2. Once sufficient epidermis strips are collected (we used one pot in Video 1), add 200 mg of Cellulase R-10 into 10 ml MES buffer in a new 50 ml tube. Mix thoroughly, so the final concentration of cellulase is 2% (w/v).

3. Carefully transfer all dissected epidermis with tweezers into the new mixture.

4. Incubate the suspension at 28 °C for two hours with gentle shaking (*e.g.*, 80 rpm on a rotating platform). During incubation, the cellulase will lyse the barley epidermal cells and release haustoria into the incubation buffer.

5. After incubation, place a 70 µm nylon mesh sieve over a 50 ml tube. Slowly pour the buffer through the filter while using forceps as a barrier to retain larger pieces of undigested plant material.

6. Once all the buffers are collected, centrifuge in a swing out rotor at 3,270 *x g* for 10 min, at 4 °C.

7. After centrifugation, the pellet should contain haustoria.

8. Pour off the supernatant and retain the pelleted haustoria.
   Note: After this step, the haustoria can either be kept at -80 °C for long-term storage or stained immediately to visualize (continue with Procedure C).

C. WGA-Alexa Fluor 488 staining (optional for quality control)
   1. Gently resuspend the haustorial pellet in 300 µl of PBS buffer (pH 7.4) (Recipe 1).
   2. Add WGA-Alexa Fluor 488 into the mixture to a final concentration of 10 µg/ml.
   3. Mix well by inverting the tube several times.
   4. Load the mixture onto a microscope glass slide and carefully place a cover glass on top.
   5. Visualize under the GFP filter with an epifluorescence microscope.
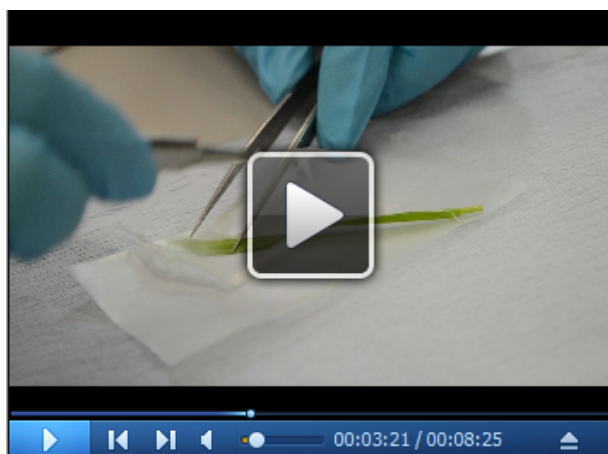

Video 1. Visualization of the protocol as a video recording of the procedures

Data analysis

Representative results: The protocol described above provides an efficient enzymatic way to obtain *B. graminis* haustoria from the host plant, barley. All materials obtained using this protocol, including epiphytic mycelia-contained acetate stripes, haustoria-present epidermal peels and purified haustoria, can be stored at -80 °C for long-term storage till needed for further analysis. One pot of heavily infected barley (roughly 80 leaves) yields approximately 1.5 g of epiphytic material (N.B. this includes the cellulose acetate film, which constitutes the major part of this fraction, by

weight), 0.3 g of epidermis and 3-4 x 10^5 haustoria (the actual number of haustoria obtained will depend strongly on the extent to which the leaves were infected). The quality of isolated haustoria can be observed by epifluorescence microscopy after staining by WGA-Alexa Fluor 488. Most of the isolated haustoria are highly branched and intact (Figure 1A). Sometimes the separated haustorium is still surrounded by the plant-derived extrahaustorial membrane, as shown in Figure 1B (arrow). Plant fragments which are small enough can also pass through the 70 μm nylon mesh sieve, so limited amount of impurities are present in pelleted haustoria complex, for example chloroplast (Figure 2, arrow).
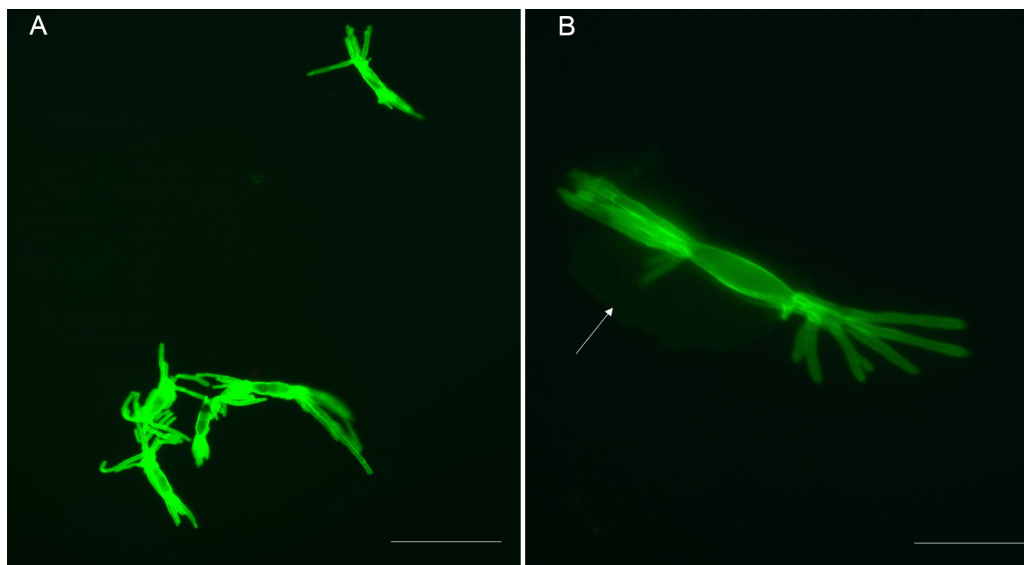
Figure 1. Isolated haustoria visualized after staining with WGA-Alexa Fluor 488. A. Stained haustoria (fluorescent green) visualized by epifluorescence microscopy and observed through a "GFP" filter. Multiple intact and mature haustoria. Scale bar = 40 μm. B. This shows both the multidigitated haustorium and a perihaustorial membrane surrounding the haustorium (arrow). Scale bars = 10 μm.

Figure 2. An isolated haustorium with some impurities. The pellet formed after filtration and centrifugation not only consists of haustoria, but also some plant debris, for example chloroplasts (autofluorescent red, arrow). These impurities could be chloroplasts and cell wall fragments. Scale bar = 10 μm.

We tested several options for overall digestion time in cellulase: 1 h, 2 h, 3 h and overnight. Overnight incubation yielded the highest number of haustoria: approximately 5 x 10^5 (this was considered the maximum amount of haustoria we can achieve), but also more fragments of plant material which reduced the purity of isolated haustoria. One-hour incubation only released less than 30% of the maximum obtained after overnight digestion. Digestions of 2 h and 3 h incubation yielded 3-4 x 10^5 haustoria, which accounts for > 80% of the maximum.

Discussion: Powdery mildew fungi are challenging to study because they have an absolute requirement for a host plant to grow and develop. This is especially true of the haustoria, the feeding structures which develop inside the host plant epidermal cells (Catanzariti *et al.*, 2006; Micali *et al.*, 2011). Several affinity isolation methods of powdery mildew haustoria have been previously published, including one modified method on *B. graminis* (Mackie *et al.*, 1991; Micali *et al.*, 2008). However, the method demonstrated for *B. graminis*, which combined gradient centrifugation and multiple filtrations, produce a pellet with 80% haustoria; the rest were reported to be cell wall fragments and chloroplasts (Godfrey *et al.*, 2009). Isolating protoplast from different plants in previous studies is mostly achieved by a combination of enzymes, including cellulose (Sun *et al.*, 2013; Wu *et al.*, 2017). To obtain relatively pure and intact haustoria from barley powdery mildew, we developed a rapid and effective method which relies on the dissection of infected epidermis from the rest of the leaf followed by degradation of the plant cell walls and lysis of the epidermal cells using cellulase.

Maximizing the number of haustoria present in infected leaves is important when developing the protocol. Barley cv. Golden Promise was chosen to maintain the fungi due to its high susceptibility

to powdery mildew. An appropriate number of seeds were sown to ensure there are enough plants while the density of the seedling is not too high to interfere with a saturating inoculation of the primary leaves. In these studies, we used approximately 3 g of seeds for a 10 cm-diameter pot. In order to ensure a high infection rate, conidia that are older than one day need to be removed from the plants used as a source of the inoculum. In this way, we ensure that only fresh conidia (< 1 day old) are used, leading to high percentage of successful germination, penetration and finally haustoria formation. The effectiveness of epidermis dissection is critical for the success of haustoria isolation. The young leaves are easier to dissect: there is a trade-off between more advanced stages of infection (yielding more haustoria) and younger leaves (yielding more intact epidermis). In practice, we have found that using plants 7 days post inoculation (dpi) as an ideal compromise. In addition, the epidermis of the abaxial side of primary leaf is the easiest to dissect compared to either the adaxial epidermis, or to the epidermis of other leaves. We therefore routinely dissect only the abaxial epidermis of the primary leaves.

To optimize the condition of haustoria isolation, several different incubation times and enzyme concentrations have been tested. Increasing concentration of cellulase (higher than 2.5%) and a combination of various enzymes (cellulase and macerozyme) have been proven effective in degrading cell walls, however, they were found to be costly and led to low viability of isolated structures (Wu *et al.*, 2017). Accordingly, we used 2% of cellulase to break down the cell walls for releasing haustoria. The time of incubation in cellulase was critical to obtain high yield of isolated haustoria and low contamination from plant derived structures. After comparing different incubation times, we suggest that 2 h is a suitable compromise, for most purposes.

This protocol utilized WGA-AF 488 to stain isolated haustoria rather than the traditional method of using Coomassie blue. The lectin moiety binds to fungal chitin and rendered the fungal structures visible as green fluorescent structures due to the Alexa 488 fluorophore clearly distinguishable from plant derived material. If required, it would be possible to visualize the contaminating plant cell wall residues by staining with Calcofluor White.

We believe this protocol may be applied to other powdery mildews with suitable modifications.

Recipes

1. 1x PBS buffer
   8 g NaCl
   0.2 g KCl
   1.44 g Na_2HPO_4
   0.24 g KH_2PO_4
   Dissolved in 800 ml distilled water
   Adjust the pH to 7.4 with HCl
   Add distilled water to a total volume of 1 L

Acknowledgments

Competing interests

The authors declare that there are no competing interests in the work described here.

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