

Novel *Phakopsora pachyrhizi* Extracellular Proteins Are Ideal Targets for Immunological Diagnostic Assays

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Phakopsora pachyrhizi, the causal agent of Asian soybean rust (ASR), continues to spread across the southeast and midsouth regions of the United States, necessitating the use of fungicides by producers. Our objective in this research was to identify ASR proteins expressed early during infection for the development of immunodiagnostic assays. We have identified and partially characterized a small gene family encoding extracellular proteins in the *P. pachyrhizi* urediniospore wall, termed PHEPs (for *Phakopsora* extracellular protein). Two highly expressed protein family members, PHEP 107 and PHEP 369, were selected as ideal immunodiagnostic targets for antibody development, after we detected PHEPs in plants as early as 3 days postinfection (dpi). Monoclonal antibodies (MAbs; 2E8E5-1 and 3G6H7-3) generated against recombinant PHEP 369 were tested for sensitivity against the recombinant protein and extracts from ASR-infected plants and for specificity against a set of common soybean pathogens. These antibodies should prove applicable in immunodiagnostic assays to detect infected soybeans and to identify ASR spores from sentinel surveillance plots.

Phakopsora pachyrhizi, the causal agent of Asian soybean rust (ASR), continues to spread across the southeast and midsouth regions of the United States following introduction in 2004 (25). Due to a lack of resistance to the pathogen in commercial cultivars of soybean, producers must rely on fungicides to combat the disease. In order to make timely, cost-effective decisions regarding the applications of fungicides throughout the growing season, growers, extension agents, and crop consultants need inexpensive, presymptomatic diagnostic decision tools. Our objectives in this research were to identify ASR extracellular proteins expressed early during infection and apply them to the development of *P. pachyrhizi*-specific immunological reagents for sensitive diagnostic assays.

We recently applied a proteomic approach to identify proteins expressed during germination of *P. pachyrhizi* urediniospores using two-dimensional gel electrophoresis (2DE) and matrix-assisted laser desorption ionization with tandem time of flight (MALDI-TOF/TOF) mass spectrometry of tryptic peptides (17). In that study, we identified a set of extracellular soluble proteins present in urediniospores, prior to and during germination. The peptides were used to identify corresponding cDNA clones from an expressed sequence tag (EST) sequence database derived from germinating *P. pachyrhizi* urediniospores (22). The clones were found to be members of a gene family which we have named PHEP (for *Phakopsora* extracellular protein), containing at least six members. Two highly expressed proteins, PHEP 107 and PHEP 369, were selected for further study as possible targets for immunodiagnostic assays.

We describe here the characterization of the PHEP protein family and development of immunospecific polyclonal (PAb) and monoclonal (MAb) antibodies raised against recombinant PHEP family members with potential as diagnostic reagents for early detection of *P. pachyrhizi*.

MATERIALS AND METHODS

Rust spores. Urediniospores of *Phakopsora pachyrhizi* or *Phakopsora meibomia* (Table 1) from the international collection maintained at the For-

eign Disease-Weed Science Research Unit were used in this study. Approximately 0.6 g of each individual spore isolate was germinated under sterile conditions for 18 h in a Pyrex dish containing 600 ml of water supplemented with 50 µg/ml of ampicillin and 50 µg/ml of streptomycin.

Protein extraction. For Western blots, soluble protein fractions from fungal spores were extracted as follows: 0.6-g samples were ground in liquid nitrogen, suspended in phosphate-buffered saline (PBS) (24) supplemented with 3 mM dithiothreitol (DTT) and 0.2% sodium dodecyl sulfate (SDS), heated at 95°C for 5 min, and centrifuged at 14,000 × g for 5 min, and supernatant was collected. Aliquots were stored at -20°C. Protein quantification was performed as described by Markwell et al. (19) using bovine serum albumin (BSA) as a standard.

Soybean pathogens. Extracts from soybean pathogens and infected plants for indirect enzyme-linked immunosorbent assay (ELISA) were generated as described by Baysal-Gurel et al. (3).

Plant material. Soybean (*Glycine max* cv. Williams 82) and Fordhook bush lima bean (*Phaseolus lunatus*) were grown in potting soil under containment greenhouse conditions (18 to 30°C, 12-h day length, supplemented when needed with lighting from 800-W metal halide lamps at 24 inches). At the 2- to 3-trifoliolate-leaf stage, soybeans and lima beans were spray inoculated with 0.375 mg/ml of *P. pachyrhizi* isolate TW72-1 and *P. meibomia* isolate Puerto Rico, respectively. After inoculation, plants were placed in a dew chamber at 21°C overnight and returned to the greenhouse. One-square-inch infected leaf samples were collected at 0 (preinoculation), 3, 5, 7, and 14 days postinfection (dpi), flash frozen in liquid nitrogen, and stored at -80°C. Leaf sample soluble proteins were extracted as described above.

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TABLE 1 *Phakopsora* isolates used in this study

Species	Isolate	Country (state) or territory/yr	GenBank accession no. ^a
<i>Phakopsora pachyrhizi</i>	TW 72-1	Taiwan/1972	JQ284010
<i>Phakopsora pachyrhizi</i>	TH 02-1	Thailand/2002	JQ284011
<i>Phakopsora pachyrhizi</i>	IN 73-1	India/1973	JQ284012
<i>Phakopsora pachyrhizi</i>	SA 01-1	South Africa/2001	JQ284013
<i>Phakopsora pachyrhizi</i>	PG 01-3	Paraguay/2001	JQ284014
<i>Phakopsora pachyrhizi</i>	AL 04-1	U.S. (AL)/2004	JQ284015
<i>Phakopsora meibomiaie</i>	PR	Puerto Rico/1976	

^a GenBank accession numbers for PHEP 369 genomic sequences.

PHEP genomic sequences. Genomic DNA was extracted from the *P. pachyrhizi* isolates listed in Table 1, using the DNeasy plant minikit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. Full-length PHEP 369 genes were generated by PCR using the primer set 5'-ATG GGA AAA GTT ATC ATC AAT GTG-3' and 5'-CTT TCC AGC CTT TGC TTT TTC ATC-3'. Fragments were directly sequenced using the BigDye Terminator version 3.1 cycle sequencing kit (ABI, Foster, CA) according to the manufacturer's protocols.

Production of recombinant PHEP (rPHEP) proteins and generation of antibodies against rPHEP 107 and rPHEP 369. Recombinant proteins were produced under contract (National Cancer Institute—Frederick, Protein Expression Laboratory, Ft. Detrick, MD). Open reading frames (ORFs) for PHEP 107 and PHEP 369 were generated by reverse transcriptase PCR (RT-PCR) with RNA isolated from germinating spores of *P. pachyrhizi* isolate TW72-1 using the primer sets 5'-ATG GGA AAA GTT ATC ATC AAT GTG-3' and 5'-CTT TCC AGC CTT TGC TTT TTC ATC-3' for PHEP 107 and 5'-ATG GGA AAA GTT ATC ATC AAT GTG-3' and 5'-CTT TCC AGC CTT TGC TTT TTC ATC-3' for PHEP 369. Both ORFs were cloned into pDest-521 expression vector containing an amino-terminal His-6 tag (Invitrogen, Carlsbad, CA) and overexpressed in *Escherichia coli* BL21(DE3) cells. Proteins were purified by immobilized metal affinity chromatography using HisTrap Phast columns (GE Healthcare, Piscataway, NJ) according to the manufacturer's protocols. Purified recombinant proteins were used to generate polyclonal antibodies (PAb) in New Zealand rabbits using a commercial provider (Bushover Biologicals, Vassalboro, ME). After the 4th bleed, titer was judged to be appropriate for experiments and production bleeds. Immunoglobulin G (IgG) fractions were purified on immobilized protein A using the manufacturer's protocols, diluted to 1 mg/ml, and stored at -80°C. Recombinant protein PHEP 369 was also used to generate monoclonal antibodies (MAbs) in mouse using a commercial provider (GenScript, Piscataway, NJ) applying standard protocols for murine hybridoma generation, selection, and screening (14). Cell line culture medium samples were screened with rPHEP 369, and IgG fractions were purified by the manufacturer from 1 liter of cell culture medium and stored at -80°C at a dilution of 3 mg/ml. ELISA was used to titer MAbs against rPHEP protein to arrive at working dilutions of 1:5,000 for Western blot analyses.

Western blotting. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 4 to 12% bis-Tris gels (Invitrogen) and transferred to 0.2- μ m-pore-size nitrocellulose membranes using a semidry blotter apparatus (Owl Separation Systems, Woburn, MA) according to the manufacturer's guidelines. After transfer, blots were blocked in 3% (wt/vol) dry milk in PBS-0.02% (vol/vol) Tween 20 (PBS-Tw) for 1 h and probed with anti-rPHEP PAb at 1:5,000 and anti-rPHEP 369 MAbs at 1:5,000 overnight at 4°C (29). Blots were washed 3 times for 5 min in 100 ml PBS-Tw. Blots were then probed with horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit IgG (Sigma Chem Co., St. Louis, MO) at 1:10,000 for 1 h, washed 3 times in 100 ml PBS-Tw, and detected using Super Signal West Pico chemiluminescent substrate (Thermo Scientific, Rockford, IL) according to the manufacturer's protocol.

TABLE 2 MAb PHEP 369 ELISA and Western blot reactions with extracts of common soybean pathogens, related rust fungi, and infected plants

Pathogen	Source	Reactivity ^a	
		MAb 2E8E5-1	MAb 3G6H7-3
<i>P. pachyrhizi</i>	Ungerminated spores	+++	+++
<i>P. pachyrhizi</i>	Germinated spores	+++	+++
<i>P. pachyrhizi</i>	Inoculated soybean	+++	+++
<i>P. meibomiaie</i>	Ungerminated spores	+++	-
<i>P. meibomiaie</i>	Germinated spores	-	-
<i>P. meibomiaie</i>	Inoculated lima bean	+	-
<i>Uromyces appendiculatus</i>	Germinated spores	-	-
<i>Uromyces appendiculatus</i>	Inoculated common bean	-	-
<i>Puccinia punctiformis</i>	Germinated spores	-	-
<i>Puccinia graminis</i>	Ungerminated spores	-	-
<i>Puccinia graminis tritici</i>	Inoculated plant material	-	-
<i>Fusarium graminearum</i>	Inoculated plant material	-	-
<i>Peronospora manshurica</i>	Inoculated plant material	-	-
<i>Pseudomonas syringae</i> pv. <i>phaseolicola</i>	Inoculated plant material	-	-
<i>Xanthomonas campestris</i> pv. <i>glycines</i>	Inoculated plant material	-	-
<i>Ustilago tritici</i>	Inoculated plant material	-	-
<i>Erysiphe polygoni</i> DC	Inoculated plant material	-	-
<i>Septoria lycopersici</i>	Inoculated plant material	-	-
<i>Pseudomonas syringae</i>	Inoculated plant material	-	-
<i>Cercospora soja</i>	Inoculated plant material	-	-

^a Absorbance readings of less than 0.049 (equivalent to background values) are designated -, readings between 0.050 and 0.300 are designated +, those between 0.310 and 0.800 are designated ++, and those greater than 0.810 are designated +++.

ELISA. Samples for indirect ELISA were prepared as described by Baysal-Gurel et al. (3), diluted 1:1 (vol/vol) in carbonate coating buffer, and used at 40% (wt/vol) for plant and 1% (wt/vol) for plant pathogens. One hundred microliters of each sample was added to a polyvinyl chloride plate and incubated overnight at 4°C. Plates were washed 2 times in 200 μ l PBS, blocked in 100 μ l of 3% dry milk in PBS at room temperature for 1 h, and washed 3 times in 200 μ l PBS. One hundred microliters of primary antibody at a 1:5,000 dilution was added per well and incubated at room temperature for 2 h. Plates were washed 3 times in PBS, and alkaline phosphatase (AP)-conjugated goat anti-mouse antibody was added at a concentration of 1:2,000 and incubated at room temperature for 2 h. Plates were then washed 3 times in PBS, 100 μ l of AP substrate was added, and absorbance was read at 405 nm in a microtiter plate reader at 15, 30, and 60 min. The results reported in Table 2 are from the 30-min reading.

Immunofluorescence localization. Spores were germinated for 4 to 18 h on glass slides in a moist chamber and incubated in 100 μ l primary antibody at 1:500 in PBS-Tw for 1 h at room temperature. Slides were washed 3 times by swirling in 25 ml PBS-Tw in a petri dish on a rocking platform. Secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG [H+L] and Alexa Fluor 514 goat anti-mouse IgG [H+L] [2 mg/ml; Invitrogen, Carlsbad, CA]) was added at a 1:1,000 dilution in PBS-Tw and incubated at room temperature for 1 h. Control slides (no primary antibody) were treated and analyzed as well. Slides were washed 3 times by swirling in 25 ml PBS-Tw in a petri dish on a rocking platform, and fluorescence was visualized on a Nikon E 600 fluorescence microscope using a Nikon B2E/C filter set optimized for fluorescein isothiocyanate (FITC) (excitation of 465 to 495, emission of 515 to 555, barrier of 505 nm) (Nikon, Tokyo, Japan). Image capture was performed with a Diagnostics Instruments real-time monochrome charge-coupled-device (CCD) camera system using SPOT Advanced software version 3.2.4 (Diagnostics Instruments, Sterling Heights, MI).

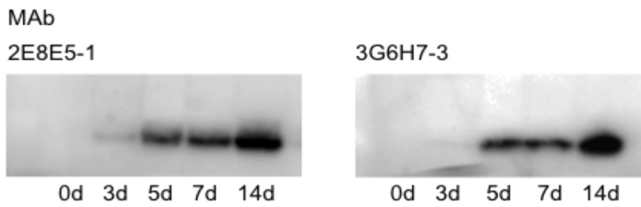


FIG 3 Western blot analysis of ASR-infected soybean probed with MAb1 and MAb3. Twenty-four-day-old soybean plants, cultivar Williams 82, were inoculated with isolate TW72-1 and collected at days 0 (uninoculated), 3, 5, 7, and 14 postinoculation. Gels were loaded with 30 μ g per lane total protein.

Motif searches applying nonclassical (leaderless) secretion algorithms (5, 6) produced neural network output scores exceeding the normal threshold given for SecretomeP motif searches (12) and also revealed putative carbohydrate and phosphate-binding domains, which may explain why multiple forms of PHEP 369 were identified on 2DE. Lectin blots performed by probing urediniospore proteins separated by 2DE with concanavalin A provided preliminary evidence for mannose-binding domains on PHEP 369 (see Fig. S3 in the supplemental material).

Kyte-Doolittle plots (not shown) displayed clear evidence of hydrophobicity that was evident in regions of the PHEP genes rich in alanine, isoleucine, leucine, and valine (15). Hydrophobic regions were localized to the most conserved regions, while the highest variability was evident in the most hydrophilic domains. Antigenicity plots generated using the ABIE Pro online tool (version 3.0; Chang Biosciences, Castro Valley, CA) revealed numerous peptide sequences as prime candidates for antibody design.

Antibody reactivity and specificity. Polyclonal antibodies were generated against rPHEP 107 and rPHEP 369 proteins, and IgG fractions were purified for characterization studies. Recombinant proteins could be detected on dot blots by chemiluminescence detection at levels as low as 100 pg at an antibody dilution of 1:5,000. By Western blot analysis, we found that the anti-rPHEP 107 and anti-rPHEP 369 PABs were not completely specific. Anti-rPHEP 107 PAB was highly reactive with rPHEP 107 and moderately reactive against rPHEP 369. By comparison, anti-rPHEP 369 PAB was highly reactive with rPHEP 369 but only slightly with rPHEP 107 (Fig. 2).

Monoclonal antibodies were then generated against the purified rPHEP 369 protein. Six MAb cell lines were selected after two rounds of screening with rPHEP 369 protein. Two MAb cell lines were specific to rPHEP 369 (3G6H7-3 and 3G6E11) and did not cross-react with rPHEP 107, whereas two MAbs (2E8E5-1 and 2E8H6) reacted with both recombinant proteins. Western blots

illustrated the reactivity and specificity for 2 MAbs with rPHEP 107 and 369 (Fig. 2). Using ELISA to determine the sensitivities of MAbs, 300 pg of rPHEP 369 protein was detected at a dilution of 1:3,000 of either 2E8E5-1 (MAb1) or 3G6H7-3 (MAb3) when used as primary antibodies, and 1 ng of rPHEP 369 protein was detected at a dilution of 1:26,000 of either MAb1 or MAb3 when used as primary antibodies. PHEP 369 in leaf soluble protein extracts from soybean inoculated with 0.375 mg/ml of *P. pachyrhizi* isolate TW72-1 could be detected by Western blotting within 3 dpi using MAb1 and within 5 dpi using MAb3 (Fig. 3).

The specificities of MAb1 and MAb3 to extracts from plants infected with common U.S. soybean pathogens, extracts of cultured common soybean pathogens, and extracts of spores of related rust fungi were evaluated using ELISA and Western blot analysis and are illustrated in Table 2. MAb1 and MAb3 did not react with extracts from plants infected with common U.S. soybean pathogens, extracts of cultured common soybean pathogens, or extracts of spores of related rust fungi. MAb1 was reactive against *P. meibomia* ungerminated spores and slightly reactive against *Phaseolus lunatus* (lima bean) infected with *P. meibomia* collected at 14 dpi during sporulation with numerous pustules (Fig. 4) but did not react with germinated spores of *P. meibomia*.

Immunolocalization and immunofluorescent assays (IFA).

An electron microscopy study was undertaken to establish the localization of PHEP proteins in *P. pachyrhizi* urediniospores. Recombinant PHEP PABs were chosen for the study because of their reactivity to spores.

Gold-labeled PABs localized PHEP 369 to echinulations (spines) on the surface of ungerminated *P. pachyrhizi* urediniospores (Fig. 5). Gold particles are evident in the spines protruding from the urediniospore wall. Overall, we observed that most spines contained multiple particles.

Immunofluorescence assays with urediniospores prior to and during germination confirmed the localization of PHEP 369 to echinulations on the *P. pachyrhizi* urediniospore surface using PAB 369 (Fig. 6A, B, C) and MAb 2E8E5-1 (Fig. 6D, E, F). Controls without primary antibody showed minimal autofluorescence.

Protein functional analysis. Considering the sequence analysis of PHEP family members and absence of any known homologues, the functional role of PHEP proteins in fungal development, infection, and interaction with the host is unclear. Attempts were undertaken to establish whether high concentrations of r107 or r369 proteins applied to a soybean leaf surface could induce a phenotypic response. Neither rPHEP 107 nor rPHEP 369 induced a hypersensitive or other visible phenotypic response when infiltrated at quantities up to 1 μ g into leaves of susceptible soybean

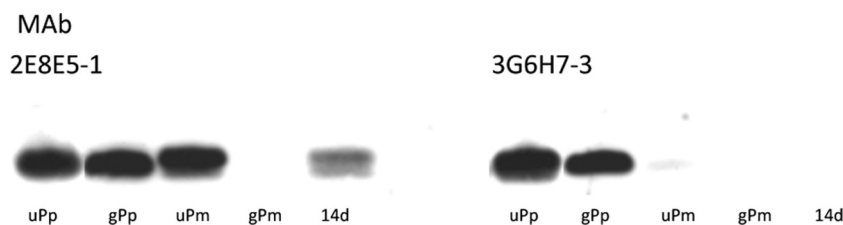


FIG 4 Western blot analysis of *Phakopsora* proteins in urediniospores and infected plants probed with MAb1 and MAb3. Urediniospore proteins were loaded at 200 ng/lane (*P. pachyrhizi*) and 1,000 ng/lane (*P. meibomia*) total protein; infected lima bean leaf extracts were loaded at 30 μ g/lane total protein. Abbreviations: uPp and gPp, ungerminated and germinated *P. pachyrhizi* urediniospore proteins, respectively; uP and gPm, ungerminated and germinated *P. meibomia* urediniospore proteins; 14d, *P. meibomia*-infected lima bean 14 days after infection.

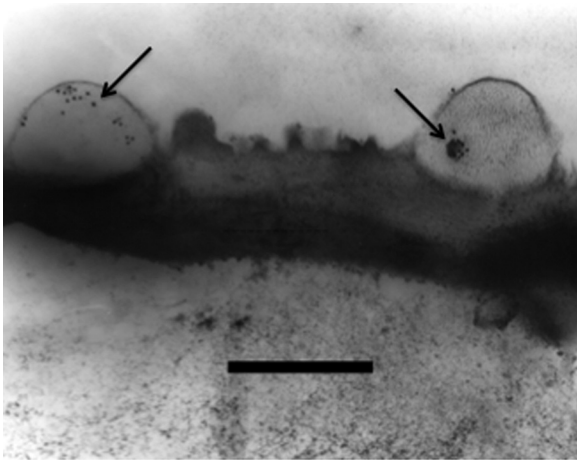


FIG 5 Immunocytochemical localization of PHEP 369 protein in echinulations (spines) of the *P. pachyrhizi* urediniospore wall using MAb1.

cultivar Williams (see Fig. S4 in the supplemental material). When rPHEP 107 and rPHEP 369 were added to inoculum at the same concentration, they did not prevent subsequent infection (see Fig. S5 in the supplemental material).

We then asked whether the presence of PAb r369 during spore germination could disrupt critical stages of the infection process. In experiments incubating *P. pachyrhizi* urediniospores with PAb 369, we observed no consistent effect of the recombinant protein on urediniospore germination (see Table S1 in the supplemental material) or adhesion to detached leaves (see Table S2 in the supplemental material).

DISCUSSION

We have identified a small gene family of extracellular proteins that are localized to the *Phakopsora* cell wall. PHEP 107 and 369 are unique low-molecular-weight proteins with no significant similarity to any amino acid or DNA sequence entries in public databases. Evidence reported in this study suggests that these extracellular proteins are associated with *P. pachyrhizi* urediniospore walls. We found it surprising that no homologues exist in the genomes of other rust fungi.

None of the PHEP family members have RxLR motifs characteristic of some fungal effector peptides (7, 13). No evidence was found for a role in recognition by the host or subsequent virulence during infection, and inhibition assays with antibodies did not indicate a role in germination, growth, or appressorium formation. Although we are not able to assign a function to the two PHEP family members described in this study, we cannot rule out a role in cell wall expansion during growth and development of the germinating urediniospore, as our antibodies may not have penetrated the cell wall during inhibition assays with live, unfixed leaf tissue. The apparently high titers of PHEP 107 and 369 as indicated by spot intensity by 2DE (17) may suggest a possible structural role for these proteins in the echinulations of the spore wall and the spore germling cell wall.

Secreted fungal proteins have largely been studied from the perspective of their roles in adhesion, pathogenesis, and virulence, with early studies focusing on lytic and degradative enzymes with roles in penetration of plant host cuticle and cell wall components (23). Recent progress has been made in genetic and proteomic cataloguing of fungal secretomes (8, 9, 16, 31). Few studies have identified and definitively localized extracellular proteins to the

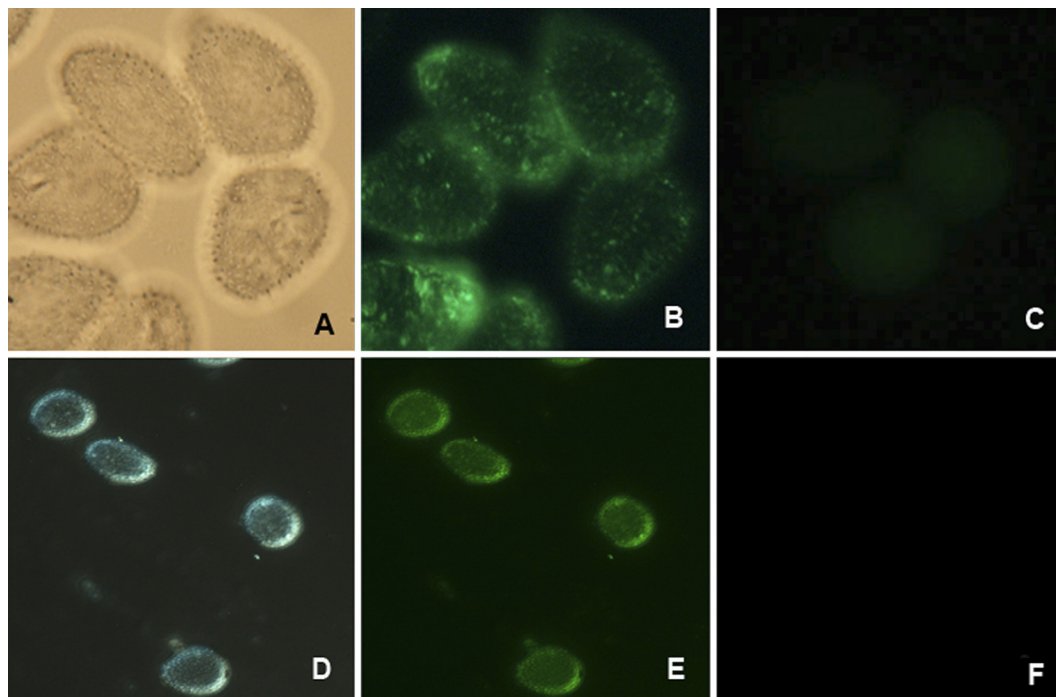


FIG 6 Immunofluorescence of germinating spores with MAbs and PABs. (A) MAb1, white light; (B) MAb1, fluorescence; (C) MAb1, control (no primary antibody), fluorescence; (D) MAb3, phase contrast, backlighting; (E) MAb3, phase contrast, fluorescence.; (F) MAb3, control (no primary antibody), phase contrast, fluorescence.

cell wall of obligate fungal pathogens (21) and defined a functional role to the protein(s) (1, 26, 28).

While extracellular proteins in fungal spore and cell walls have been common targets for specific immunochemical diagnostic reagents, few have been directed against surface protein targets in obligate rust, bunts, and smut fungi (11, 18).

Antibodies made to PHEP proteins provide ideal candidates for the development of *P. pachyrhizi* species-specific immunodiagnostic assays. To our knowledge, rPHEP PABs and MABs are the first antibodies designed to react against a set of unique *P. pachyrhizi* proteins. Antibodies produced in previous studies were generated against complex mixtures of intact urediniospores and pulverized germinated spores (3, 20, 30). Based on our results, MAB 3G687-3 is species specific for *P. pachyrhizi*, and MAB 2E8ES-1 is genus specific for *Phakopsora*. While both MAB cell lines detected ASR in presymptomatic leaves, it must be noted that the artificially high infection rates we employed to produce soybean leaf samples for ELISA and Western blot analysis may not represent diagnostic samples typically encountered under field conditions with low to moderate disease pressure.

The antibodies should prove applicable in ELISA and handheld lateral flow diagnostic assays with infected soybeans, although this may be dependent upon the level of infection. Given the excellent reactivity in immunofluorescence assays, the MABs should prove as highly specific probes to identify fungal spores trapped in soybean rust sentinel surveillance plots. Both MABs should also prove useful as tools to quantify fungal biomass in infected leaves, with potential application in screening soybean lines for slow rusting and resistance.

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