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GENETIC CROSSTALK AND ITS EFFECTS ON VIRULENCE AND MATING IN *USTILAGO MAYDIS*.

By

John Desmarais

Submitted in Partial Fulfillment of the Requirements for Graduation *summa cum laude* and for Graduation with Honors from the Department of Biology

University of Louisville

Louisville, Kentucky

March 25, 2018

Abstract

Ustilago maydis is a model organism for study of fungal mating and host infection. Two compatible haploid mating types must mate to form a dikaryon in order for the fungus to infect its host, corn (*Zea mays*). There are a variety of genetic mechanisms that regulate mating and infection in the fungus, many of which coregulate both processes. The aim of this study was to investigate how alteration of certain regulatory proteins in *U. maydis* affects these basic processes as well as how alteration of genes involved in signaling pathways can affect the expression of one another. Primarily, the focus was on exploring the role of the *ptn1* gene. This gene was disrupted and therefore found to be involved in tumorigenesis and teliospore development during infection. Additional disruption of the *ust1* and *unh1* genes, which play opposite roles in tumorigenesis, was attempted on these *ptn1* deletion strains of *U. maydis* using a high fidelity CRISPR-Cas9 system to examine the influence of these genes on the *ptn1* pathway. Further experiments were intended to generate *ptn1* deletion mutant strains of *U. maydis* in which the *pdc1* gene, encoding a major protein involved in cellular regulation, was simultaneously overexpressed. Phenotypes of *pdc1* overexpression mutants of *U. maydis* were examined for both effects on *U. maydis* cells in culture and during plant infection. *Ptn1* deletion strains were found to be more susceptible to cell wall stress, displayed lesser aerial hyphae formation during mating, and showed reduced infection symptoms including reduced tumor size and reduced number of tumors. Overexpression of *pdc1* led to no changes in mating or virulence in wild type strains but may exert an added effect on *ptn1* deletion strains and their corresponding phenotypes.

Introduction

U. maydis maydis is an obligate fungal parasite of *Zea mays* (corn) and is one of the most useful genetic model organisms for the study of fungal host-pathogen interaction mechanisms. Like many other fungal species, *U. maydis* must undergo a dimorphic change from two compatible haploid mating types to an infectious dikaryotic form to infect its host. Infection by the fungus elicits the production of galls or tumors in all above-ground parts of the plant and inside these galls the reproductive spores of *U. maydis* develop. In mammals, the *PTEN* signal transduction pathway is involved in the regulation of biological processes such as programmed cell death, metabolism, cell growth, cell proliferation, and suppression of tumorigenesis. The *PTEN* ortholog in *U. maydis*, *ptn1*, is believed to be involved in a signal transduction cascade that regulates mating, tumorigenesis and spore development during infection. An ongoing study in the lab is aimed at investigating the role of the *ptn1* signal transduction pathway in plant infection by *U. maydis* through functional characterization of the *ptn1* gene. Mutant strains of *U. maydis* in which *ptn1* has been deleted display a decrease in virulence, including a decrease in tumorigenesis and tumor size. Moreover, there is a severe reduction in spore production in the tumors that do result from infection by the *ptn1* mutants (Cid et al. 2008; Vijayakrishnapillai et al. 2018).

Additional literature revealed two other genes involved in infection and tumor production by *U. maydis*, *unh1* and *ust1*. The *unh1* gene is similar to *ptn1* in that it is required for tumor maturation and spore development (Doyle et al. 2016); in contrast, *ust1* prevents spore formation in haploid fungal strains and places a limit on tumorigenesis and tumor size during infection (Chacko et al. 2009; García-Pedrajas et al. 2010). Other experiments have found that *ust1* gene

deletion disruption mutants display abnormal morphology and pigmentation, such that haploid fungal strains without a copy of the gene appear grittier and darker and diploid double deletion strains produce darker, more melanized tumors during infection (Islamovic et al. 2015). An aim of this study was to examine how deletion of these genes would affect virulence and mating of mutant *U. maydis* strains in which the *ptn1* gene was already deleted.

Mutant strains of *U. maydis* were made where *ptn1* was deleted using a homologous recombination construct with a hygromycin resistance marker. CRISPR-Cas9 deletion constructs were used to attempt *ptn1* expression disruption as well (Schuster et al. 2016, 2018; Shi et al. 2017). Further experiments intended to generate double mutants where expression was reduced for both *ptn1* and either *ust1* or *unh1*. Changes in mating and virulence of these mutant strains were predicted and have been assessed by assays on charcoal media and controlled plant infection in order to observe any possible crosstalk between the *ptn1* pathway and either the *ust1* or *unh1* genes. Initial attempts at these double deletion experiments found that the plasmid used for generating the Cas9 construct was not functioning correctly. Therefore, these experiments were put on hold and focus was shifted to another set of experiments.

14-3-3 proteins are involved in signal transduction and cell cycle regulation, among other cellular maintenance functions. In other fungal systems, such as yeast, determining the exact role of each 14-3-3 protein in cellular processes has proved difficult, as many isotypes exist for each protein and each protein can have numerous binding partners (Kakiuchi et al. 2007). *U. maydis*, however, only possesses one 14-3-3 protein homolog, known as Pdc1. This presents an opportunity to study a single 14-3-3 protein's function within a fungal system. Pdc1 protein specifically is believed to regulate a signal transduction cascade that interacts with *ptn1* by regulating the function of a signaling protein that is required for normal cellular growth and

viability, Rho1. Similarly, Pdc1 is required for viability in haploid cells. Although the physical interaction between the Rho1 signaling protein, Pdc1, and Ptn1 has been documented, the exact effects of Pdc1 on *ptn1* transcriptional activity are still unclear (Pham and Perlin 2010). To examine the effects of *pdc1* on *ptn1*, *pdc1* overexpressor strains of *U. maydis* were generated. This upregulation of *pdc1* activity is predicted to exert an effect on symptoms associated with *ptn1* deletion.

Materials and Methods

DNA Isolation

The mutant *U. maydis* colonies were inoculated into YEPS (yeast extract-peptone-sucrose) media and allowed to shake overnight at room temperature. The fungal genomic DNA was isolated by microextraction using PCI (25 phenol:24 chloroform:1 isoamyl alcohol) followed by ethanol precipitation. The samples were vacuum dried and TE (10 mM Tris/1 mM EDTA, pH 8.0) solution was added to allow for proper storage (Hoffman and Winston 1987). Presence of DNA was confirmed by electrophoresis in agarose gels or measurements using a Nanodrop 2000 spectrophotometer.

PCR Specifications

Polymerase chain reaction (PCR) was used to amplify plasmid and fungal genomic DNA. PCR was carried out in a T100 ThermalCycler (Bio-Rad Laboratories, Hercules, CA, USA). For PCR amplification reactions, Platinum Taq GreenHot Start DNA Polymerase (Life Technologies Corporation) was used. (Vijayakrishnapillai et al. 2018). The relevant PCR steps and

temperatures for each step are shown in Table 1. Primers used for gel screening of transformants and target primers used in attempted CRISPR-Cas9 gene disruption are shown in Table 2.

Table 1. Polymerase Chain Reaction Conditions.

Step	Duration	Temperature	Description
1	4 minutes	94 °C	Initial denature
2	30 seconds	94 °C	Cycle denature
3	30 seconds	60 °C	Annealing of primers
4	3 minutes	72 °C	DNA Elongation (1 minute per 1 kilobase pairs)
5			Repeat steps 1-4 34 times
6	5 minutes	72 °C	Final elongation
7	Hold	4 °C	Termination

Table 2. Primers used in study.

Primer Name	Sequence	Use (Relevant Fig.)
pdc Int primer	5'-AGTCACCCTTCATCTTGTGG-3'	Confirm *pdc1* overexpression (Fig. 8)
EndOtef primer	5'-AGATTCGCCCGCTTTCTTCT-3'	Confirm *pdc1* overexpression (Fig. 8)
Hyg Sph1 newRT	5'-TTTGCATGCCTCAGGCCTCATGTTTGACA-3'	Confirm presence of *hyg* cassette (Fig. 4)
Hyg Mfe1 Lt	5'-TTTCAATTGTGGCCGAACGTGGTAACTAC-3'	Confirm presence of *hyg* cassette (Fig. 4)
PTEN QRT LT	5'-TTTGGTTTCATTGCCGTATCTTC-3'	Confirm presence of *ptn1* gene (Fig.4)
PTEN INT RT	5'-TAGGTAAGAGGTGGGCGATG-3'	Confirm presence of *ptn1* gene (Fig.4)
ptn1 Target	5'-GATCGTCATGACGCTTGTCG-3'	CRISPR-Cas9 Gene Disruption
ust1 Target	5'-GGAATCGGCAACAGCAGCTG-3'	CRISPR-Cas9 Gene Disruption
unh1 Target	5'-GCGAGGGTGATGGAGTCGGA-3'	CRISPR-Cas9 Gene Disruption

Gene Disruption Construct Development

Constructs for the disruption of *ptn1* (see Fig. 1) or for the overexpression of *pdc1* (see Fig. 2) were generated in vitro and plasmids bearing these were transformed into *Escherichia coli* strain DH5α using cells that had been made chemically competent for DNA transformation (Chung et

al. 1989) and selection on agar plates containing 200 µg ampicillin per ml (Wang et al. 1988). The hygromycin-resistance-conferring *ptn1* deletion construct was isolated and purified from the transformed bacterial cells using standard alkaline lysis protocols for plasmid purification (Sambrook and Russell 2006). The isolated construct was transformed into *U. maydis* protoplasted cells exposed to the relevant DNA by plating onto YEPS plates with a final concentration of 150 µg hygromycin per ml (Bösch et al. 2016). Hygromycin is a fungicide that acts by inhibiting protein synthesis. The plates were incubated at 26 °C for 3-5 days before colony growth was assessed. Suspected transformant colonies that were conferred hygromycin resistance were re-plated onto YEPS-hygromycin agar plates and stored for subsequent experiments.

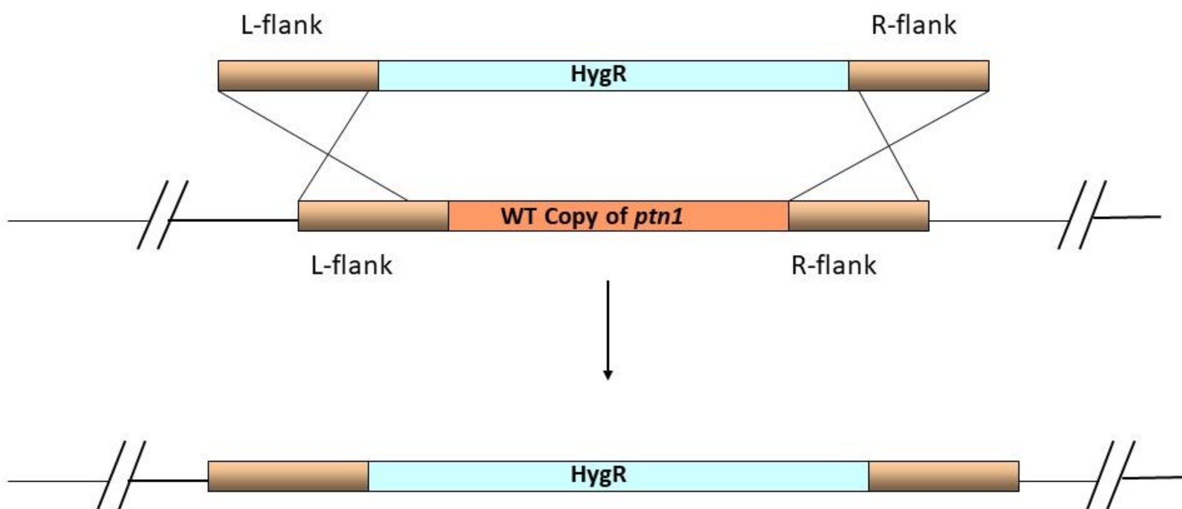

Fig. 1. Mechanism of *ptn1* deletion construct. A plasmid containing a hygromycin resistance marker (HygR) flanked by DNA homologous to flanking regions of the *ptn1* gene was transformed into *U. maydis* to delete *ptn1* by homologous recombination of the plasmid into the fungal genome.

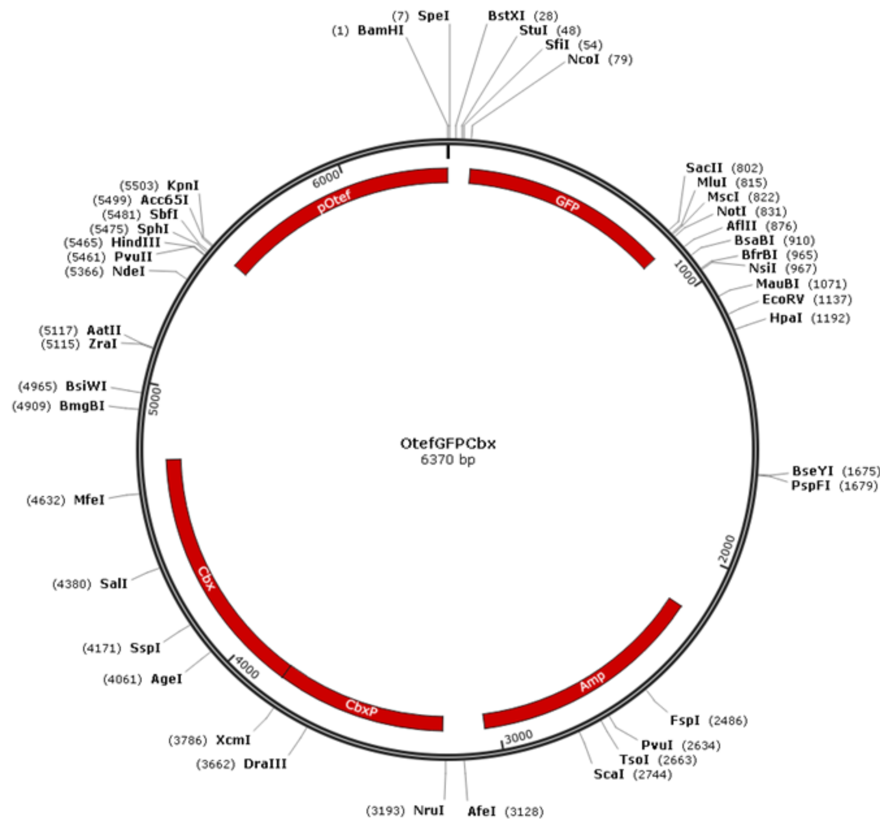

Fig. 2. Sample construct map for *pdc1* overexpression. A construct such as this with *hyg* in place of *cbx* and *pdc1* in place of *gfp* was cloned into *U. maydis* for constitutive expression of the *pdc1* gene by the *otef* promoter.

CRISPR-Cas9 Construct Development

E. coli colonies were provided with a CRISPR-Cas9 plasmid via bacterial transformation (Wang et al. 1988) (see Fig. 3). The plasmid DNA was isolated via an alkaline lysis protocol (Sambrook and Russell 2006). Putative sites for targeted mutation of each of the *ptn1*, *ust1* and *unh1* genes were selected using the E-CRISP online tool (www.e-crisp.org) to select for the highest fidelity for sgRNA cut sites by the ultimate Cas9 construct. Primers containing the genome information for each cut site was obtained from an outside company (Eurofins Scientific, Louisville, Kentucky). Cut sites on the Cas9 vector for restriction enzymes Msc1 and Xba1 were identified to open the vector for introduction of the sgRNA target site genome information. Primers for each target site for each respective gene targeted for disruption were designed with Msc1 and

Xba1 restriction cut sites. These primers were purified from agarose gel by GENECLEAN® II Kit (Qbiogene, Inc., Carlsbad, California) and amplified by PCR. Target primers and amplified Cas9 vector DNA were digested together by Msc1 and Xba1 to introduce the target sites into the vector. The final construct was then transformed into *U. maydis* cells by plating an STC-polyethyleneglycol solution containing the protoplasted cells along with the construct DNA onto YEPS agar plates with a final concentration of 150 µg/mL hygromycin (Bösch et al. 2016). The plates were incubated at 30 °C for 3-5 days before colony growth was assessed. Suspected transformant colonies were regularly replated onto YEPS-carboxin plates to gradually eliminate Cas9 construct DNA to reduce the likelihood of off-target effects due to Cas9.

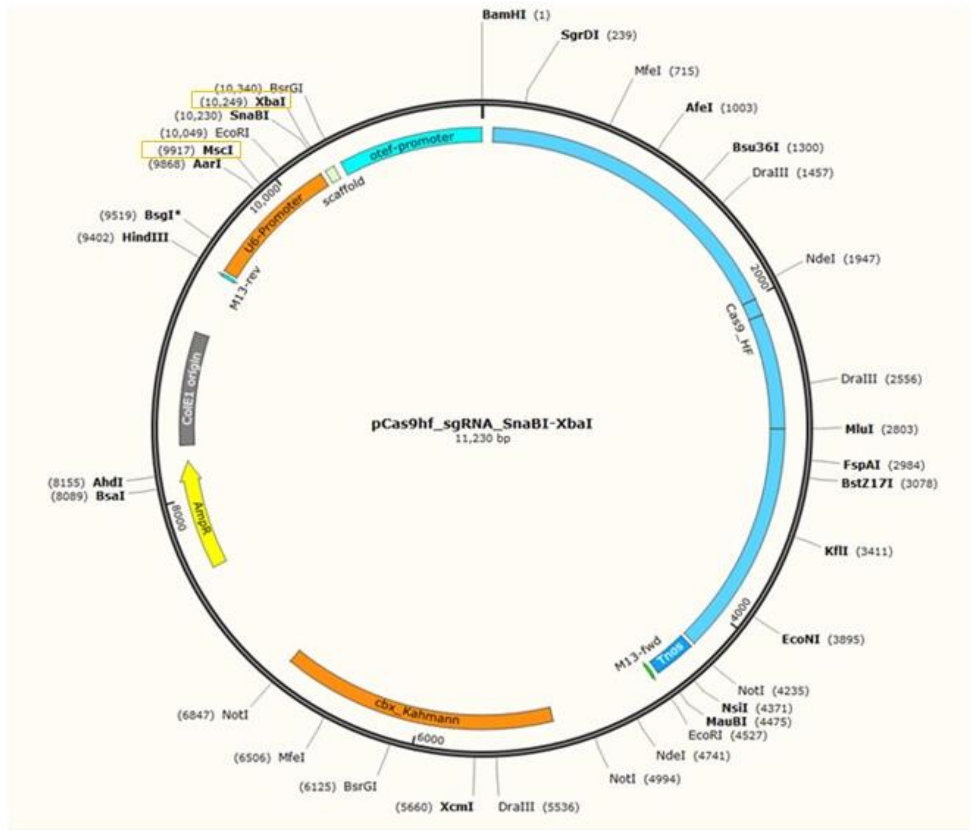

Fig. 3. Cas9 Construct for Attempted Gene Deletions. Target DNA sequences for each gene were inserted into the vector in the space between the highlighted enzymes. The construct would then be guided to the complementary sequence in the targeted gene to cut the DNA, resulting in disruption of the target gene.

Stress Tests

Cultures (4 mL) of *U. maydis* in YEPS media, shaken at 260 rpm, were grown overnight at room temperature. Cells were grown to an optical density at 600 nm (OD_{600}) of 0.8 and cells were resuspended in sterile dH_2O to an OD_{600} of 1.6. The parent inoculum and three sequential 1:10 serial dilutions of the fungal strains were spotted onto media containing 1 mM Congo Red and observations were made after incubation at 26 °C for 48 hours (Ram and Klis 2006). Congo Red is a cell wall stressor that acts by binding to the proteins that form the fungal cell wall. This prevents complete lateral attachment of cell wall proteins, compromising rigidity of the cell wall (Kopecká and Gabriel 1992).

Mating Assay

Cultures (4 mL) of *U. maydis* in YEPS media, shaken at 260 rpm, were grown overnight at room temperature. Cells were grown to an OD_{600} of 0.8, and cells were resuspended in sterile deionized water to an OD_{600} of 1.6 prior to spotting. 10 µL aliquots of haploid strains of opposite mating type background were co-spotted onto PDA charcoal plates and mating reactions were observed after incubation at 26 °C for 24 hrs. and 48 hrs (Gold et al. 1997). A positive mating reaction produced a white “fuzz” phenotype of aerial hyphae production.

Plant Infection and Infection Assessment

Cultures (4 mL) of *U. maydis* in YEPS media grown overnight at room temperature were diluted using 50 mL fresh YEPS liquid media and grown overnight again to an OD_{600} of 1.0, harvested and resuspended in sterile dH_2O to an OD_{600} of 3 prior to infection. Plant infection was

performed by inoculating week-old maize seedlings (Golden Bantam seeds, Bunton Seed Co., Louisville, KY and W. Atlee Burpee & Co., 62 Warminster, PA). Haploid strains of opposite mating types were mixed prior to plant infection. One-week old seedlings of *Zea mays* were infected with the indicated strains of *U. maydis* by injecting the seedlings with inoculum containing a mixture of compatible strains. Mock injections were performed by injecting water into the seedlings as a control. The virulence of each treatment group was scored by a disease index (DI) on a scale of 0 to 5, where 0 = No Symptoms, 1 = Chlorosis, 2 = Small leaf tumors, 3 = Large leaf tumors or small stem tumors, 4 = Large stem tumors with bending, and 5 = Death. Thirty plants were used in each experiment with plants scored for disease symptoms on 14 and 21 days post-infection (dpi). A haploid *a1b1* background mutant strain crossed with a haploid *a2b2* mutant strain was compared in each case to the corresponding wild type cross. The data was analyzed using Kruskal-Wallis test followed by post-hoc comparison (Gold et al. 1997; Vijayakrishnapillai et al. 2018).

Results

ptn1 Deletion Strains

The hygromycin homologous recombination construct produced *ptn1* deletion (Δ*ptn1*) strains in three haploid mating type backgrounds of *U. maydis.* The 1/2 (a1b1), 2/9 (a2b2), and FB1 (a1b1) Δ*ptn1* mutant strains were confirmed as transformants by PCR amplification using primers specific to the *ptn1* gene and primers specific to the substituted *hyg* (hygromycin-resistance) gene. Confirmed transformants displayed no band for *ptn1* and a band for *hyg* when compared to control (WT) strains of the same background, which show a band for *ptn1* and no band for *hyg* (see Fig. 4).

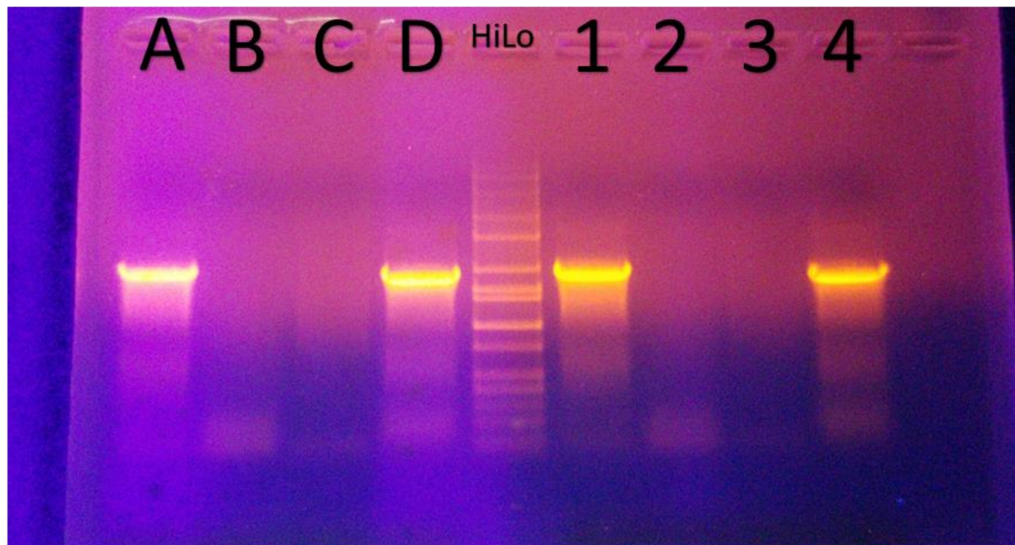

Fig. 4. Screening of putative *U. maydis* Δ*ptn1* mutants. PCR was used to screen for bona fide deletion of *ptn1* with the *hyg* cassette. A) FB1 Δ*ptn1* + primers for hygromycin resistance gene. B) FB1 wild type + primers for hygromycin resistance gene. C) FB1 Δ*ptn1* + primers for *ptn1* gene. D) FB1 wild type + primers for *ptn1* gene. 1) 1/2 Δ*ptn1* + primers for hygromycin resistance gene. 2) 1/2 wild type + primers for hygromycin resistance gene. 3) 1/2 Δ*ptn1* + primers for *ptn1* gene. 4) 1/2 wild type + primers for *ptn1* gene. HiLo is a DNA size standard (Minnesota Molecular).

Stress tests of the mutant strains were performed on Congo red media to assess growth differences from the wild type. These tests showed less growth and more susceptibility to cell wall stress for each mutant strain when compared to WT strains (see Fig. 5).

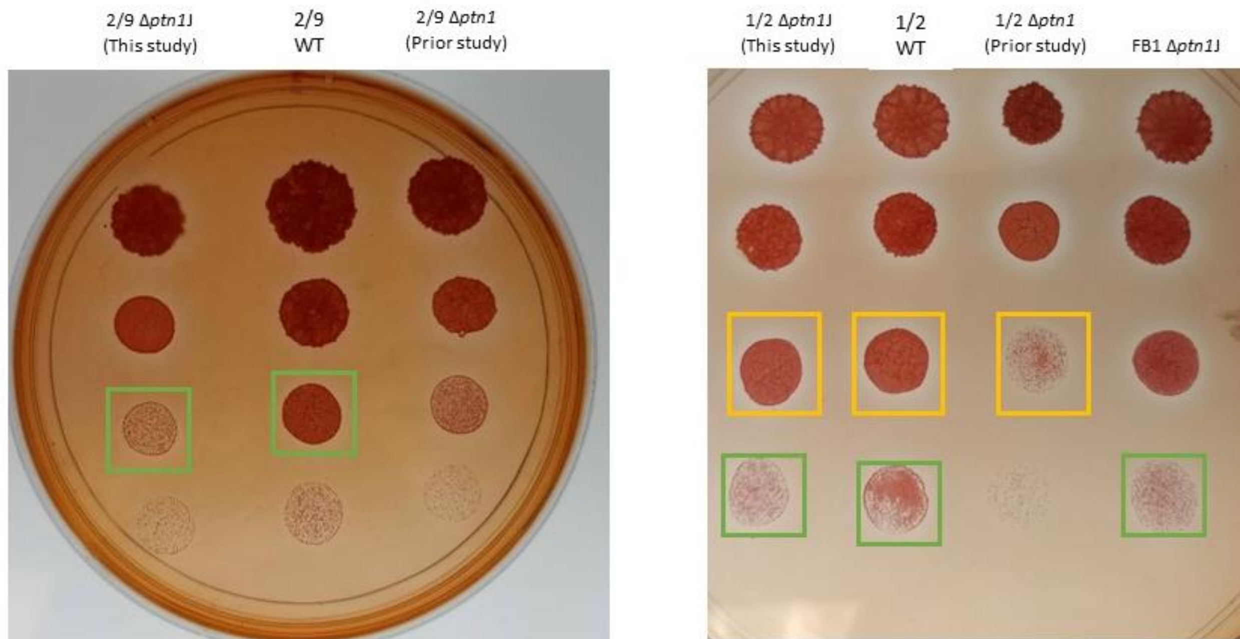

Fig. 5. Δ*ptn1* Mutant Stress Tests. *Ptn1* deletion mutants displayed increased cell wall susceptibility, as evidenced by lower growth on Congo Red media than wild type. Growth differences are shown by the squares.

Additionally, mating assays on charcoal media showed that mutant strains displayed lower mating affinity and growth when crossed with WT strains bearing an intact *ptn1* gene, and extremely lower mating and growth when compatible Δ*ptn1* mutants were crossed. This supports previous findings that *ptn1* plays an important role in mating, and even having one copy of the gene missing has consequences for mating (see Fig. 6).

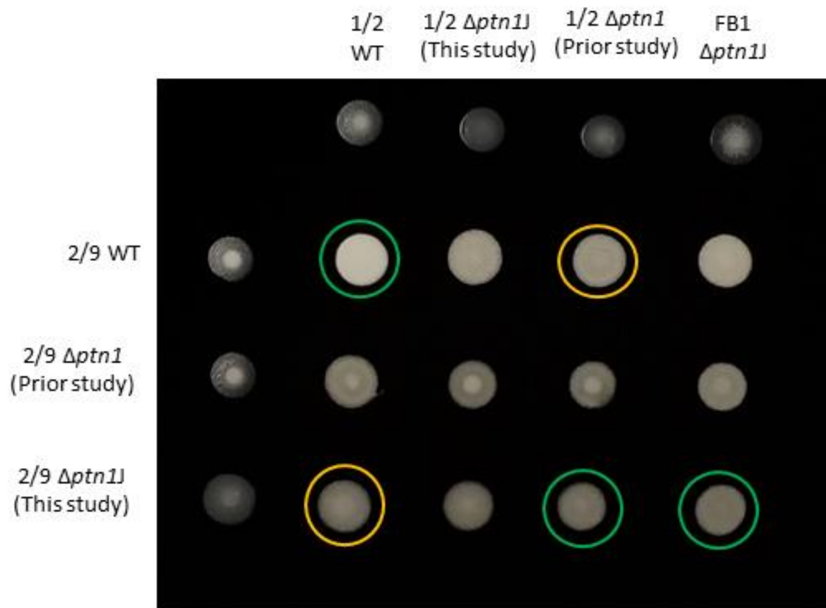

Fig. 6. Mating assay trials for Δ*ptn1* mutant strains.

Mating assay for Δ*ptn1* strains. Crosses involving Δ*ptn1* strains did not display as much aerial hyphae development during mating when compared to wild type. Differences in mating between strains are show by the circles.

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The disruption of *ptn1* gene expression has adverse effects on the virulence of *U. maydis* as well. Plant infection trials consisting of groups of plants infected with each compatible mutant mating pair (1/2 x 2/9 and FB1 x2/9) yielded significantly less advanced phenotypical markers of infection (number and size of galls) for mating pairs that consisted of one or more mutant strains compared to WT infection (see Fig. 7).

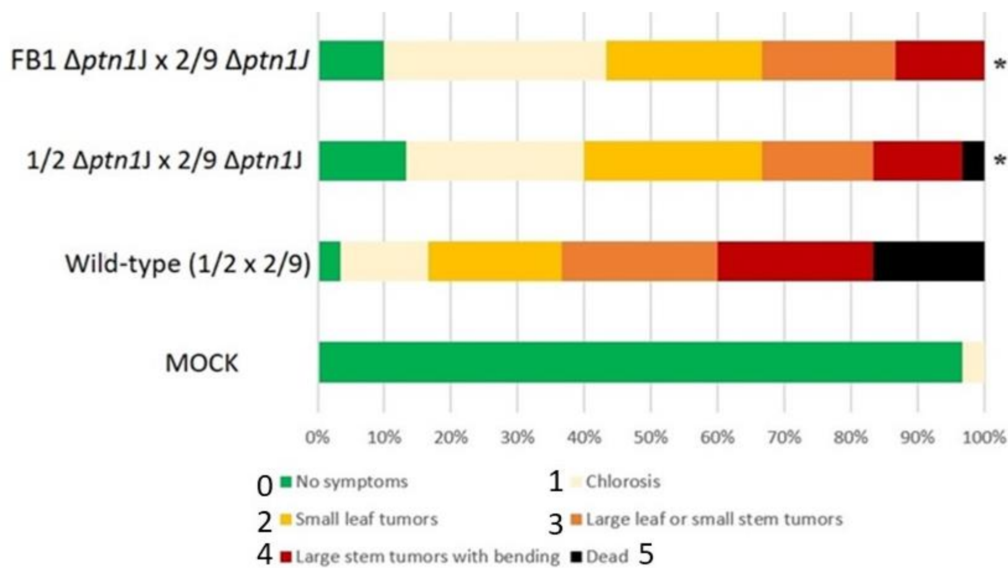

Fig. 7. Plant infection data for Δ*ptn1* strains. Corn seedlings were infected with various strains of *Ustilago maydis* and infection severity was scored on a disease index from 0 (no infection) – 5 (plant death). Compatible 1/2 x 2/9 and FB1 x 2/9 Δ*ptn1*J mutant pairs were used in infection. Δ*ptn1*J strains displayed significantly decreased virulence when compared to wild type. * indicates $p < 0.05$.

<u>Attempted gene disruptions using CRISPR/Cas9</u>

Target cut sequences for each of the *ust1* and *unh1* genes were selected and cloned into a high fidelity CRISPR-Cas9 deletion construct in order to disrupt gene expression at those loci. The construct was introduced into *E. coli* and later isolated via plasmid minipreps. The *ust1* target construct was transformed into the 1/2 and 2/9 mating type backgrounds via PEG-mediated transformation and suspected transformants were screened by PCR amplification of the disrupted region. PCR screening could not confirm any transformants and sequencing attempts did not provide adequate reads for interpretation. Concurrent experiments in the lab using the plasmid used here for the Cas9 construct have also been unsuccessful. These CRISPR-Cas9 deletion attempts have been halted until new, reliable plasmid vector is obtained.

<u>*Pdc1* Overexpressor Strains</u>

In order to investigate the influence of *pdc1* on *ptn1*, overexpressor strains in which the constitutive *otef* promoter drove *pdc1* gene transcription were generated in the 1/2 and 2/9 mating type backgrounds. These mutants were confirmed by electrophoresis on agarose gel using

primers specific to *otef* and *pdc1* (see Fig. 8). Wild type strains of *U. maydis* did not display a band as they do not have an *otef* promoter.

Fig. 8. *Pdc1* OX Gel Confirmation. *Pdc1* overexpressor transformants were confirmed using end primers specific to the *otef* promoter and internal *pdc1* gene primers. Wild type strains that lack *otef* do not display a band. A) 1/2 *pdc1* OX. B) 2/9 *pdc1 OX*. C) *otef-pdc1* containing plasmid. D) 1/2 wild type. E) 2/9 wild type.

Stress tests of these strains were performed to examine if any changes in growth were brought about by overexpression of *pdc1*. Stress tests of both overexpressor strains show no phenotypic change when compared to the wild type strains, suggesting that cell wall viability was not compromised by *pdc1* overexpression (see Fig. 9).

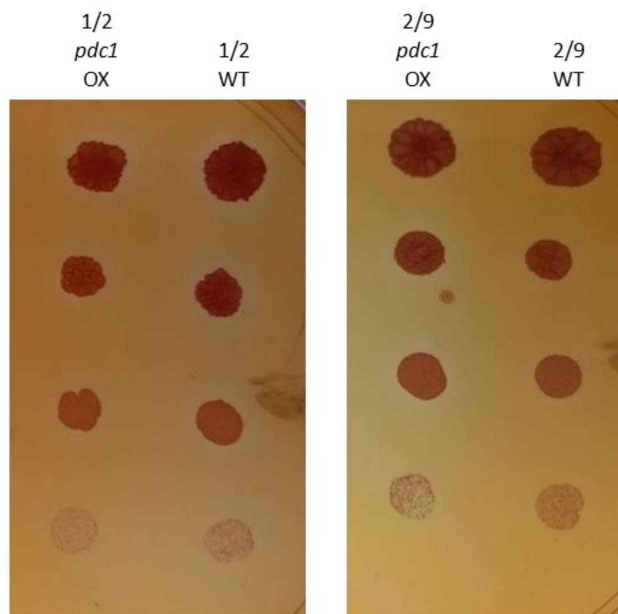

Fig. 9. *Pdc1* OX Mutant Stress Tests. *Pdc1* overexpressor mutants displayed no differences in growth from the wild type.

Additionally, mating assays of both strains displayed no change in mating filamentation when crossed with each other or when crossed with compatible wild type strains that have a normal copy of the *pdc1* gene (see Fig. 10). These tests imply that *pdc1* overexpression does not directly affect the typical mating response of *U. maydis*.

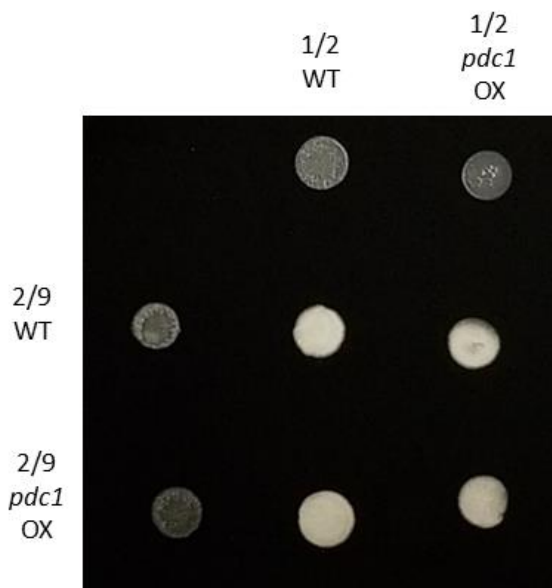

Fig. 10. Mating assay for *pdc1* OX strains. Compatible haploid wild type and *pdc1* OX mutant strains of *U. maydis* were crossed. No differences in mating in the mutant strains from wild type were observed.

Plant infection testing of the *pdc1* overexpressor strains was carried out in order to assess if there were differences in infection severity compared to wild type. The mutant strains were not found to have a significant difference in virulence when compared to wild type. These preliminary results suggest that constitutive expression of *pdc1* does not significantly attenuate virulence in *U. maydis*. However, these results are based on a single set of plant infection. More replicates must be carried out in order to confirm these results (see Fig. 11).

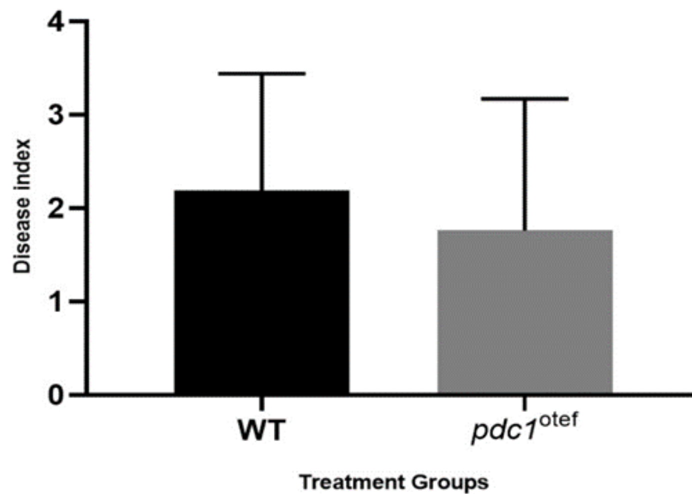

Fig. 11. Plant infection for *pdc1* OX strains. Mutant strains displayed no significant reduction in virulence compared to wild type. More replicates must be performed to confirm results.

Discussion

This study attempted to show how alteration of *ust1*, *unh1*, and *pdc1* gene expression affected Δ*ptn1* strains of *Ustilago maydis*. Deletion of the *ptn1* gene in the 1/2, 2/9, and FB1 backgrounds of *U. maydis* were successful and confirmed by PCR amplification. These mutant strains were found not only to be more susceptible to the cell well stressor, Congo red, but also to produce a reduced "fuzz" white phenotype normally associated with compatible mating types during mating assay trials. Due to the apparent role of *ptn1* in mating, it was thought that deletion of even one copy of the *ptn1* gene was enough to attenuate the mating response; this prediction was supported by the mating assays in this paper. Moreover, it appears that mating pairs for which both copies of the *ptn1* gene were absent displayed a more attenuated mating response than the mating pairs where one partner had a copy of the gene. Thus, the normal mating response appears to require both copies of the gene.

If *ptn1* contributes to virulence of the fungus, Δ*ptn1* strains of *U. maydis* should display a lessened ability to infect corn as well. Plant infection trials for compatible Δ*ptn1* mutants showed a significant decrease in pathogenicity of infection, displaying smaller and less numerous tumors. Additionally, relatively less mature teliospores were shown to develop within the tumors created

by the mutant strains (Dalton 2016; Vijayakrishnapillai et al. 2018). This supports previous studies showing that *ptn1* is necessary not only for tumorigenesis, but also for further teliospore development in the tumors caused during infection. This attenuation of virulence caused by deletion of the *ptn1* gene was of interest in suggesting subsequent experiments to evaluate other contributing genes that might further reduce virulence or to rescue this reduced virulence. Thus, I tried to disrupt two other genes, *unh1* and *ust1*, by a CRISPR-Cas9 system.

As previously stated, *ust1* acts as a suppressor of tumorigenesis in haploid strains of *U. maydis*. Its regulatory action is relaxed after the dimorphic switch during mating to allow for tumor formation during infection. Previous studies have found that haploid *ust1* deletion mutants display different phenotypes than the wild type fungus, including a darker pigmentation and grittier appearance. Additionally, mutant strains produce teliospore-like structures in liquid culture (García-Pedrajas et al. 2010). Diploid double deletion mutants have also been shown to produce darker and more numerous tumors during infection (Islamovic et al. 2015). Since Δ*ust1* mutants have been shown to display greatly increased virulence, double deletion mutants in which *ptn1* and *ust1* are both deleted were intended to be created by CRISPR with the prediction that deletion of *ust1* action could complement deficiencies in infection brought about by *ptn1* deletion.

Additionally, *unh1* was selected for its similarity in function to *ptn1*, influencing tumorigenesis, teliospore development, and meiosis during infection. *Unh1* deletion strains of *U. maydis* have been found to have reduced teliospore development during infection, with teliospores that did reach maturity displaying abnormal discoloration (Doyle et al. 2016). Strains in which both *ptn1* and *unh1* were disrupted by CRISPR were to be generated to see if *unh1* exerted any influence on the *ptn1* pathway by examining whether double mutant strains

displayed even further attenuated infection symptoms than the Δ*ptn1* strains. Trials were attempted using the high fidelity CRISPR-Cas9 deletion construct and proceeded to the point of insertion of target cut sites for each gene into the construct; subsequent isolation of the complete construct was successful. However, transformations of the construct into *U. maydis* yielded no candidate colonies that were confirmed to be deletion mutants. These experiments were put on hold until a more reliable plasmid could be used to create the construct can be obtained, which was beyond the timeline of this study. This remains an area of interest for future experiments.

The effects of *pdc1* on *ptn1* was another area of interest for this study. Pdc1 has been shown to be involved in the Rho1-mediated signal transduction pathway and its gene product is believed to interact with Rho1 protein during the signaling process. Further support for the interaction between Pdc1 and Rho1 has been found in experiments showing that overexpression of *rho1* mitigated the adverse effects of *pdc1* deletion strains of *U. maydis* (Pham et al. 2009). This study aimed to further investigate the role of Pdc1 in the Ptn1 signal transduction pathway by overexpressing *pdc1* and examining changes in mating, cell growth, and infection. *Pdc1* overexpression mutants did not display changes in mating or cell growth when compared to wild type. Based on one set of plant infection data, overexpression mutants displayed a slight reduction in virulence that was not found to be statistically significant. Additionally, more replicates of infection using these mutant strains must be carried out to validate these results. These experiments serve to further inform another ongoing study in the laboratory involving *U. maydis* strains that have both *ptn1* deletion and *pdc1* overexpression mutations. Preliminary findings of that study suggest that these dual mutation strains of *U. maydis* may actually display reduced virulence of infection relative to both wild type and Δ*ptn1* strains (unpublished). Further

experimentation is required to observe other differences of the dual mutants from the wild type fungus.

Conclusions and Limitations

This study attempted to show the effects of genetic disruption and overexpression on the biological processes of *U. maydis*. It also attempted to investigate how certain gene products involved in signal transduction pathways pertaining to virulence interact with one another. It was found that *ptn1* deletion strains of *U. maydis* show not only reduced pathogenicity, but also reduced mating and growth ability as well. It was planned to further mutate these *ptn1* deletion mutants by CRISPR-Cas9 gene disruption of two other genes of interest, *ust1* and *unh1*. It was predicted that deletion of *ust1* in *ptn1* deletion strains of *U. maydis* might recover some of the pathogenicity of the fungus, whereas it was predicted that deletion of *unh1* in *ptn1* deletion strains would lead to a further reduction in virulence than a *ptn1* deletion alone. However, these experiments could not be completed presently due to the CRISPR-Cas9 construct being ineffective in generating these mutations. This remains as an open area of study for future experiments.

Pdc1 overexpression mutant strains of *U. maydis* were found not to display significant differences in mating, cellular growth, or virulence from wild type. However, as previously stated, more plant infection trials must be carried out in order to properly understand effects of *pdc1* overexpression on virulence since the present study only includes data from one set of infection trials. The other ongoing study in the Perlin lab that is examining *pdc1* overexpression in *ptn1* deletion mutants will be able to further examine how the signal transduction pathways involving these genes interact. One future direction for this study is the final generation of dual

deletion mutations of *ptn1*/*ust1* and *ptn1*/*unh1* using CRISPR-Cas9 in *U. maydis* along with all relevant mating, cellular growth, and infection testing of the dual mutant strains. Another future direction of this study is to generate dual *pdc1* overexpression/*ptn1* deletion mutants of my own on which to perform all relevant tests. Meeting these future goals will facilitate increased understanding of these important regulatory genes and their interactions with one another and will further elucidate important basic genetic mechanisms in *U. maydis.*

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