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Identifying phenotypic effects in Sporisorium reilianum and Ustilago maydis lacking the alternative oxidase gene.

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IDENTIFYING PHENOTYPIC EFFECTS IN *SPORISORIUM REILIANUM* AND *USTILAGO MAYDIS* LACKING THE ALTERNATIVE OXIDASE GENE.

By

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Abstract

Ustilago maydis and *Sporisorium reilianum* are both dimorphic fungi that cause infection in the host plant, corn (*Zea mays*). In order for infection to be successful, compatible haploid mating types must form a dikaryon that later leads to the development and dispersal of teliospores. In order to sustain growth, energy is produced via the electron transport chain within the mitochondrion. Host plants often produce harsh reactive oxygen species, resulting in the need to use an alternative respiration pathway such as employing an alternative oxidase. Therefore, this study attempted to investigate the role of the *aox* gene in pathogenicity for these fungi. This was achieved by disrupting the gene of interest and assessing mating, response to stress, and virulence. Virulence was found to be lower in seedlings infected with *U. maydis* deletion strains, suggesting *aox* could play a role in pathogenicity. Mating assays and stress tests did not show any differences in growth. Unfortunately, data could not be collected from *S. reilianum* infections, whose assessment requires full development of infected plants, but further study will investigate virulence and teliospore germination rates. There was a slight difference in mating, but this could not be quantified and should be compared to mating during infection as a future point of investigation. Additionally, there were no differences in morphology or growth ability of deletion strains when compared to wild type during stress tests.

Introduction

Ustilago maydis and *Sporisorium reilianum* are both dimorphic smut fungi that cause disease on maize/corn (*Zea mays*). The name "smut fungus" stems from the dark teliospores characteristically produced inside of the tumors or galls (caused by *U. maydis*) or spore sacs (*S. reilianum*) that form on the tassels/cobs. Despite these being related species, *U. maydis* produces tumors containing teliospores on all above-ground tissue including the stems, leaves, tassels, and ears. In contrast, *S. reilianum* produces its teliospores only in the tassels or ears of infected maize plants. Additional symptoms include chlorosis, or yellowing, of the leaves, and buildup of anthocyanin, a pigment which causes a red or purple coloring of leaves (Martinez, Roux, Jauneau & Dargent, 2002). Although less agriculturally relevant due to hybrid corn strains, these organisms now serve as models for understanding host-plant interactions as well as the relationship between mating and virulence.

These two related fungi also have similar mating systems, with a few important differences. *U. maydis* requires that its haploid mating partners each have two compatible mating loci within their respective genomes in order for pathogenic development to occur. The *a* locus encodes a pheromone as well as a pheromone receptor for the compatible partner. These versions (a1 or a2) must be different in the mating partners (Banuett & Herskowitz, 1994). Once the haploid cells have met this requirement and initiated contact, they must then determine whether they are also compatible at a second locus, the *b* locus. As many as 25 different alleles have been identified at this locus. In mating partners, the *b* locus must also be different in order to allow the bE and bW proteins to form a transcription factor that upregulates genes for pathogenicity by binding to the DNA (Banuett & Herskowitz, 1994). An example of compatible haploid wild type strains is the combination of strains FB1 (a1b1) and FB2 (a2b2), where both the *a* and *b* alleles are different between the two potential mating partners. An important difference from *U. maydis* is that

Sporisorium has three possible *a* types (a1-a3) with two pheromone genes each, making the individual pheromones recognizable to only strains with one of the other *a* alleles (Schirawski, Heinze, Wagenknecht & Kahmann, 2005). The *b* locus also exists with at least five identified alleles. The two wild types strains of *Sporisorium reilianum f. sp. zeae* for this project are 5-1 with the a2 allele and 5-2 with the a1 allele.

In eukaryotic organisms, the mitochondria function in converting carbon sources to energy in the form of adenosine triphosphate, or ATP. Following glycolysis and the tricarboxylic acid cycle, the electron transport chain creates a gradient using protons that are used to produce ATP when pumped through the enzyme ATP synthase. The fungal mitochondrial electron transport chain is composed of four protein complexes, numbered I through IV, where oxidation and reduction reactions occur to create a proton gradient (Juárez, Guerra, Martínez & Pardo, 2004).

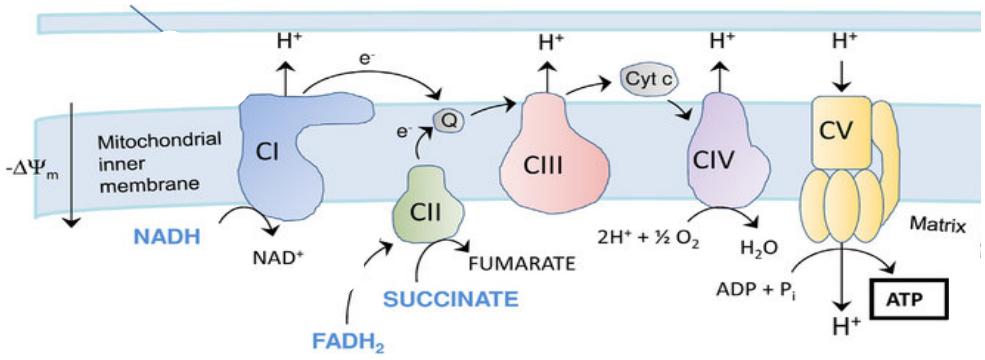

Figure 1: Electron transport chain. The classic electron transport chain, located within the inner membrane of the mitochondrion, uses four complexes to pump protons (H^+) into the intermembrane space. At those protons pass through complex V or ATP synthase, adenosine triphosphate (ATP) is produced and can be used as a form of energy.

However, both complexes III and IV can be inhibited and if this occurs, little to no ATP is produced due to the lack of a terminal electron acceptor. However, many plants, fungi, algae, and some protists contain an alternative oxidase (AOX) which serves as the terminal electron acceptor (Rogov, Sukhanova, Uralskaya, Aliverdieva & Zvyagilskaya, 2014). This allows respiration to continue without being linked to ATP production, as energy produced is released as heat.

An alternative oxidase has been identified in *U. maydis*, and preliminary work for this project bioinformatically identified a putative *aox* gene in *S. reilianum* (Juárez, Guerra, Martínez & Pardo, 2004). This alternative oxidase was identified by using the Basic Local Alignment Search Tool (BLAST) furnished by the National Center for Biotechnology Information. It allowed for comparison between the *U. maydis* amino acid sequence and possible regions in the *Sporisorium* genome encoding an orthologue. The results indicated a protein in the ferritin-like superfamily, specifically an AOX. This suggested an alternative oxidase is likely present and could play an important role for the fungus as it infects corn. In *U. maydis*, AOX was regulated when under oxidative stress such as the presence of Antimycin A, an inhibitor of complex III. Specifically, it may prevent the buildup of reactive oxygen species (ROS) including the superoxide anion radical and hydrogen peroxide (Sierra-Campos, Velázquez, Matuz-Mares, Villavicencio-Queijeiro & Pardo, 2009). This suggests the presence of an alternative oxidase allows the fungus to survive in a range of conditions which is especially relevant as some plants produce ROS as a defense mechanism and the presence of an AOX may aid in pathogenicity, as has been observed in other fungi, including pathogens of humans (Poloni & Schirawski, 2016).

For example, *Aspergillus fumigatus* is an airborne pathogenic fungus that results in an infection called aspergillosis. When the *aox* gene was nearly completely repressed via RNA interference (RNAi) techniques, the mutants showed reduced infection rates due to the presence of macrophages, a type of white blood cell involved in immune response. These macrophages employ ROS as a means of killing the conidia, or spores, that cause infection (Magnani et al., 2008). Additionally, in *Cryptococcus neoformans*, the existence of an *aox* gene and functional pathway were confirmed before continuing experimentation in live animal models that could be extrapolated to better understand human infection by this human pathogen. Once infection using the mutant strain was done, this also suggested the importance of AOX in order to survive the

onslaught of ROS produced by macrophages (Akhter et al., 2003). A third example is in the human pathogen *Paracoccidioides brasiliensis*; when AOX was inhibited, viability and vitality of cells was reduced (Hernández et al., 2015). Although not plant pathogens, these examples suggest AOX could be important for surviving ROS as well as during the morphological change.

Based on these studies and the potential consequences of lacking AOX, it was targeted for gene disruption. However, in order to see any phenotypic effect, deletion was necessary in both compatible mating types for each species of fungus. Once the gene was disrupted, the mutants could be exposed to various cell stressors and mating assays performed to look for any differences in growth. Infection of maize, however, was expected to show the largest difference in overall fitness, primarily measured by degree of pathogenicity. Following infection, the germination rates of teliospores were to be investigated but this testing could not yet be completed.

Materials & Methods

DNA Isolation

For each mating type, the strains were inoculated into 4 mL of YPS (yeast extract-peptone-sucrose) and shaken overnight at 28˚ C. Fungal genomic DNA was isolated in order to provide a source for regions flanking the gene (hereafter referred to as, up and down flanks) that would be used to assemble the Δ*aox* construct. The DNA was extracted using PCI (25 phenol:24 chloroform:1 isoamyl alcohol) followed by 100% ethanol precipitations and 70% ethanol wash steps. After precipitation, samples were dried via vacuum and stored in TE (10 mM Tris/1 mM EDTA, pH 8.0) for long term stability (Hoffman & Winston, 1987). Successful DNA extraction was confirmed by electrophoresis on an agarose gel.

PCR Protocol

Polymerase chain reaction (PCR) was used to amplify the fungal genomic DNA of interest, including the up and down flanks surrounding the *aox* gene. It was also used to amplify the relevant pieces from plasmids in order to assemble the construct. The reaction was carried out in a T100 ThermalCycler (Bio-Rad Laboratories, Hercules, CA, USA). To amplify the DNA, Platinum Taq GreenHot Start DNA Polymerase (Life Technologies Corporation) was used. The steps of the reaction and temperatures are shown below in Table 1. Primers used throughout the experimental procedure in Gibson overlap, screening of potential transformants, and screening for targeted insertion can be found in Table 2.

Table 1. PCR Protocol[a]

Description	Temperature (˚C)	Duration	
Initial Denaturing Step	94	4 minutes	
Denaturing Step	94	30 seconds	Repeat 34x
Annealing Primers	60	30 seconds	
DNA Elongation	72	2 minutes 30 seconds	
Final Elongation	72	5 minutes	
Termination	4	∞	

[a]Standard reaction conditions, for flanks and selectable marker amplification.

Table 2. PCR Primers

Primer Name	Sequence	Use (Relevant Figure)
1. Ori + Amp Fw	5'-GGGTCGTTTAATTAATATTGAAAAAGGAAGAG-3'	Gibson Down Flank & Ori + Amp
2. Down R	5'-TTCAATATTAATTAAACGACCCTGCTACGAAC-3'	Gibson Down Flank & Ori + Amp
3. Up F	5'-GCCTTTTGATATCTCTCATTCGTTGCTCAT-3'	Gibson Ori + Amp & Up Flank
4. Ori + Amp Rv	5'-AATGAGAGATATCAAAAGGCCGCGTTGCTG-3'	Gibson Ori + Amp & Up Flank
5. Hyg 1507 F	5'-GGGTGATTGTTAAAACGACGGCCAGTGAAT-3'	Gibson Up Flank & Hyg
6. Up R	5'- CGTCGTTTTAACAATCACCCATCCTTGCTC-3'	Gibson Up Flank & Hyg
7. Down F	5'- CAATTCCACAAGCACCTCGATACGACAAGC-3'	Gibson Hyg & Down Flank
8. Hyg 1507 R	5'-TCGAGGTGCTTGTGGAATTGTGAGCGGATA-3'	Gibson Hyg & Down Flank
9. Sr AOX Up F	5'- TTTTGATATCAAGCATGGTGACGAGGAGAT-3'	Confirm Construct (Fig. 4)
10. Sr AOX Dn R	5'- TTCAATATTAATTAAAGGTGATGAAGGAACGAACG-3'	Confirm Construct (Fig. 4)
11. Um AOX Up F	5'-GCCTTTTGATATCCGCAATCACGAACGAAAAC-3'	Confirm Construct (Fig. 4)
12. Um AOX Dn R	5'-TTCAATATTAATTAAGGTATGCCTCAGCTCAAAGG-3'	Confirm Construct (Fig. 4)
13. Sr AOX Outflank Dn R	5'- GAGAGAGAGAGGAGCGCAAA-3'	Targeted Insertion (Figs. 4 & 6)
14. Um AOX Outflank Dn R	5'-AGACAAAGCGTGGATAAACTCA-3'	Targeted Insertion (Figs. 4 & 6)
15. 1507Hyg1006Out	5'- TTGTCTCGCTTCCTTTAGCC-3'	Targeted Insertion (Figs. 4 & 6)
16. Sr AOX Outflank Up F	5'- CCCAGAAGTAAACCGTCTCG-3'	Targeted Insertion (Figs. 4 & 6)
17. Um AOX Outflank Up F	5'- ACTTGTCCCTTCCTCGTCCT-3'	Targeted Insertion (Figs. 4 & 6)
18. 1507 Hyg5OUT	5'- TTGTCTCGCTTCCTTTAGCC-3'	Targeted Insertion (Figs. 4 & 6)
19. SRZ AOX qRT F	5'-GAAAAGACCGTGGCTCTCC-3'	qRT-PCR; confirming construct
20. SRZ AOX qRT R	5'- GTGCTTCACTGGCATCGTC-3'	qRT-PCR; confirming construct
21. Um AOX qRT F	5'- CTTACAACGCCCTACCATCG-3'	qRT-PCR; confirming construct
22. Um AOX qRT R	5'- TTTGAGGTGGAATGGAGAGG-3'	qRT-PCR; confirming construct

Quantitative real time polymerase chain reaction (qRT-PCR) was used to confirm the construct was assembled correctly and ensure *aox* was not being expressed. The reaction was carried out in a StepOne thermal cycler (Applied Biosciences, Foster City, CA, USA) using Evagreen flurorescent dye (Biotium, Fremont, CA, USA). The reaction mixture is detailed in table 3 and the protocol is found in table 4.

Table 3. qRT-PCR reaction mixture

Evagreen Dye	6.4 µL
H_2O	20.8 µL
Primer 1	0.8 µL
Primer 2	0.8 µL
Complementary DNA (cDNA)	3.2 µL
Total	32 µL

Table 4. qRT-PCR protocol

Temperature (˚C)	Duration	
95	10 minutes	
95	15 seconds	} Repeat 35x
60	1 minute	

Gene Disruption Construct Development

Gibson overlap was used to create the Δ*aox* construct following successful PCR amplification of the individual pieces. The pieces were each amplified, separated via agarose gel electrophoresis, and purified out of the gel before being re-amplified to ensure they were pure. Purification from agarose was done using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA). With all of the purified pieces, the Gibson overlap method allowed for the construct to be built in one reaction using overlapping primers between pieces to be joined (see Fig. 3).

In order to do 4-6 piece fragment assembly, 0.2-0.5 pmols of DNA fragments were needed. Readings on a Nanodrop 2000 spectrophotometer were taken to determine DNA concentration of each piece. Then, the following equation was used in order to determine the necessary amount to add for each piece.

$$\text{pmols needed} = \frac{0.1(\text{Size x } 650)}{1000}$$

The reaction was run at 50˚ C for one hour using the NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs, Ipswich, MA, USA). The successful reaction resulted in a circularized plasmid which was confirmed by DNA sequencing.

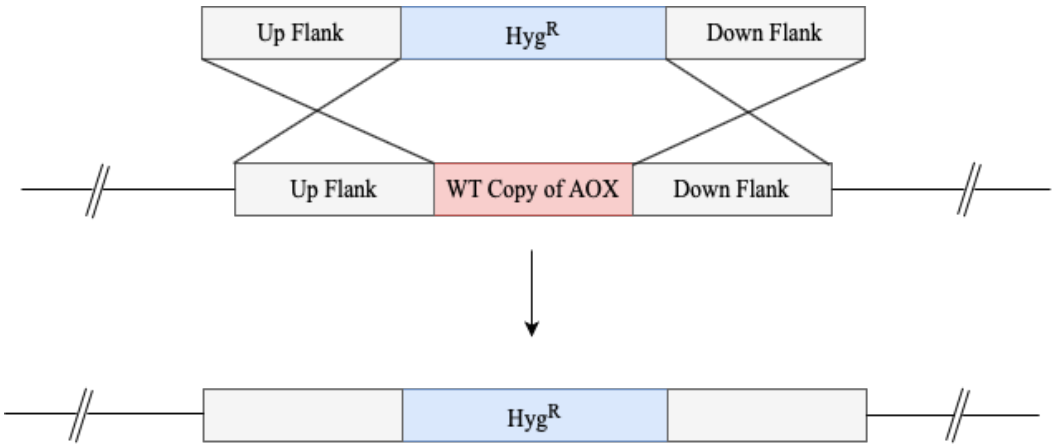

Figure 2: Mechanism of Δ*aox* Construct. The construct containing a hygromycin resistance marker (Hyg^R) flanked by DNA homologous to flanking regions of the *aox* gene was first cloned into a minimal plasmid replicon (oriAmp); this was used as a template for PCR amplification of the Δ*aox* Construct, which was then transformed into *U. maydis* and *S. reilianum* to delete *aox* via homologous recombination with the fungal genome.

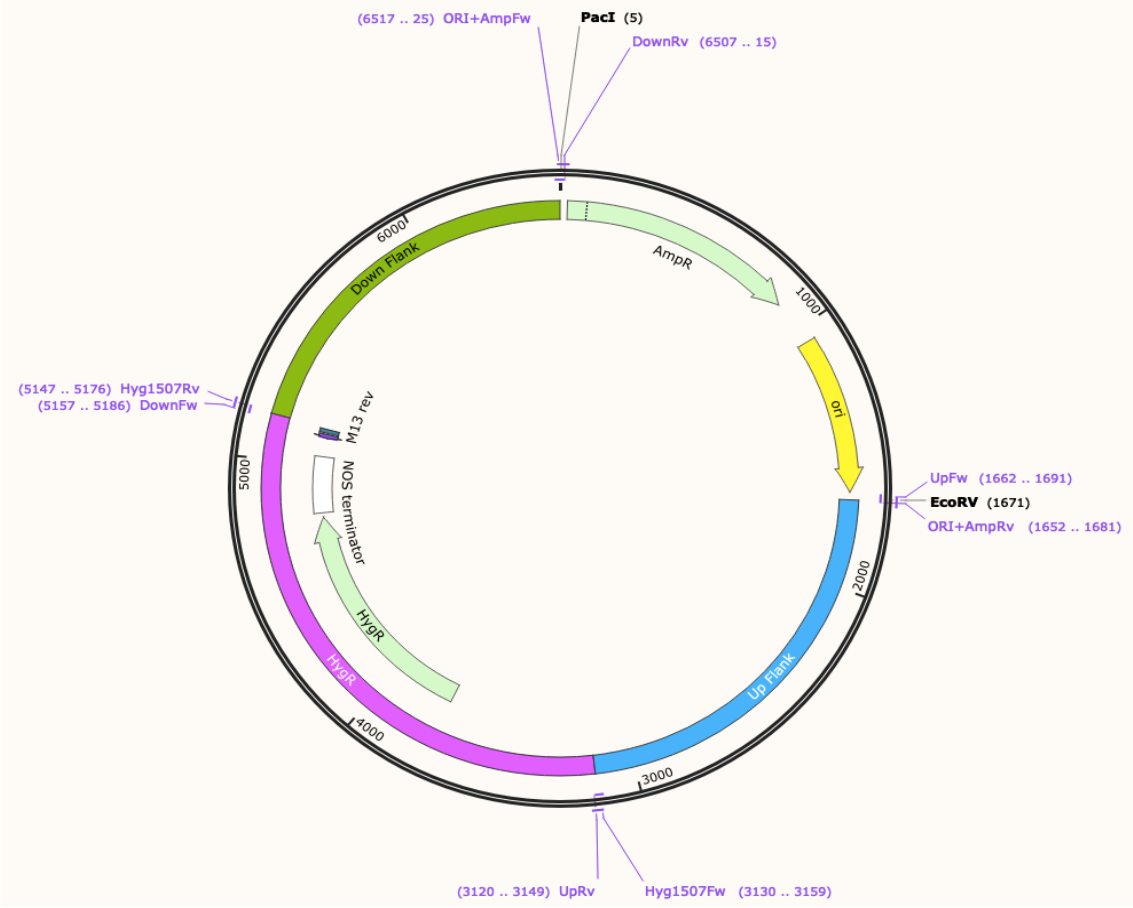

Figure 3: Δ*aox* construct in oriAmp plasmid. This construct was assembled via Gibson overlap and *E. coli* transformants were selected on ampicillin. The Hyg^R was used to select for potential fungal transformants.

Polyethylene Glycol (PEG) Transformations

Transformations of both *U. maydis* and *S. reilianum* were done using protoplasts for each required mating type (FB1, FB2, 5-1, and 5-2). Protoplasts were used because the fungal cell wall is sturdy, making it difficult for DNA to enter the cell. By enzymatically stripping the cell of its wall with Novozyme (2.5 mg/mL), DNA could enter while the cell maintained its ability to regenerate the cell wall. The deletion construct was transformed into *U. maydis* and *S. reilianum* by plating a STC-polyethyleneglycol mixture with the protoplasts and construct DNA onto YPS plates containing hygromycin B (100-200 µg/mL). Plates were kept upright and incubated at least 4 days

at 28˚ C before picking colonies to put onto fresh YPS plates containing hygromycin B. Colonies that grew were also streaked onto YPS plates without antibiotic.

Identifying Transformants

Potential transformants were identified based on their ability to grow on (100-200 μg/mL) hygromycin plates. However, in order to confirm the colonies that grew were transformants, overnight cultures from the YPS plates were made and then gDNA extractions performed. PCR using a series of diagnostic primers was done in order to confirm presence of the construct and replacement of the *aox* gene of *U. maydis* and *S. reilianum* strains.

Mating Assays

Cultures (4 mL) of *U. maydis* in YPS media were shaken at 260 rpm overnight at 28˚ C until they reached an OD_{600} (absorbance at 600 nm) of 0.8. Then, the cells were resuspended in sterile deionized water to an OD_{600} of 1.6 before spotting on the plates. 10 μL aliquots of compatible/opposite mating types were co-spotted onto PDA (potato dextrose agar) charcoal plates and observed for mating after incubation at 28˚ C for 24 and 48 hours (Gold, Brogdon, Mayorga & Kronstad, 1997). A positive mating reaction was signaled by a white $fuzz^+$ phenotype and aerial hyphae production.

For *S. reilianum*, precultures were made and shaken at 28˚ C overnight at 260 rpm. Once these grew, a 50 mL main culture was inoculated and grown to OD_{600} of 1. 5 mL of each compatible mating type was pipetted into a flask with 30 mL of PD media and again shaken overnight. The next day, 10 μL aliquots from each flask were pipetted onto a water agar plate (2% agar with DI water) and cultivated at 28˚ C for two days before being transferred to room

temperature. When filamentation became visible for the wild type strains, the colonies were viewed under the microscope.

Infection and Assessment

U. maydis was grown as 4 mL cultures in YPS media overnight at 28˚ C and then diluted with 50 mL of fresh YPS and grown again overnight to an OD_{600} of 1.0. These were then resuspended in sterile dH_2O to an OD_{600} of 3.0 before infection. Compatible haploid mating strains (FB1 x FB2 and FB1Δ*aox* x FB2Δ*aox*) were mixed prior to infection and injected into one-week old seedlings (Golden Bantam seeds, Bunton Seed Co., Louisville, KY and W. Atlee Burpee & Co., 62 Warminster, PA) via hypodermic needle (Chavan & Smith, 2014). Mock injections with sterile water as well as an uninfected group were maintained as controls. Following two weeks of growth, virulence of infection was assessed by a disease index on a scale ranging from 0 to 5, where 0 = No Symptoms, 1 = Chlorosis or anthocyanin production, 2 = Small leaf tumors, 3 = Medium tumors or > 1 leaf with a tumor, 4 = Stem tumor, and 5 = Death. There were 35 plants in each experimental group for *U. maydis*, with an additional 8 plants in each control group.

For *S. reilianum*, overnight cultures of individual strains were grown in YPS at 28˚ C while shaking at 260 rpm before being transferred to a 20 mL flask of YPS and grown to OD_{600} of 0.7. Once at an OD_{600} of 0.7, the cultures were centrifuged for 15 minutes at 3500 rpm until a pellet formed. The supernatant was poured off and the pellet resuspended in 2.5 mL of ddH_2O. The two compatible mating types were mixed and were injected into two-week-old seedlings (Tom Thumb seeds, High Mowing Organic Seeds, Wolcott, VT, USA) using a 1 cc hypodermic syringe. Strain combinations were also checked on a water agar plate to ensure mating was successful. The crosses listed in table 3 were used. Symptoms usually do not appear until the corn has reached maturity

after approximately 8 weeks but can be evaluated using a standardized scale based on severity of symptoms similarly to that of *U. maydis*.

Table 5 *S. reilianum* crosses

Cross	Mating Type	Number of Plants
5-1 x 5-2	a2b2 x a1b1	20
5-1 Δ*aox* x 5-2 Δ*aox*	a2b2 x a1b1	20
5-1 x CXI2	a2b2 x a3b3	20
5-1Δ*aox* x CXI2Δ*aox*	a2b2 x a3b3	20
5-2 x CXI2	a1b1 x a3b3	20
5-2Δ*aox* x CXI2Δ*aox*	a1b1 x a3b3	20
Mock Infection with Water	----------	21
No Manipulation	----------	36

Oxidative Stress Tests

Overnight cultures (4 mL) of *U. maydis* were grown in YPS and shaken at 260 rpm at 28˚ C. Cells were grown until an OD_{600} of 0.8 and resuspended in dH_2O to an OD_{600} of 1.6. Undiluted inoculum and 5 additional sequential 1:10 serial dilutions of the fungal strains were spotted onto media containing 1 mM Congo Red, a cell wall stressor. Other stressors used included 1 mM hydrogen peroxide, 1 M NaCl, and 1 M sorbitol. Observations of growth were made after incubation at 28˚ C for 48 hours (Ram & Klis, 2006). A similar protocol was used for *S. reilianum* and all stress tests were completed.

Results

aox Deletion Mutants

The *aox* gene was deleted via homologous recombination in haploid mating types FB1 (a1b1) and FB2 (a2b2) of *U. maydis* and 5-1 (a2b2), 5-2 (a1b1), and CXI2 (a3b3) in *S. reilianum*. Primer pairs

9/10 and 11/12 were used to amplify inward from the flanks to confirm the presence of the construct. To confirm the construct was inserted in the correct location, primers 13-18 were used. These attached to sites outside of the construct and continued in toward the hygromycin resistance cassette, where the other primer attached (see Fig. 4). This meant transformants showed a band due to the presence of the hygromycin resistance gene while wild type did not (see Fig. 6). Additionally, qRT-PCR (primers 19/20 and 21/22) was used to further confirm the construct was assembled properly and prevented the expression of the *aox* gene.

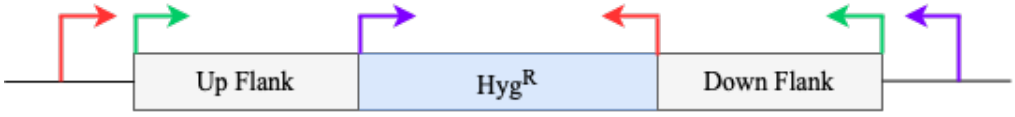

Figure 4: Confirming transformants. Diagnostic primer pairs were used in order to determine if colonies were transformants (green primers). The red and purple primers were used to assess if the construct was inserted in the correct genomic location.

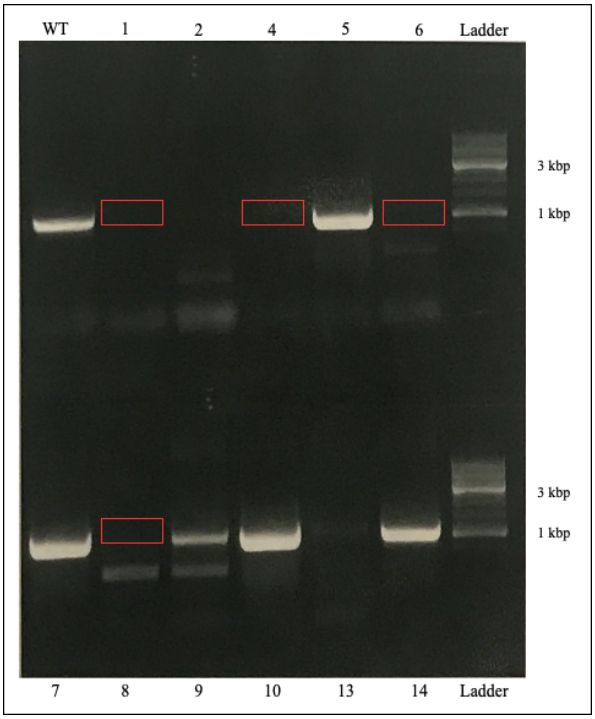

Figure 5: Confirming transformants. Potential transformants (for *S. reilianum* strain 5-1) were confirmed by the lack of a band at ~1200 base pairs, corresponding to the *aox* gene. Wild type (WT) was run as a positive control. Numbers correspond to colonies from Hyg plates.

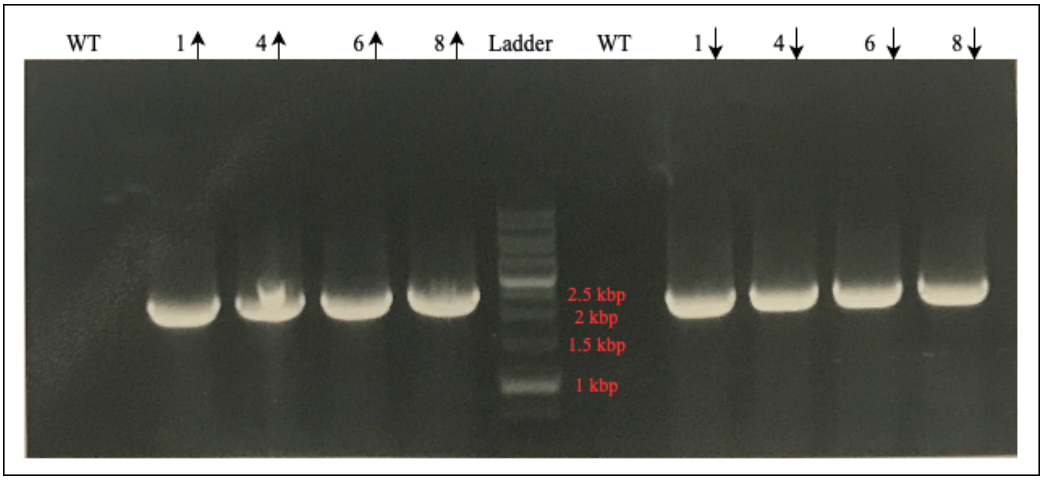

Figure 6: Confirming genomic location of insertion. Targeted insertion was tested via primer pairs that annealed outside of the construct and went in toward *hyg*. WT was used as a negative control and should not have shown a band. 5-1 transformants are shown.

Mating Assays

Mating assays for *U. maydis* were conducted on charcoal media and the production of the white fuzz^{+} phenotype indicated a positive mating reaction. Expected mating was shown when

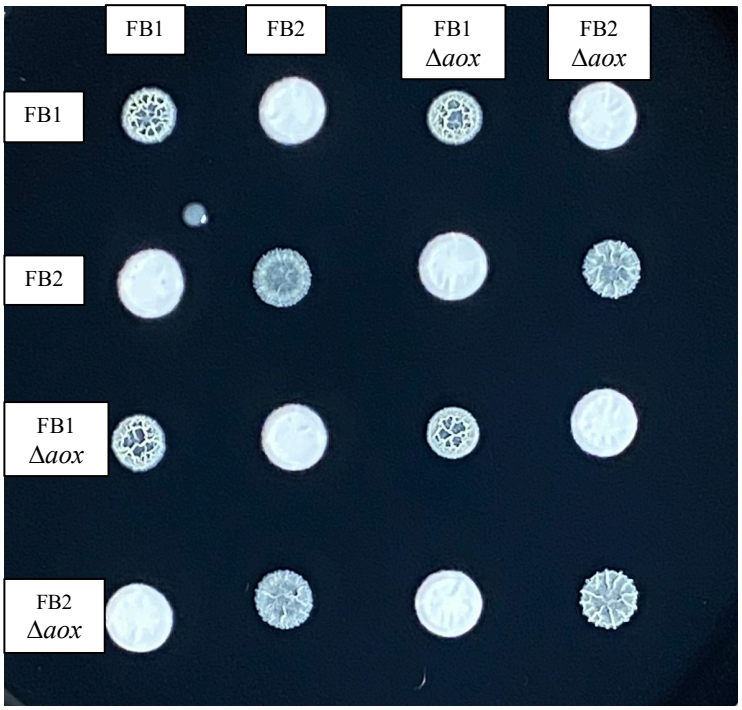

Figure 7: *U. maydis* mating assay. Mating was assessed on charcoal PDA media by co-spotting compatible haploid mating types. No discernable mating differences were seen in Δ*aox* strains compared to wild type.

compatible mating types were crossed for wild type, wild type with Δ*aox*, and compatible *aox* deletion mutants. This suggests *aox* does not play a significant role in mating capabilities.

S. reilianum mating assays were not carried out on charcoal media and instead relied on microscopy following the mixing of compatible haploid mating types. Individual strains were also used as controls to ensure mating did not occur. First, conjugation tubes were observed under 40x bright field microscopy. No haploid strains showed conjugation tubes, but all mated pairs did. Then, using a higher-powered bright field microscope, the haploid strains and mated pairs were observed for aerial filamentation after growing two days at 28˚ C. The haploid mating types did appear different, but this was due to differences in cell density and no individual strain showed a significant amount of filamentation.

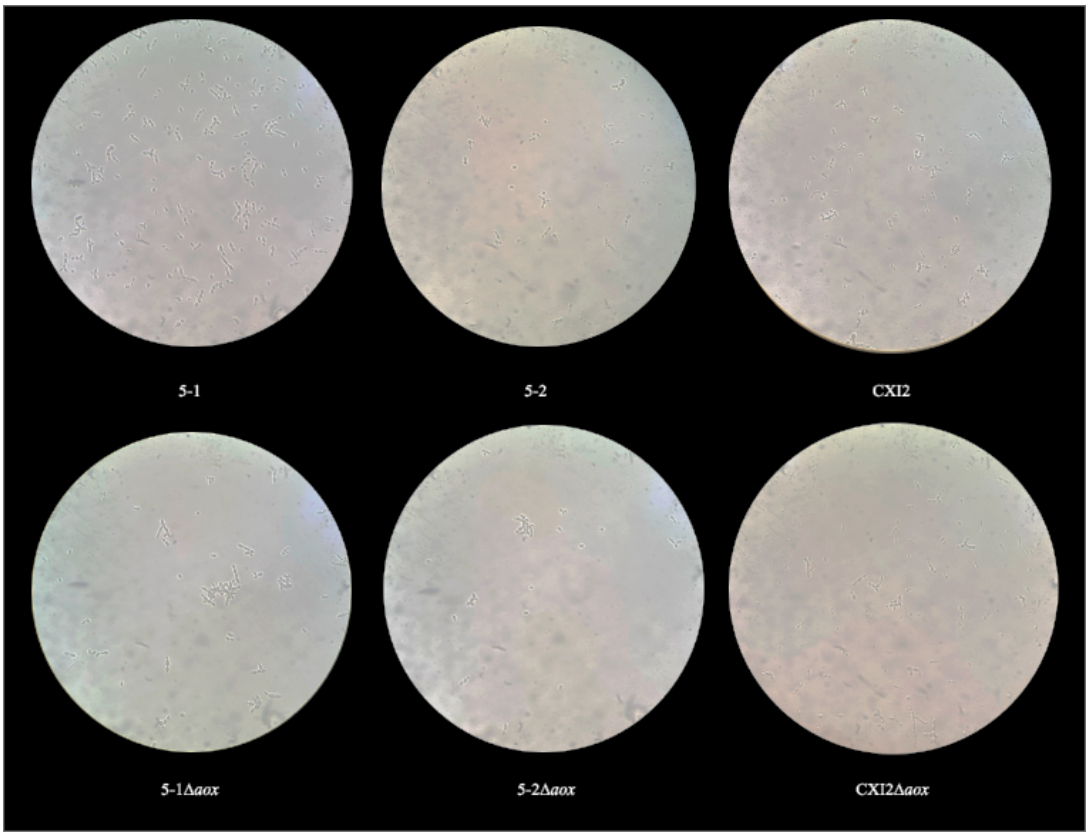

Figure 8: SRZ haploid strains conjugation. Haploid strains listed above were pipetted onto a water agar plate and checked for formation of conjugation tubules at 40x magnification. These haploid strains did not show the formation of conjugation tubules.

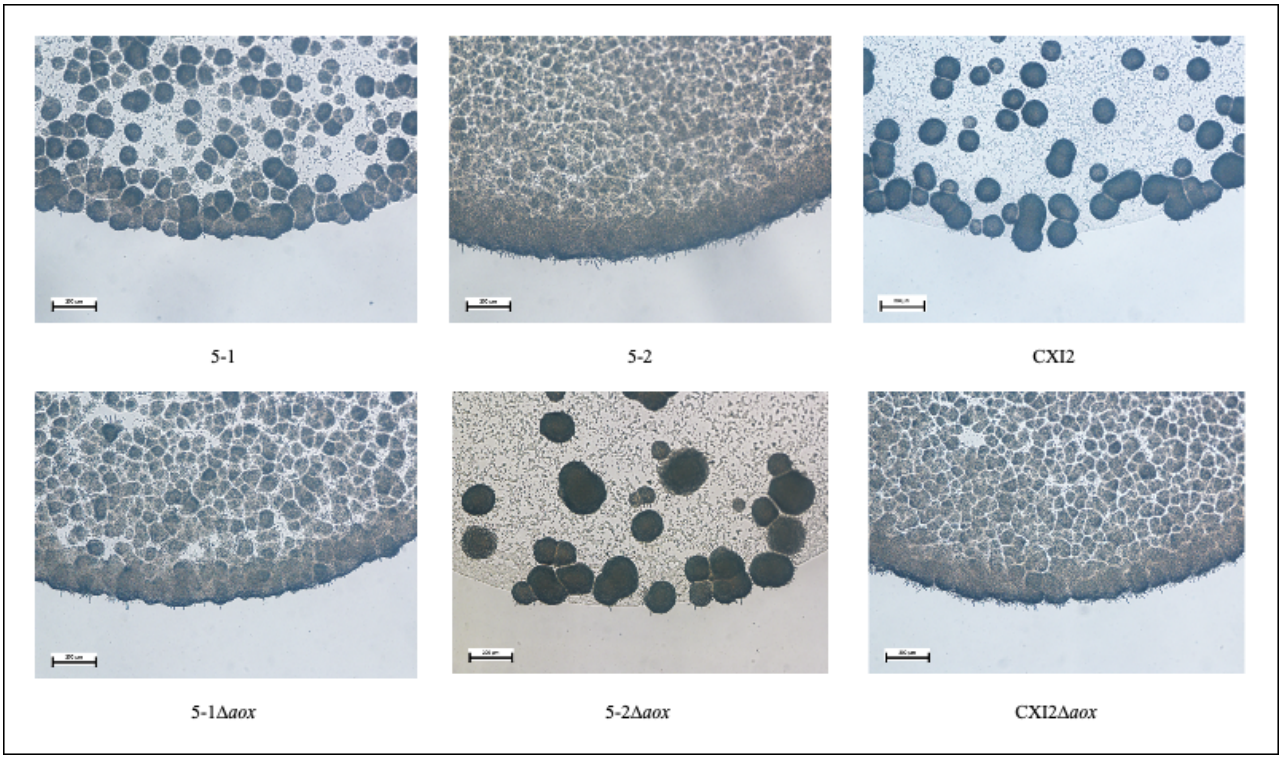

Figure 9: SRZ haploid strain hyphae. Following growth at 28˚ C, the haploid strains were checked for hyphae formation. Aerial hyphae were not present because mating could not occur. Scale: 200 µm.

When compatible haploid mating types were mixed and spotted on water agar, formation of conjugation tubules was evident at 40x magnification. Although there was a potential difference, there was not a convenient way to quantify this other than infection data. This difference was not as clear once hyphae were examined, but the ultimate assessment would have resulted from infection data which could not be completed before this report. Additional replicates of all mating assays should be performed to confirm this potential difference. Future assessments might be able to employ a haemocytometer and counting multiple microscopic fields. Infection data would also be necessary to compare differences in mating to differences in symptoms.

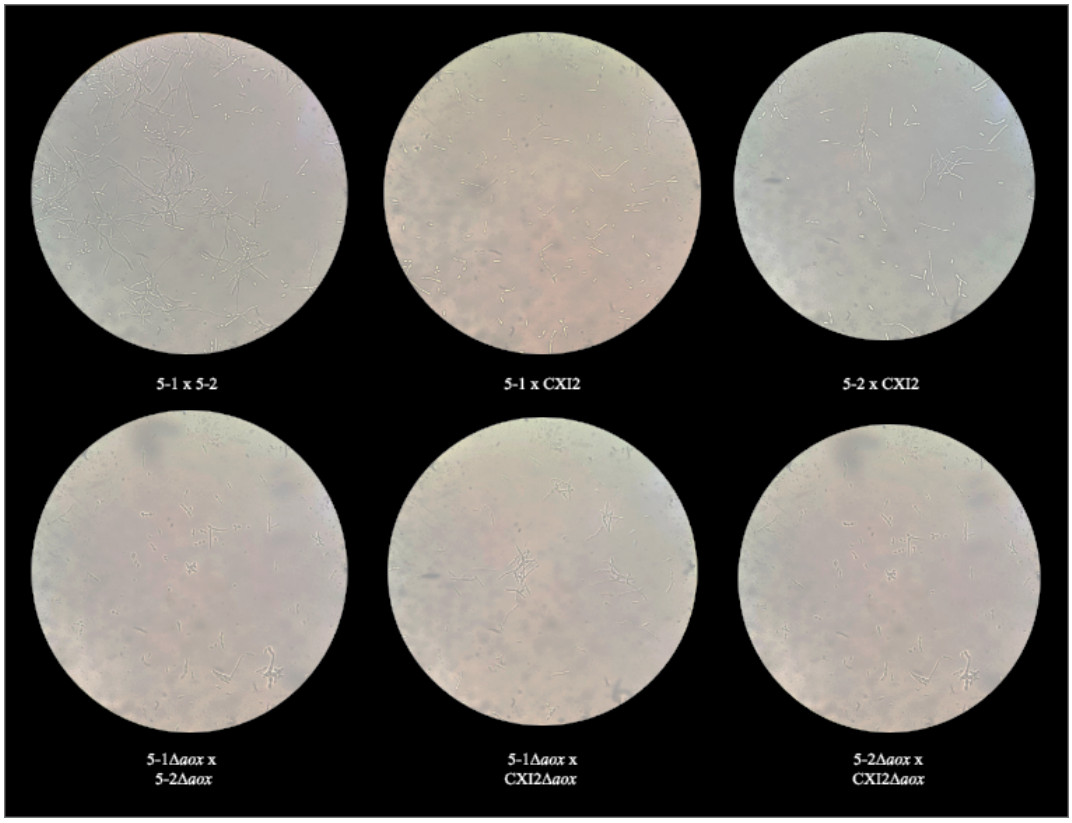

Figure 10: SRZ compatible haploid strain conjugation. Compatible haploid strains were mixed on water agar plates and checked for formation of conjugation tubules at 40x. There was a qualitative difference when comparing wild type crosses with Δ*aox* crosses, but this could not be quantified.

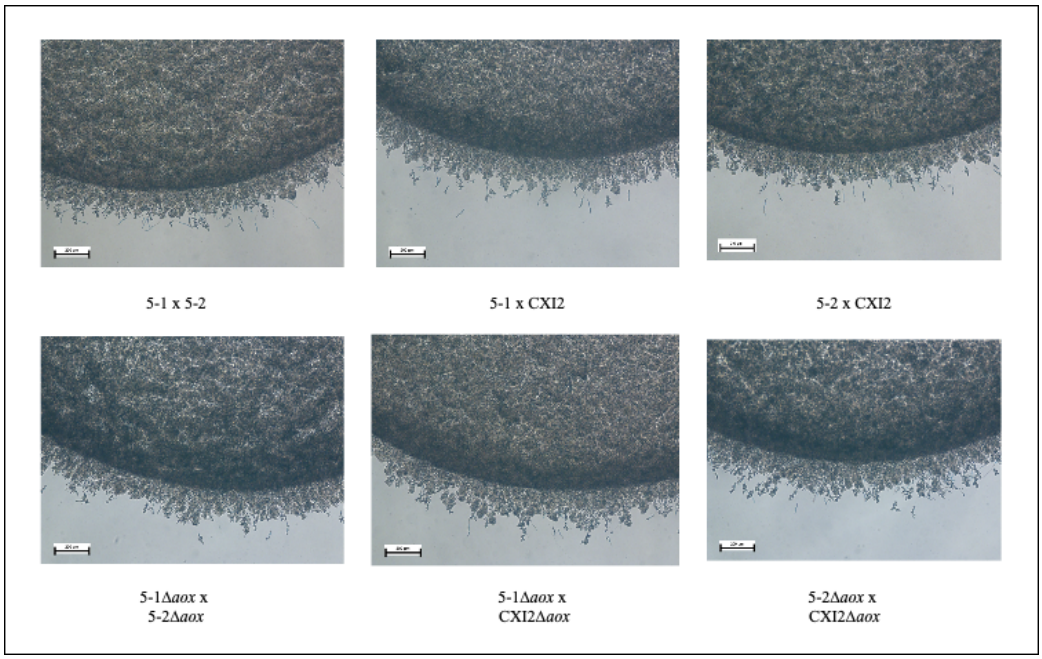

Figure 11: SRZ compatible haploid strains hyphae. Following growth at 28˚ C for two days, the compatible strains were checked for hyphae formation. Aerial hyphae were present in all crosses but showed only a slight difference in growth among wild type and Δ*aox* crosses. Scale: 200 µm.

Infection and Attempted Assessment

Infection symptoms for *U. maydis* began to appear approximately one week following infection but continued to worsen as the plants further matured. At two weeks post infection, they were scored for symptoms using the standard scale. The group that was not manipulated showed no symptoms as did the mock infection group injected with sterile water only. However, the two infected groups both showed symptoms with a difference in overall virulence when comparing the FB1 x FB2 (WT) and FB1Δ*aox* x FB2Δ*aox* groups (see Fig. 12). The disease index (DI) of FB1 x FB2 was 1.7 as compared to the double mutant infections with a DI of 0.8. Analysis done using a Kruskal-Wallis test in Prism (GraphPad Software, San Diego, CA, USA) found significance with p = 0.0003 (Daniel, 1990).

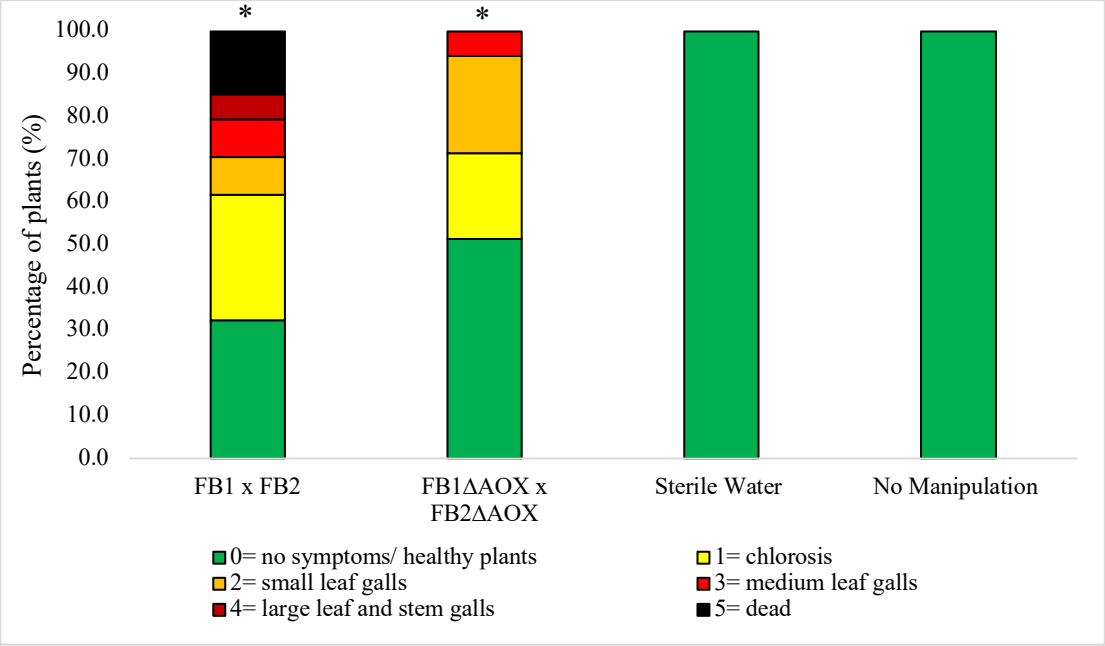

Figure 12: Plant infection data for *U. maydis* Δ*aox* strains. Corn seedlings were infected with two compatible mating types of *U. maydis* and the infection scored using a disease index from 0 (healthy) to 5 (plant death). Δ*aox* strains displayed significantly reduced virulence when compared to wild type. * indicates p<0.001

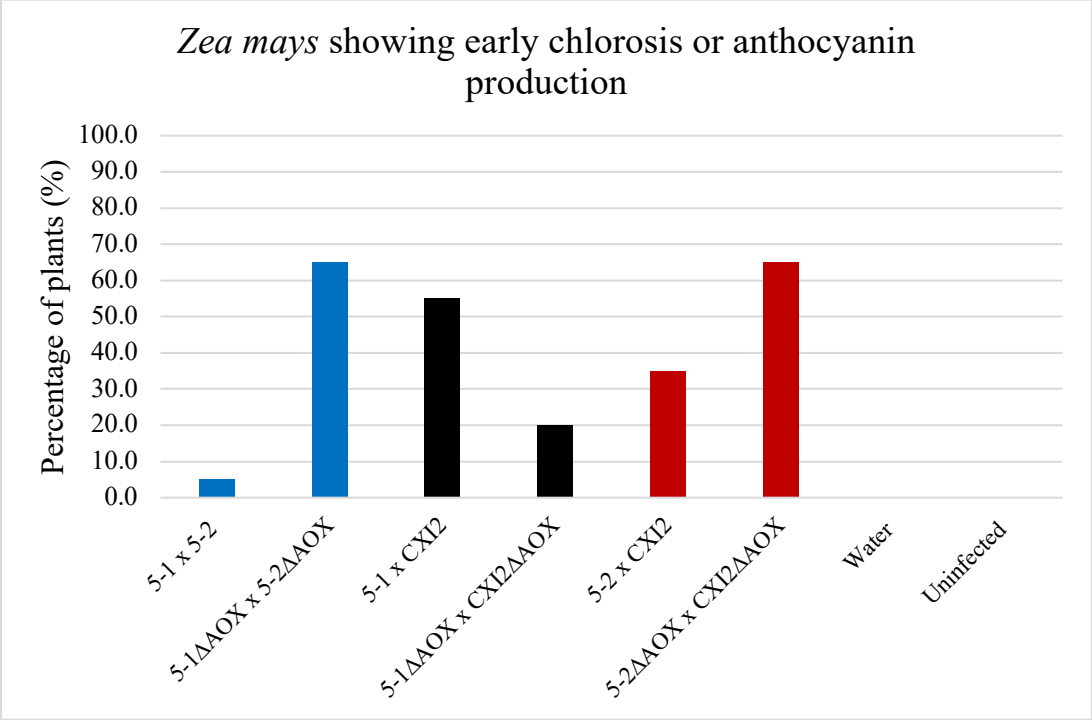

Figure 13: *Zea mays* showing early symptoms. Corn plants infected with *S. reilianum* showed the earliest symptoms of infection before the plants reached maturity. There was no clear, consistent pattern in differences regarding the wild type and mutant crosses.

S. reilianum does not show symptoms of infection until the corn plant reaches maturity at 7-8 weeks old. However, symptoms were visible on many plants before 7-8 weeks had passed, prompting further investigation of this phenomenon. Symptoms for all plants appeared mild, but chlorosis or anthocyanin production was evident; in contrast to *U. maydis* infections, no tumors were present. Although this could suggest higher virulence when the *aox* mutants were crossed, there were early symptoms present in both wild type and double mutant crosses. There were differences within the same mating groups, but these occurred in opposite directions, indicating no consistent differences. However, this may suggest differences in mating type crosses that could be studied in future investigations. The corn had not yet reached full maturity at the time of presenting these results and therefore further analysis was not possible, including assessing teliospore germination rates.

Oxidative Stress Tests

The stress tests performed on *U. maydis* did not indicate any differences in growth in response to Congo Red, hydrogen peroxide, NaCl, or sorbitol. This suggests the Δ*aox* strains could cope as well with the osmotic or oxidative stresses imposed by these agents.

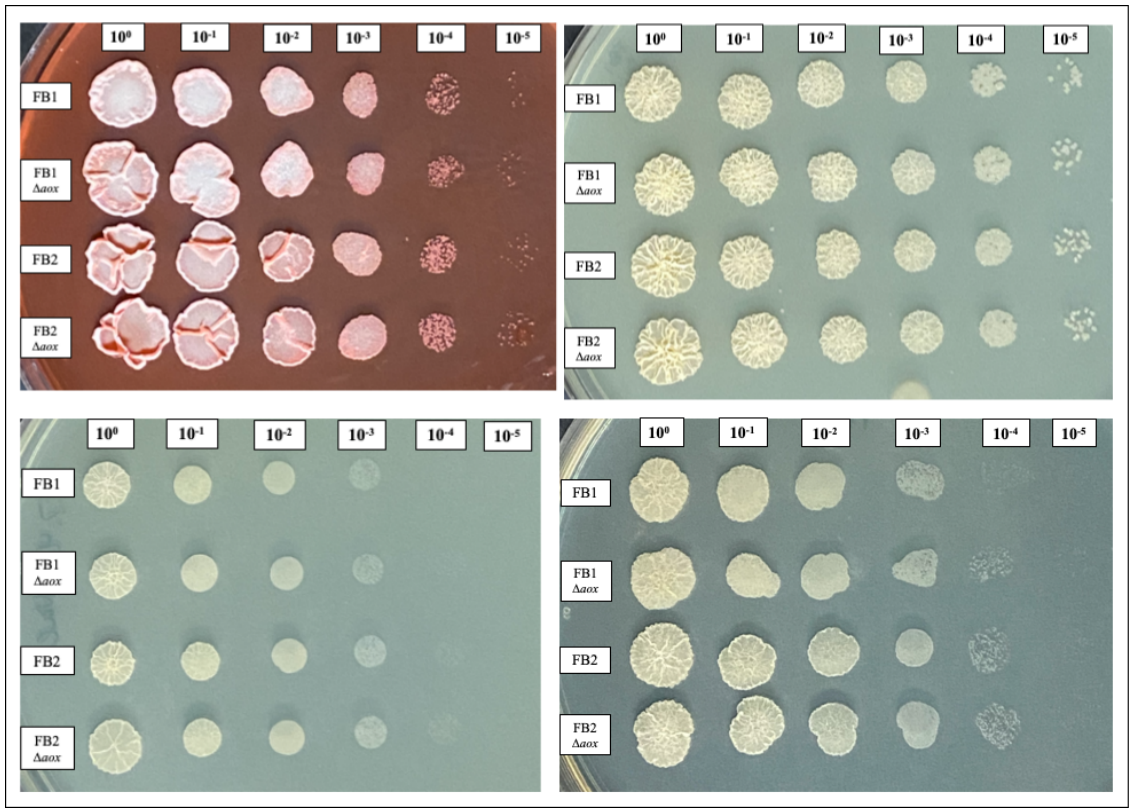

Figure 14: *U. maydis* stress tests. Top (L to R): 1 mM Congo Red and 1 mM H_2O_2. Bottom (L to R): 1 M NaCl and 1 M sorbitol. Growth differences were not displayed in Δ*aox* strains as compared to wild type.

S. reilianum also showed no difference in growth when exposed to these oxidative or osmotic stress agents. However, further assessment of mutant strains in both types of fungus should be examined via a Clark electrode to better understand the respiration patterns of the mitochondria and look for any phenotypic effects.

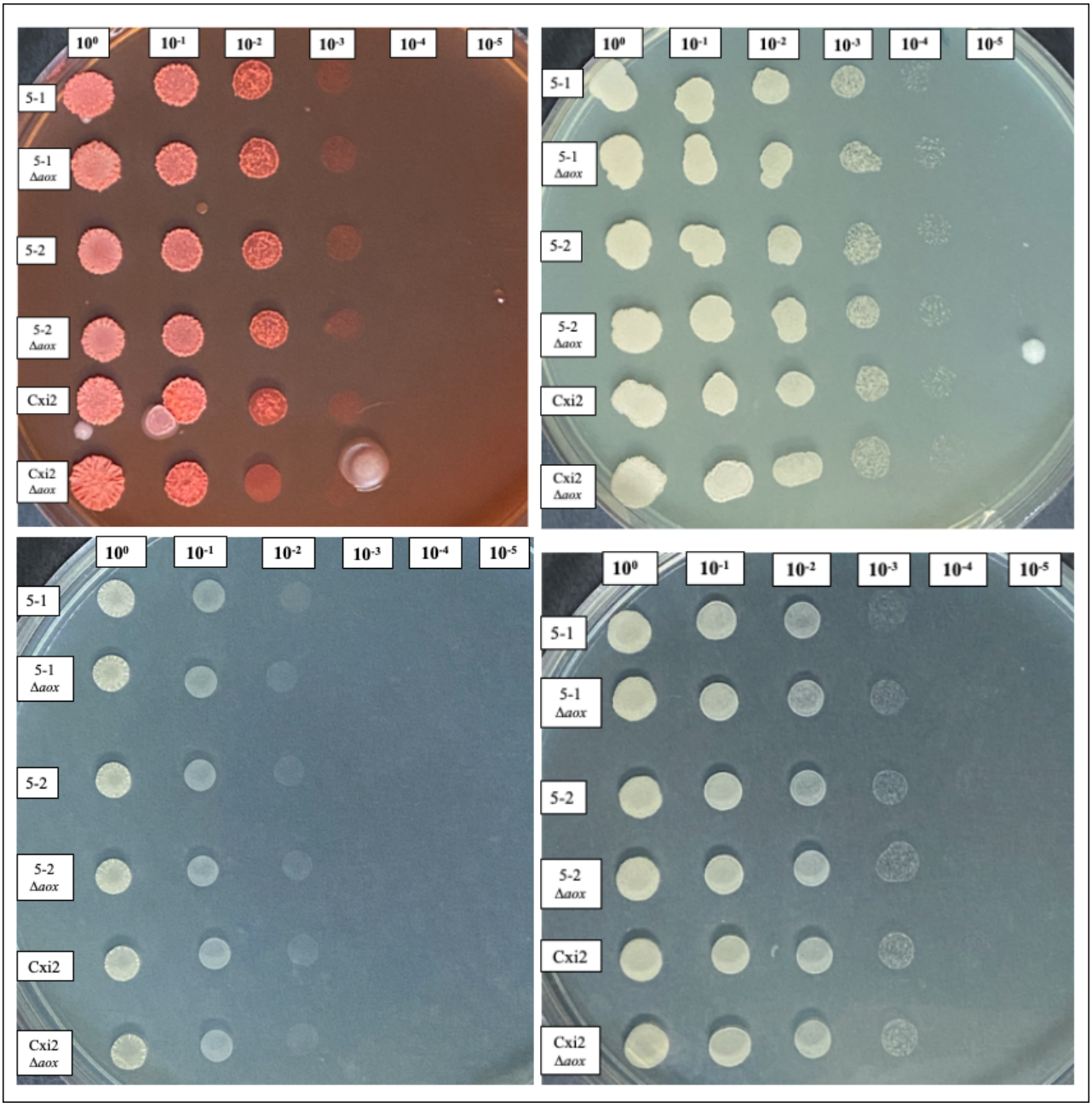

Figure 15: *S. reilianum* stress tests. Top (L to R): 1 mM Congo Red and 1 mM H_2O_2. Bottom (L to R): 1 M NaCl and 1 M sorbitol. Growth differences were not displayed in Δ*aox* strains as compared to wild type.

Discussion

This study attempted to investigate the impact of deleting the *aox* gene in both *U. maydis* and *S. reilianum* and how this affected phenotypes specifically relating to virulence on the host plant, *Zea mays*. Deletion of the gene was successfully achieved in the FB1 and FB2 backgrounds of *Ustilago*

and the 5-1, 5-2, and CXI2 backgrounds in *Sporisorium*. Successful deletion was confirmed via PCR and qRT-PCR (results not shown), ensuring the gene was not actively being expressed.

U. maydis mutant strains did not show any differences in mating on charcoal PDA media. The fuzz^{+} phenotype was unchanged, suggesting the Δ*aox* mutants were not altered in their mating ability. However, this is expected considering the *aox* gene is involved in mitochondrial respiration when the fungus faces ROS such as superoxide and hydrogen peroxide. There was also a significant difference in virulence between wild type strains and the mutant strains when infecting corn ($p < 0.001$). This directly contradicts data published that suggested *aox* was needed for coping with respiratory stress, but not necessary for pathogenicity (Cárdenas-Monroy et al., 2017). These results therefore must be replicated in order to ensure any existing differences in virulence are accurately represented. If *aox* does contribute to virulence, decreases in disease severity should be evident in the mutant strains.

There were also no differences in the stress tests despite the role of *aox* and its involvement in cellular respiration. It would have been interesting to see a change in growth in the hydrogen peroxide stress test, but there may be additional changes to the respiration profile that were not adequately assessed by this test. These smaller changes may be observed in future studies if a Clark-type electrode were used and protein complexes within the electron transport chain were inhibited. There are several known inhibitors of complexes III and IV and the alternative oxidase protein should be inhibited via salicylhydroxamic acid.

S. reilianum mutant strains showed a potential difference in mating, but it could not be quantified. Additional methods that would allow for quantification would be useful as a means of comparison with infection data. This could have implications if the mating assays and infection data contradicted. Without infection data at this time, there are three outcomes that may result based on the severity of symptoms between the wild type and mutant crosses. If the mutant crosses

result in less virulence, this would suggest alternative oxidase is necessary for pathogenicity as it is in other fungi such as *C. neoformans* (Akhter et al., 2003). The alternative is increased virulence in the deletion mutants. This could implicate *aox* as necessary for the pathogen because without this protein, it would be more likely to kill the host plant. Killing the host plant at a higher rate could be detrimental and minimize its ability to proliferate. The final scenario would result in the same levels of virulence in both wild type and mutant crosses, suggesting *aox* is not necessary for pathogenicity. This would agree with the paper published on *U. maydis* which is closely related and could indicate similar roles of the gene in both (Cárdenas-Monroy et al., 2017).

As with *U. maydis*, there were no differences in stress tests outcomes. Again, further testing should be done using a Clark-type electrode in order to gather a respiration profile for the wild type and mutant strains as well as compatible mating types. This could show a phenotype not observed within the scope of these studies.

Conclusions and Limitations

The goal of this study was to investigate the role of *aox* in pathogenicity in both *U. maydis* and *S. reilianum*. It attempted to evaluate the impact of deletion of this gene and infecting *Zea mays* with both wild type and mutant crosses. *U. maydis* seedlings infected with the mutant combination presented with decreased virulence but did not show differences in growth in mating assays and stress tests. Because these results contradict published data, the trials should be repeated with a larger sample size to ensure the conclusions are accurate. In addition to replicating the results seen in *U. maydis*, the corn should be grown beyond seedlings to allow for the full scope of symptoms to develop which might broaden the conclusions. It would be expected that smaller or less frequent tumor development would occur in the mutants if *aox* is necessary for virulence.

There may also be differences in teliospore germination rates. Other areas of study include making mutants in other backgrounds such as 521 (a1b1), 518 (a2b2), 1/2 (a1b1), and 2/9 (a2b2).

S. reilianum data for stress tests did not show any differences but could be repeated using a disk-diffusion method to quantify the zone of inhibition for a variety of cell stressors. Unlike *U. maydis*, there was slight contrast between wild type and mutant crosses in the mating assays. These can be compared to data once available from the current infections. If there are not differences in mating as shown by the assays, this could be an area of further study. For both types of fungus, further understanding of their respiration profiles can be gained by using a Clark electrode to give real-time data regarding their respiration outputs. Once optimized, these data could also point to phenotypic differences. By striving for these future goals, more can be discerned regarding the impact of *aox* on respiration and virulence of two related fungi, *U. maydis* and *S. reilianum*.

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