Immunodiagnostic assays targeted to urediniospore wall proteins of Asian soybean rust

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INTRODUCTION

Phakopsora pachyrhizi, the causal agent of Asian soybean rust (ASR), continues to expand in range in the southeast and mid-south regions of the U.S., resulting in increased fungicide applications for producers.

Our objectives in this research were to identify ASR protein targets for development of immunodiagnostic assays, preferably expressed *in planta* at all stages of the infection process.

We previously identified and characterized a small family of extracellular proteins in the *P. pachyrhizi* urediniospore wall, termed PHEPs (for PHakopsora Extracellular Protein), representing potential candidates for immunodiagnostic reagents.

APPROACH

One protein family member, PHEP 369, was selected as an ideal immunodiagnostic target after localization studies confirmed its extracellular location in the spore wall, and Western blot analysis using polyclonal antisera raised against PHEP 369 detected PHEP 369 in soybean plants as early as 3 days after infection.

Monoclonal antibodies ('MAbs') 2E8E5-1 and 3G6H7-3 generated against recombinant PHEP 369 were tested for sensitivity against the recombinant protein and ASR-infected plant extracts, and for specificity against a set of viral, bacterial and fungal soybean pathogens.

MATERIALS AND METHODS

Production of Recombinant Proteins and Monoclonal Antibodies – A PHEP 369 full-length open reading frame was generated by RT-PCR using RNA from P. pachyrhizi isolate TW 72-1. The PCR product was cloned into the His6-tagged vector pDest-521 (Invitrogen). Proteins were purified by immobilized affinity chromatography using HisTrap columns (GE Healthcare). The purified recombinant protein was used to generate monoclonal antibodies in mouse (GenScript, Piscataway, NJ.). IgG's were purified from one liter of cell culture media and stored at -80° C.

Spore and Plant Material – Extracts from urediniospores of selected rusts, cultured soybean pathogens and infected plants were used in experiments. Samples were ground in liquid nitrogen, suspended in PBS supplemented with 3mM DTT and 0.2% SDS, heated at 95° C for 5 minutes, centrifuged at 12,000g for 5 minutes and supernatant was collected. Aliquots were stored at -20° C. Protein quantification was performed using the Markwell assay (Markwell, et.al. 1978) with BSA as a standard.

Western Blotting - Proteins were separated by SDS-PAGE on 4-12% Bis-Tris gels and transferred to 0.2 µM pore size nitrocellulose membranes using a semi-dry blotter apparatus (Owl Separation Systems, Woburn, MA) according to manufactures guidelines. After transfer, blots were blocked in 3% (w/v) dry milk in PBS-0.05% (v/v) Tween-20 for 1 h, and probed with anti-PHEP 369 MAbs at 1:5000. Blots were washed 3X for 5 minutes in 100 mL PBS-0.05% Tw. Blots were then probed with goat anti-mouse IgG conjugated HRP (Sigma Chem. Co., St. Louis, MO) at 1:10,000 for 1 h and detected by chemilumenescence.

Immunofluorescence Localization of Proteins – Spores were germinated for 4 to 18 hours on glass slides in a moist chamber, then incubated in 100uL primary antibody at 1:500 in PBS-0.1% (v/v) Tw for 1 hr at room temperature. Slides were washed 3X in 25 mL PBS-Tween in a Petri Dish on a rocking platform. Secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG (H+L)(2 mg/ml) – Invitrogen) was added at 1:500 dilution in PBS-Tw and incubated at RT for 1 hr. Slides were washed 3X in 25 mL PBS-Tw and visualized on a Nikon E 600 fluorescence microscope using a B2E/C filter set optimized for FITC (Excitation= 465-495, Emission=515-555, Barrier 505 nm). Image capture was performed with a Diagnostics Instruments RT monochrome CCD camera Spot camera system using SPOT Advanced software v. 3.2.4.

ELISA – Samples were extracted in PBS, diluted one to one in carbonate coating buffer and used at a concentration of 40ug/100ul for plant and 1ug/100ul for plant pathogens. One hundred microliters of each sample was added to a PVC plate and incubated overnight at 4°C. Plates were washed 2X in 200ul PBS, blocked in 100ul of 3% dry milk in PBS at room temperature for 1 h and washed 2X in 200ul PBS. Primary antibody was diluted to 1:5000, 100ul was added pre well and incubated at room temperature for 2 h. Plates were washed 2X in PBS and secondary antibody was added at a concentration of 1:2000 and incubated at room temperature for 2 h. Finally plates were washed 2X in PBS and absorbance was read at 405nm using alkaline phosphotase substrate.

Figure 1. MAb PHEP 369 reaction with recombinant PHEP 369 and 107.

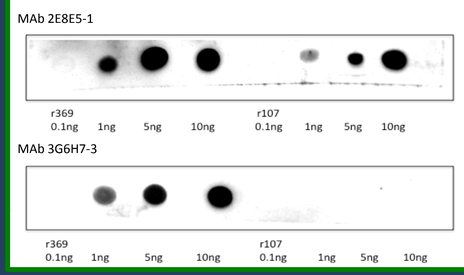
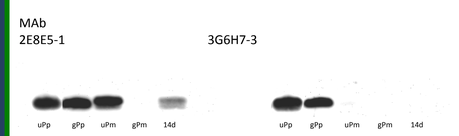


Figure 2. MAb PHEP 369 reaction with extracts from spores of *P. pachyrhizi, P. meibomiae*, and *P. meibomiae*-infected lima bean.



Note: u - ungerminated spores, g – germinated spores, Pp – *P. pachyrhizi*, Pm – *P. meibomiae*

Figure 3. MAb PHEP 369 reaction with extracts of *P. pachyrhizi*-infected soybean.

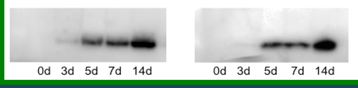


Table 1. MAb PHEP 369 ELISA and Western Blot reactions with extracts of common soybean pathogens, related rust fungi and infected plants.

		REACTIVITY	
PATHOGEN	**SOURCE**	**MAb 2E8E5-1**	**MAb 3G6H7-3**
P. pachyrhizi	ungerminated spores	+++	+++
P. pachyrhizi	germinated spores	+++	+++
P. pachyrhizi	inoculated soybean	+++	+++
P. meibomiae	ungerminated spores	+++	-
P. meibomiae	germinated spores	-	-
P. meibomiae	inoculated lima bean	+	-
Uromyces appendiculatus	germinated spores	-	-
Uromyces appendiculatus	inoculated common bean	-	-
Puccinia punctiformis	germinated spores	-	-
Puccinia graminis	ungerminated spores	-	-
Puccinia graminis tritici	inoculated plant material	-	-
Rose rust	inoculated plant material	-	-
Fusarium graminearum	inoculated plant material	-	-
Downy mildew	inoculated plant material	-	-
Pseudomonas syringae pv. phaseolicola	inoculated plant material	-	-
Xanthomonas campestris pv. glycines	inoculated plant material	-	-
Ustilago tritici	inoculated plant material	-	-
Erysiphe polygoni DC	inoculated plant material	-	-
Septoria lycopersici	inoculated plant material	-	-
Pseudomonas syringae	inoculated plant material	-	-
Cercospora sojina	inoculated plant material	-	-

RESULTS

MAb 2E8E5-1 demonstrated high specificity to r369 and r107 proteins (Figure 1), *P. pachyrhizi* spores, *P. pachyrhizi* infected soybean, *P. meibomiae* ungerminated spores and *P. meibomiae* infected lima bean (Figure 2 and Table 1).

MAb 3G687-3 was specific to r369 protein (Figure 1), extracts from *P. pachyrhizi* spores and infected soybean (Figure 2 and Table 1). MAb 3G687-3 did not react with r107 protein, extracts from *P. meibomiae*, related rust fungi or any of the soybean pathogens tested.

Interestingly, MAb 2E8E5-1 reacted with ungerminated spores and infected leaf material - but not germinated spores - of *P. meibomiae.*

Both MAbs detected *P. pachyrhizi* isolate TW72-1 in soybean plants within 3 days post inoculation using Western Blot (Figure 3).

Using ELISA, 300pg of r369 protein was detected using both MAbs at a dilution of 1 to 3,000 primary antibody. One ng of r369 protein was detected at a dilution of 1 to 26,000.

No other related rust fungi , soybean pathogens or infected plants reacted with either MAb by western blot or ELISA.

Figures 4A-D. Immunofluorescence of *Phakopsora pachyrhizi* urediniospores treated with MAb PHEP 369.

Figure 4A – MAb 2E8E5-1 FITC conjugate

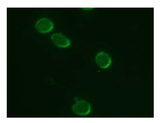


Figure 4C – MAb 3G6H7-3 FITC conjugate

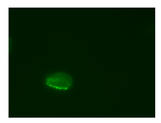


Figure 4B – MAb 2E8E5-1 white light

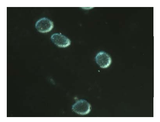
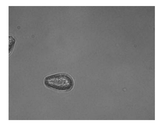


Figure 4D – MAb 3G6H7-3 white light



CONCLUSIONS

Based on our results, MAb 3G687-3 is species-specific for *P. pachyrhizi*, and MAb 2E8ES-1 is genius-specific for *Phakopsora*.

Both MAb cell lines detected ASR in pre-symptomatic leaves.

FUTURE DIRECTIONS

These antibodies will prove applicable in ELISA and hand-held lateral flow diagnostic assays with infected soybeans, and to identify fungal spores trapped in national soybean rust sentinel surveillance plots.

Both MAbs should prove useful as tools to quantify fungal biomass for soybean resistance screening.

The MAbs also should prove useful in elucidating the role of PHEPs in infection, germination, adhesion, penetration, and host recognition.

COLLABORATORS

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- Western Illinois University: Herb Edwards

SUPPORT

- Iowa Soybean Association
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- USDA-ARS
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- DOE- Joint Genome Institute

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