2012

Determining the Virulence of Corn Pathogen *Fusarium verticillioides* Utilizing Deletion Mutants Lacking Genes FV_00027 or FV_NPS6

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DETERMINING THE VIRULENCE OF CORN PATHOGEN *FUSARIUM VERTICILLIOIDES* UTILIZING DELETION MUTANTS LACKING GENES FV_00027 OR FV_NPS6

A Thesis Submitted

in Partial Fulfillment

of the Requirements for the Designation

University Honors with Distinction

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May 2012

This Study by: Abigail Zieman

Entitled: Determining the virulence of corn pathogen *Fusarium verticillioides* utilizing deletion mutants lacking genes Fv_00027 or Fv_NPS6

has been approved as meeting the thesis or project requirement for the Designation

University Honors with Distinction or University Honors (select appropriate designation)

April 27, 2011
Date

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5/7/12
Date

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Abstract

Fusarium verticillioides is an important fungal pathogen of corn. Many pathogens have virulent genes which are genes that increase infectivity of the pathogen. After a bioinformatics study, it was determined that genes Fv_00027 and Fv_NPS6 are potential virulent genes in *F. verticillioides*. To determine if either gene contributes to the virulence of the fungus, deletion mutants of *F. verticillioides* were created lacking either Fv_00027 or Fv_NPS6 genes. These deletion mutants were utilized in an optimized corn assay to determine the effect of infectivity on corn. To test these deletion mutants, corn seeds infected with *F. verticillioides* were grown on germination paper and harvested. The assay included both negative (no fungus) and positive (wild type *F. verticillioides*) controls. The seedling weights and shoot weights were collected for each treatment and a computer program was used for the statistical analysis.

Acknowledgements

Thank you to my research advisor Dr. Nalin Goonesekere of the University of Northern Iowa Chemistry and Biochemistry Department for his guidance and support throughout this process. I would also like to thank Dr. Marek Sliwinski from the University of Northern Iowa Biology Department for his advice and for graciously letting me use his lab. Thank you to Dr. Simet from the University of Northern Iowa Chemistry and Biochemistry Department for the use of his lab. Thank you to Divya Chouhan for the use of her thesis and for training me in lab. Thank you to Boi Lam Hong for his technical assistance with running experiments. This research was supported by a SOAR award from the College of Humanities Arts and Sciences as well as an Intercollegiate Academics Fund research award. Finally, thank you to all my friends and family for their support throughout this entire process.

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1. Introduction

The *Fusarium verticillioides* fungus has been identified as a symptomless endophyte (Bacon C. W., 1992; Bacon C. W., 1996), implying that there is a beneficial host-fungal relationship of benefit to plants in the Gramineae or grass family (Rice, 1990). It is when this relationship is disrupted, either by abiotic or biotic factors that the fungus becomes pathenogenic, resulting in higher levels of mycotoxin production (Desjardins, 1998). A couple of these factors or stressors that have been shown to cause pathenogenicity are moisture stress (Bacon C. W., 2001) and tissue damage (Yates, 2000).

Moisture stress and tissue damage may cause disease of the plant from *F. verticillioides* infection, which can be asymptomatic, or cause severe rotting of the plant. Whichever way the fungus presents itself is dependent upon the growing conditions. The strain that is of primary focus in this study, *Fusarium verticillioides* also known as *F. moniliforme*, produces toxins called fumonisins, which are toxic to the plant as well as to humans and several domesticated animals (Nelson, 1993). Fumonisins are toxic because they disrupt sphingolipid metabolism (Wang, 1991). Sphinogolipids are important cell membrane components involved in the regulation of cell growth and differentiation (Merrill, 1991), and so disrupting sphingolipid metabolism causes severe physiological effects. Fumonisins produced by *F. verticillioides* infection have also been classified as potential carcinogens for humans (IARC, 2002). Since these toxic fumonisins are present in asymptomatic plants, as well as symptomatic plants, this puts control of fumonisin contamination and *F. verticillioides* infection as a priority for food safety research (Brown, 2006).

Food safety research is also a priority because *F. verticillioides* is a pathogen of vegetables and grains including corn, wheat, potato, cassava, palm, banana, and pine. *F. verticillioides* is responsible for several different diseases including root, stalk and ear rot, blights, and wilts in these crops. Infection of these crops can occur at any stage of development (Brown, 2006). The versatility of this fungal pathogen makes *F. verticillioides* a dangerous crop pathogen.

There is one crop in particular that is of great importance, which is corn. *F. verticillioides* infection is the most prevalent fungal infection of corn (*Zea mays*) (Leslie, 1996; Lorang, 2001; Nelson, 1993). Corn is a major food source around the world, and is an important economical crop for Iowa. Since corn is a major staple in the diet of many people around the world, it is important to fully characterize this interaction between *F. verticillioides* fungus and corn such as determining the genes responsible for infection.

Since *Fusarium verticillioides* is the most reported fungal infection of corn the objective was to determine if predicted genes were responsible for infecting the corn plant. Two genes, Fv_00027 and Fv_NPS6, were tested using a the Corn Assay utilizing previously made deletion mutants of *F. verticillioides* to determine if either gene contributes to virulence of the fungus. The hypothesis was that the *F. verticillioides* mutants lacking either genes Fv_00027 or Fv_NPS6 are less virulent than the wild type *F. verticillioides*. To quantify these results, the Corn Assay used three treatments Positive Control, Negative Control, and a Mutant treatment. Seedling weights were averaged and then used in a statistical analysis to quantitatively show whether or not the deletion mutant affected the plant differently than either control.

2. Literature Review

The entire mechanism for *F. verticillioides* infection of corn remains a bit of a mystery. A recent hypothesis is that the conidia, or spores, of the fungus enter through a small opening in the ovary wall of the corn kernel. This area is referred to as the stylar canal. Before the corn is harvested, water carries the *F. verticillioides* spores down the surface of the silks of the corn to the kernels where the spores are funneled into this stylar canal and gain entry into the kernel. This was determined by a study done by Duncan and fellow researchers. This study also showed that there are different phenotypes of the stylar canal that may affect resistance to *F. verticillioides*. These results were obtained using a fluorescent protein tagged to transformants of *F. verticillioides* and a Scanning Electron Microscope (Duncan, 2009).

While some kernels have a natural resistance to *F. verticillioides*, one method that was recently used to control *F. verticillioides* infection was the use of bacteria. The bacteria *Bacillus amyloliquefaciens* and *Microbacterium oleovoransn* both promoted a significant reduction in fumonisin production. Paola Pereira, a research scientist from the Universidad Nacional de Rio Cuarto in Cordoba, Argentina, obtained these results by running experiments with three treatments. The first set of seeds was incubated in saline solution as a control, the second set of seeds was incubated with *B. amyloliquefaciens*, and the third set of seeds was incubated with *M. oleovorans*. The seeds were then planted in late November and harvested in the middle of March. The seeds were infected by *F. verticillioides* in the soil (Pereira, 2001).

Instead of infecting seeds with bacteria to prevent infection, one approach is to determine the genes responsible for infection. Virulence genes are genes that increase the virulence, or infectivity, of a pathogen by aiding the multiplication of the pathogen, which in this research is

F. verticillioides (Poulin, 1999). To identify other possible virulence genes in *F. verticillioides*, a bioinformatics study was performed by Dr. Nalin Goonesekere of the University of Northern Iowa Chemistry and Biochemistry department (Chouhan, 2010). After the bioinformatics study identified possible virulent genes, those genes needed to be tested. This led to the creation of the deletion mutants of *F. verticillioides* used in the Corn Assay experiments.

The deletion mutants created for the Corn Assay Experiments were knock out mutants, which means that one of the genes in the genome of the fungus was targeted and replaced with another gene; a Hygromycin Resistance Gene (HYG). The Hygromycin Resistance Gene was inserted for the purpose of screening for correct deletion mutants. Using several rounds of Polymerase Chain Reaction (PCR), linear fragments of DNA were created, which consisted of a Hygromycin Resistance Gene, flanked by the flanking regions of the target gene. This double-joint PCR product was then cloned into a TOPO XL vector to make plasmids, which were then transformed into wild type *F. verticillioides*. When the plasmid was transformed into the wild type *F. verticillioides*, homologous recombination occurred to replace the target gene with a Hygromycin Resistance Gene, which allowed for screening for possibly correct deletion mutants. The Hygromycin Resistance Gene would make the correct deletion mutants resistant to Hygromycin, an antifungal compound. The deletion mutants were also checked for accuracy using different rounds of PCR to amplify the Hygromycin Resistance Gene, and the target gene (Chouhan, 2010). The target gene was either Fv_00027 or Fv_NPS6 because those were the two genes identified as being virulent in the bioinformatics study. The two deletion mutants that were created were then for genes Fv_00027 and Fv_NPS6. Another gene that has been identified as a virulence gene by a different scientist in *F. verticillioides* is FSR1 (Shim, 2006).

To determine with certainty that the linear double-joint PCR product was correctly transformed into the plasmids, some plasmids were sent to the Iowa State University Sequencing Facility for sequencing. One set of plasmids that were sequenced showed that the double joint fragment was correctly cloned into the bacterial vector. It has already been shown that the deletion mutants lack the target gene through PCR, and the sequencing supported those results. After the creation of these deletion mutants, they were utilized in the Corn Assay.

The Corn Assay was used to try and determine if the predicted genes played a role in pathogen infectivity. The assay includes Negative (no fungus) and Positive (wild type *F. verticillioides*) controls which set the baseline for the mutant treatment. A lot of time was spent optimizing the corn assay through experiments called corn germination screens. Corn germination is dependent upon the concentration of water and oxygen that gets to the seeds as well as the temperature of the growing environment. The germination step was critical in the full Corn Assay. The corn seeds needed to germinate to determine that plant growth was affected by the fungus only, which could not be done if the seeds were dead and unable to germinate even under optimal circumstances. The Corn Assay is an important part of this research because the predicted genes needed to be tested in a real-world, but still in a controlled environment to minimize external variables. Even though the bioinformatics study predicted these genes as being virulent does not mean that affect virulence.

3. Definitions

Assay- Refers to an experiment.

Cloning- A process used insert a linear piece of DNA into a circular plasmid.

Deletion mutant- In this study will refer to *F. verticillioides* lacking one gene that has been replaced by a Hygromycin Resistance Gene.

Double-Joint Product- A linear piece of DNA composed of a Hygromycin Resistance Gene and the right (3' region) and left (5' region) flank of either target gene Fv_00027 or Fv_NPS6.

Germination- Germination was defined in this study as the point when a corn seed had a radical shooting out of the bottom of the seed and was not cracked.

Homologous recombination- A naturally occurring process in a living organism where a gene can be knocked out and replaced by another gene because the regions around the gene have homogeny, or are very similar.

Hygromycin- An anti-fungal compound.

Inoculate- To infect.

Inoculums- Inoculums are what cause infection. In the Corn Assay, it refers to the *F. verticillioides* conidian used to infect the corn seeds.

Negative Control- The Negative Control is the set of seeds that was not infected with any fungus. The point of the Negative Control is to show what happens in the absence of the *F. verticillioides*.

Polymerase Chain Reaction (PCR)- A method of DNA amplification. Primers are used to specify the specific region of template DNA that needs to be amplified.

Plasmid- A circular piece of DNA found in bacteria independent of the bacterial genome. Plasmids are utilized a lot in biochemistry because they are a way to produce a large amount of DNA very quickly and easily.

Primers- Short lengths of DNA about 18-25 bp long used in Polymerase Chain Reaction to amplify DNA.

Protocol- A protocol is a procedure or method used to conduct an experiment

Positive Control- The Positive Control is the set of seeds that was infected with the wild type *F. verticillioides*. The purpose of the Positive Control is to show what happens in the presence of wild type *F. verticillioides*.

Virulence- Ability of a pathogen to cause disease in the host organism

4. Methodology

a. Procedure for Creating Deletion Mutants

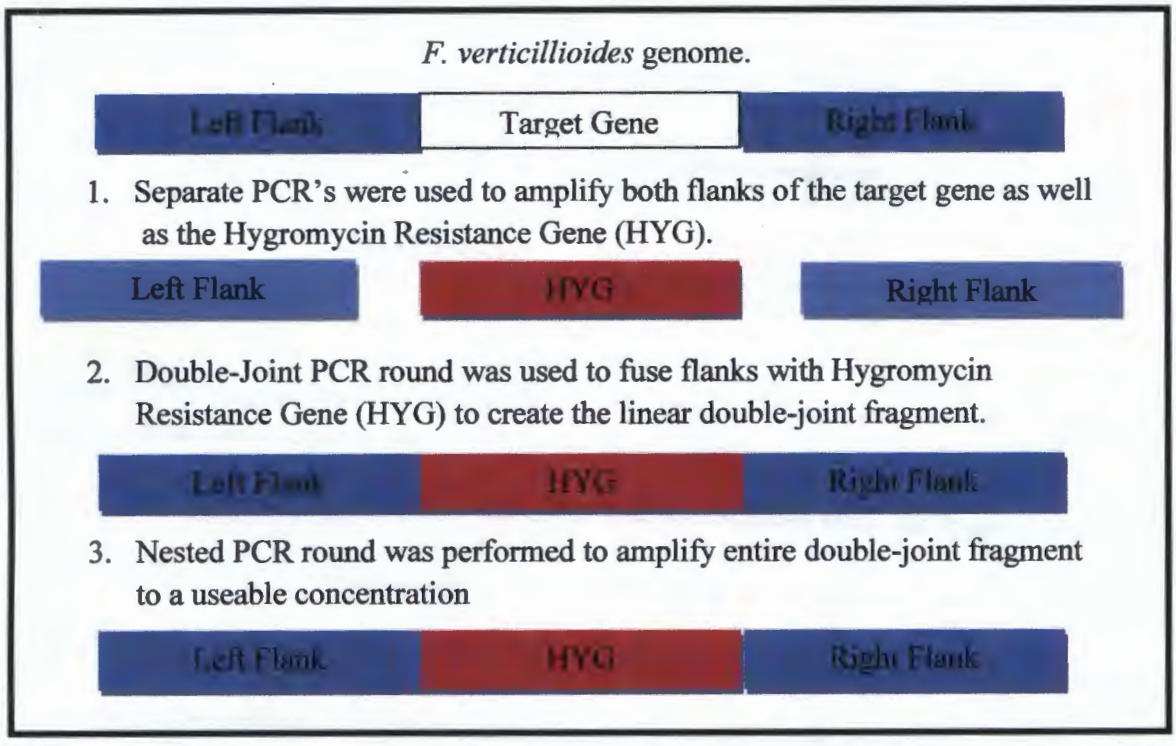

Figure 1: This diagram is an illustration of the creation of the double-joint fragment. There were three rounds of PCR performed.

Figure 2: The vector used for cloning was from the TOPO XL PCR Cloning Kit. The insert was inserted between the P_{lac} and lacZ sites. This picture is courtesy of the Invitrogen website. The full sequence of the vector is located on the Invitrogen website.

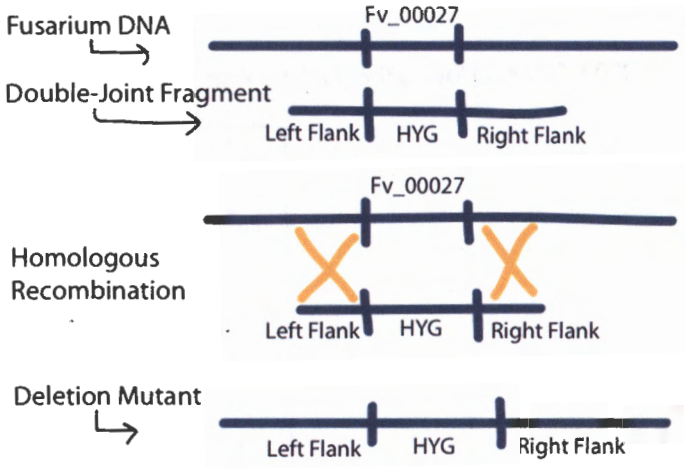

Figure 3: The diagram above is an illustration of the process of homologous recombination, which was used to create the deletion mutants of *F. verticillioides.* In this case gene Fv_00027 is the target gene.

A length of sequence both upstream and downstream from the target gene was amplified using PCR. These flanking regions were then fused to a Hygromycin Resistance Gene using the Double-Joint PCR method. Afterwards, these Double-Joint Fragments were amplified to a high enough concentration to be cloned into a bacterial vector plasmid, which was referred to as the nested PCR round. After performing an agarose gel extraction with crystal violet, the Double-Joint Fragments were cloned into a bacterial vector plasmid. Cloning was done with the TOPO XL PCR Cloning kit from Invitrogen. After the Double-Joint Fragment was successfully cloned into the plasmid, the plasmid was transformed into wild type *Fusarium verticillioides*.

Custom primers were designed for each round of Polymerase Chain Reaction (PCR) utilizing the website http://frodo.wi.mit.edu/primer3/, and were shipped from Integrated DNA Technologies (IDT) in Coralville, IA. The PCR reagents were from the TaKaRa PCR kit and the reactions were combined according to manufacturer's recommendations. The PCR cycles used are listed in Tables 1-3. After transformation of the cloned plasmids, the deletion mutants were checked with PCR. The primers used and the cycle are included in Tables 4 and 5.

Table 1: Flank and HYG PCR Cycles. X=50°C, 52°C, 54°C, 56°C, 58°C, 60°C.

Step 1	94° C	3 minutes	1 cycle
Step 2	94° C	1 minute	30 cycles
	X° C	15 seconds	
	72° C	5 minutes	
Step 3	72° C	10 minutes	1 cycle
hold	4° C	∞	

Table 2: Double-Joint PCR Cycle.

Step 1	94° C	2 minutes	1 cycle
Step 2	94° C	45 seconds	15 cycles
	58/62° C	10 minutes	
	72° C	12 minutes	
Step 3	72° C	15 minutes	1 cycle
hold	4° C	∞	

Table 3: Nested PCR Cycle. X=48°C, 52°C, 54°C, 56°C, 58°C, 60°C

Step 1	94° C	2 minutes	1 cycle
Step 2	94° C	45 seconds	35 cycles
	X° C	45 seconds	
	72° C	12 minutes	
Step 3	72° C	15 minutes	1 cycle
hold	4° C	∞	

Table 4: Primers used for deletion mutant verification.

Primer Name	Primer Sequence (5' to 3')
R_hygroshort_p96	GAA AGC ACG AGA TTC TTC GC
L_hygroshort_p96	TCT TGT CAA CCG TCT TCT CC
InternalF_00027_p104	GAA CGA TCT CTC ACC GTA
InternalR_00027_p104	GGG GAT TGA CTC GAT ACA GG
veri_LF_for_00027_p109	CTG GGC CAT CAA TAG GAC AT
veri_LF_rev_00027_p109	CAA CAT GAT GTC AGG CCA TT
veri_RF_for_00027_p109	GAC GAT GAG CGC ATT GTT AG
veri_RF_rev_00027_p109	TAG ACG CGA AGA GAT CAT CA
InternalF_NPS6_p104	TGA GAT CTC TGC AAA CCT CC
InternalR_NPS6_p104	TGT CAA CAG AAC TAG AGG CG
veri_LF_for_NPS6_p109_2	CCA AGA CTA GCG AGG CAC AT
veri_LF_rev_NPS6_p109_2	CCG TCG ATG GAT AGA CTG TT
veri_RF_for_NPS6_p109	AGA TTT CAT ACA CGG TGC CT
veri_RF_rev_NPS6_p109	TGC TGC TCA GTT CTT TGA GA

Table 5: Deletion mutant verification cycle. X=50, 52, 54, 56, 58, 60° C

Step 1	94° C	3 minutes	1 cycle
Step 2	94° C	1 minute	30 cycles
	X° C	1 minute	
	72° C	2 minutes	
Step 3	72° C	10 minutes	1 cycle
hold	4° C	∞	

Purification of DNA from the Agarose Gel Using the Promega Wizard PCR Clean Up Kit

A 0.7% agarose gel with ethidium bromide was run for 50 minutes at 110 V with the PCR products to be purified. The gel was visualized and photographed using a long-wavelength UV lamp for as short a time as possible. The DNA fragment of interest was then excised using an autoclaved spatula. The gel slice was transferred to a preweighed 1.5 ml microcentrifuge tube to obtain the weight of the gel slice.

Membrane Binding Solution was added in a ratio of 10 µl of solution per 10 mg of agarose gel slice. The mixture was vortexed unless the DNA fragment was longer than three kilobases and incubated at 55° C for 10 minutes, or until the gel slice dissolved. An SV Minicolumn was placed into a Collection Tube. Each gel slice mixture was poured into a separate SV Minicolumn/Collection Tube assembly and was set aside to incubate for three minutes at room temperature. The SV Minicolumn assembly was then centrifuged at 13,000 RPM for three minutes. To optimize the product yield, the transfer flow through in the Collection Tube was then poured back into the SV Minicolumn and incubated and centrifuged for one minute each. The column was then washed by adding 700 µl of Membrane Wash Solution, previously diluted with 95% ethanol, to the SV Minicolumn. The assembly was then centrifuged for three minutes at 13,000 RPM. The SV Minicolumn was then removed from the assembly

without wetting the bottom of the column with the flow through. The Collection Tube was emptied and wiped out to remove any ethanol. The SV Minicolumn was then replaced in the Collection Tube and the assembly was centrifuged an additional three minutes at 13,000 RPM to remove any residual ethanol.

The wash step was repeated with 500 µl of Membrane Wash Solution only the SV Minicolumn assembly was centrifuged for 7.5 minutes at 13,000 RPM in the second wash step. The SV Minicolumn was then removed again and the Collection Tube was once again emptied and wiped out with a kimwipe to remove any excess ethanol. The assembly was reassembled and then centrifuged for an additional three minutes to once again allow evaporation of any residual ethanol. The SV Minicolumn was then transferred to a clean 1.5 ml microcentrifuge tube. A 30 µl aliquot of autoclaved water was then added directly to the center of the column with a micropipette. The column was incubated at room temperature for five minutes and then centrifuged for five minutes at 13,000 RPM. The microcentrifuge tube with the eluted DNA was then stored at 4° C and a 0.7% agarose gel was run to determine concentration of the purified DNA.

Procedure for Cloning DNA primarily using the TOPO kit

A 0.8% agarose gel was prepared with 0.8 grams of agarose and 100 ml of 1X TAE buffer. After dissolving agarose in the TAE buffer, 40 µl of 2 mg/ml Crystal Violet solution was added to the agarose making it a slight purple color. The gel box and comb (8 well) were rinsed with autoclaved water before pouring the agarose gel. Once solidified, the gel was placed in the gel tank and covered with autoclaved 1X TAE buffer.

The 6X Crystal Violet Loading Buffer was then added in a ratio of 8 µl to 40 µl of PCR

product and loaded into the wells of the gel. The gel was run at 80 volts for 1.5 hours. The DNA fragment was then excised with an autoclaved spatula under a fluorescent light to visualize the thin blue PCR product band. The excised gel slices were then purified using the protocol for the Promega Wizard PCR Clean-Up Kit. The Mock PCR step was then run with purified nested PCR product (200ng), PCR Buffer, dNTPs, and noncommercial Taq from Dr. James Jurgenson from the University of Northern Iowa Biology Department. This ensured that the DNA had an extra A, which is necessary for cloning. The cycle was 72°C for 30 minutes.

The following five microliter TOPO® Cloning reaction was set up in a sterile microcentrifuge tube: four microliters gel-purified PCR product and one microliter PCR®-XL-TOPO® vector. The mixture was mixed gently and then incubated five minutes at room temperature. After the five minute incubation, one microliter of the 6X TOPO® Cloning Stop Solution was added and then the mixture was mixed for several seconds. The tube was then briefly centrifuged and placed on ice.

What followed was the transformation of plasmid into One Shot® TOP10 chemically competent cells. A volume of 2 µl of the TOPO® Cloning reaction was added to vial of One Shot® cells. The mixture was then incubated on ice for 30 minutes. The cells were then heat-shocked for 30 seconds at 42°C without shaking. The tube was then immediately transferred to ice and incubated an additional two minutes. A volume of 250 µl of room temperature S.O.C medium was added to the tube before the tube was capped and shaken horizontally at 37°C for one hour. The reaction was then placed on ice. The cells (50-150 µl) were then spread onto prewarmed LB/kanamycin plates and incubated overnight at 37°C.

While the Double-Joint Fragment did not need to be cloned into the plasmid for homologous recombination to occur, there was a greater yield of mutants when the plasmid was used. It was also determined that flank sizes of ~1000-1500 base pairs were ideal for the creation of deletion mutants. This was determined by using varying sizes of flanks. The largest flank size tested was ~4000 base pairs long. Larger flanks did not increase the efficiency of homologous recombination as originally predicted.

Method for Isolating Plasmid Using Promega Wizard Pure Yield System

A 1.5 ml aliquot of bacterial culture was centrifuged for 30 seconds at 13,000 RPM in a microcentrifuge. After the supernatant was discarded, an additional 1.5 ml of bacterial culture was added to the same tube and centrifuged for 30 seconds at 13,000 RPM. Using a micropipette, 600 µl of autoclaved distilled water was then used to resuspend the cell pellet. Next, 100 µl of Cell Lysis Buffer was added to each microcentrifuge tube and inverted six times to ensure thorough mixing. Cell lysis was indicated by the color change from opaque to clear blue. Within three minutes of adding the Cell Lysis Buffer, 350 µl of cold (4-8°C) Neutralization solution was added and mixed thoroughly by inverting the tube. The neutralization was complete when the solution changed color from clear blue to yellow. The microcentrifuge tube was then centrifuged at 13,000 RPM in a microcentrifuge for three minutes. The supernatant was then transferred to a Pure Yield minicolumn. The minicolumn was then placed into a Pure Yield collection tube and centrifuged at 13,000 RPM for 15 seconds. The flow through was discarded and the minicolumn was placed back into the same Pure Yield collection tube. The Endotoxin Removal Wash, 200 µl, was the added to the minicolumn and centrifuged at 13,000 RPM for 15 seconds. A micropipette was then used to add 400 µl of Column Wash Solution to the minicolumn and was centrifuged at 13,000 RPM for 30 seconds. The minicolumn was then

transferred to a clean 1.5 ml microcentrifuge tube. Then 30 µl of elution buffer was added directly to the minicolumn matrix and incubated at room temperature for five minutes. After the five minute incubation, the column in the microcentrifuge tube was centrifuged at 13,000 RPM for 15 seconds to elute the plasmid DNA. When the plasmid was greater than 10 Kb, the elution buffer was warmed to 50° C using a water bath prior to elution. Then 50 µl of elution buffer was added directly to the minicolumn matrix instead of 30 µl. The rest of the protocol remained the same. The microcentrifuge tubes with the eluted plasmid were then stored at 4°C. To determine concentration, the eluted plasmids were run on a 0.7% agarose gel.

Fungal Transformation Procedure

Circularized plasmid (1-5 µg) was added into 200 µl thawed protoplast suspension and the mixture was incubated for 20 minutes on ice. After the incubation, one ml of PTC (40% PEG 4000 in 1X STC) solution was added to the mixture and incubated at room temperature for another 20 minutes. The mixture was transferred to an orange capped Falcon tube and five ml of TB3 media (3g/L yeast extract, 3g/L casamino acids, 20% sucrose) was added. The entire mixture was then incubated 15 hours with shaking of 80 RPM at room temperature. Next the culture was centrifuged at 4000 xg for 5-8 minutes using an ultracentrifuge and the supernatant was discarded with a pipette. One ml of STC (1.2M sorbitol, 10 mM Tris-HCl pH 8, 50mM $CaCl_2$) was added to the hyphae along with nine ml of TB3 media with 0.7% agarose and 100 µg/ml of Hygromycin. This mixture was then poured onto a petridish. When the agarose had solidified, the plate was sealed lightly with parafilm and incubated another 15 hours upside down at room temperature. After the 15 hours the plate was overlaid by adding 10 ml of TB3 media with 0.7% agarose and 200 µg/ml hygromycin. The mutants grew within 2-4 days and then the

growing colonies were transferred to Fv complete media with 20 g/ml agar and 200 μg/ml hygromycin.

After the transformation step, it took about five days for the mutants to grow. One of the colonies on the plate was used to start a liquid culture with complete Fusarium Complete Media (For one liter: 30 g sucrose; 2 g $NaNO_3$; 1 g KH_2PO_4; 0.5 g $MgSO_4$*7 H_2O; 0.5 g KCl; 2.5 g N-Z Amine A; 0.2 ml trace elements; 10.0 ml Vitamins) for several days. Afterwards, the genome was extracted using the Fungal Genome Extraction protocol. The resulting deletion mutant genomes were verified using PCR to check for the Hygromycin Resistance Gene as well as the target gene. The mutants were defined as correct if the Hygromycin Resistance Gene was present and the target gene was not. To ensure correct insertion of the entire Double-Joint Fragment, another round of PCR was performed. This round was referred to as Deletion Mutant Verification PCR and used primers designed to amplify the region where the fungal genome meets the double-joint fragment.

Fungal Genome Extraction

A plate (Fusarium Complete Media +200 μg/ml Hygromycin) was streaked from the conidia of the deletion mutants. Liquid cultures were grown for two to three days in 40 ml Fusarium Complete Media + Hygromycin in a 250 ml Erlenmeyer flask with shaking at 28° C. Mycelium was collected by pouring the culture through filter paper and washed with sterile water, switching out filter paper when too wet to prevent tearing. Liquid nitrogen was used to freeze the mycelia to be ground into a fine powder using a mortar and pestle. The powder was suspended in 0.8 ml 2X CTAB extraction buffer (1.4 M NaCl; 100 mM Tris base pH 8.0; 2% CTAB Hexadecyltrimethylamonium bromide; 20 nM EDTA; 1% 2-ercaptoethanol (2-me)) and incubated at 65° C for 30 minutes with shaking every five minutes. Then the microcentrifuge

tube was spun at 12,000 RPM for 10 minutes and the aqueous layer was transferred to another clean microcentrifuge tube. Chloroform/isoamyl alcohol (24:1) was used in a 1:1 ratio to extract the DNA and was shaken by hand for five minutes and then centrifuged 10 minutes at 12,000 RPM. The aqueous layer was then removed and digested with RNASE A (from the Promega Wizard Genomic DNA Purification Kit) for 30 minutes at 37° C and the chloroform/isoamyl alcohol extraction was repeated. Cold isopropanol was used to precipitate the DNA. After pelleting at 12,000 RPM for two minutes the DNA pellet was dissolved in 100 µl of autoclaved water. The product was then run on a 0.7% agarose gel to determine concentration.

b. Sequencing of Deletion Mutant Plasmids

Plasmids from long term cultures were transformed into Z-Competent cells using the following protocol. 1-5 µl of plasmid DNA was added to a tube of thawed Z-Competent cells on ice and were mixed gently by finger flicking. The mixture was incubated on ice for 5-10 minutes. Afterwards four volumes of SOC medium (400 µl of SOC per 100 µl) and then shaken gently at ~90 RPM and 37° C for one hour. After this incubation the mixture was spread onto LB/kanamycin (50 µg/ml) plates that had been prewarmed for at least 30 minutes and left overnight at 37°C. One colony was used to inoculate a liquid culture with LB media + kanamycin (50 µg/ml). The culture was allowed to grow 12 hours before the plasmids were purified using the Promega Wizard Pure-Yield System according to the protocol discussed earlier. The plasmids were then mailed overnight to the Iowa State University Sequencing Facility where they performed the sequencing reactions. The sequences were then downloaded and aligned to the expected sequences using BLAST.

c. Corn Assay

The Corn Assay, an analytic method used to quantitatively measure how *F. verticillioides* infects corn, is based on another corn assay (Fuchs, 2004). The major difference between the two assays is that in this assay, the germinated corn seeds were grown on germination paper instead of using soil and pots. The suggestion to use the germination paper came from Dr. Marek Sliwinski in the University of Northern Iowa biology department. This protocol was optimized throughout this process and the most optimized protocol is outlined below. The corn assay involves sterilization, inoculation, germination, and harvest of corn seeds followed by a statistical analysis of the total weights and shoot weights of the seedlings.

The sterilization involves covering hybrid sweet corn seeds (Johnny's Selected Seeds Hybrid corn, white sweet silver queen F1) in a sterilized Erlenmeyer flask with a mixture of 0.05% Tween in sterile water, and shaking at 70 RPM for three minutes. After decanting the water, the seeds were covered with a solution of 50% Clorox bleach with Tween. The flask was then shaken at 70 RPM for 10 minutes. The seeds were washed with a large volume of water two times to remove any remaining bleach. The seeds were then covered with sterile water and incubated in a water bath at 28 °C for four hours.

After the incubation, the water was decanted off the seeds, and the seeds were then covered with water that was 60°C and put into a 60°C water bath for four minutes. Afterwards, the water was decanted off, 20 mL of room temperature water was added and the flask was covered with aluminum foil. After the seeds were left at 28°C for two days, the seeds that had germinated were selected until each treatment had 40 germinated seeds. Then 20 milliliters of

water and two microliters of inoculum were added to the seeds and the flasks were placed in the fridge for two days.

The seeds were then placed onto germination paper that had been soaked with distilled water. The paper was rolled up and then covered in saran wrap and left in a beaker with water to germinate for 10 days with occasional watering and were then harvested. At harvest, the following data was collected: the weight of the seed, the weight of the shoot, and whether the seed was infected or not. Since *F. verticillioides* infection affects the early development of the plant, from recording the above data it was possible to determine if the mutant *F. verticillioides* infects the plants as severely as the wild type *F. verticillioides*.

There were three treatments done, a Negative Control, the mutant condition, and the Positive Control. The Negative Control had seeds treated the same, except no inoculums or fungus was added to the seeds in the inoculation step. This provided the standard for how the corn seedlings grow without any infective fungus. The mutant treatment had the seeds infected with either the Fv_00027 or Fv_NPS6 deletion mutants. The Positive Control was the treatment where the corn seeds were infected with the wild type *F. verticillioides* which gave a standard for how the corn seedlings grow when infected with wild type *F. verticillioides*.

A statistical analysis of the average total weights of the corn seeds and average shoot weights from each treatment were done to determine if there was a statistically significant difference between each treatment. If the seeds treated with the mutant were statistically different from the seeds treated with the wild type *F. verticillioides* then it was determined that the mutant is less virulent. The statistical analysis was done by the computer program SPSS.

d. Protocol Optimization

There were multiple instances where the corn assay described above needed to be optimized. These protocol optimizations were referred to as germination screens. These germination screens would consist of the first part of the corn assay where the seeds are sterilized, heat shocked, and incubated for two days at 28° C. Then the seeds would be counted to determine if there was a change in germination.

Table 6: Summary of Full Length Corn Assay Protocols. Even though the protocol had been previously optimized, the protocol needed to be optimized multiple times. This table summarizes any of the changes that were made in the protocol as a result of these protocol optimization experiments. Old* refers to seeds purchased in August 2010. New* refers to seeds purchased in August of 2011.

xperiment	Inoculation Time	Brand of Bleach	Age of Seed	Parafilm/Aluminum Foil	Deletion Mutant
	Day 1	Generic	Old*	Parafilm	Fv_00027
	Day 1	Clorox (50%)	New*	Parafilm	Fv_00027
	Day 3	Clorox (50%)	New*	Parafilm	Fv_00027
	Day 1	Clorox (50%)	Old*	Parafilm	Fv_00027
	Day 3	Clorox (50%)	Old*	Aluminum Foil	Fv_00027

5. Results and Discussion

a. Creation of Deletion Mutants

The methodology used to create the deletion mutants utilized a cloning step, which was shown to be important for deletion mutant creation. It was tested whether the Double-Joint Fragment could be transformed directly into the *F. verticillioides*. It was shown that there was a higher yield of deletion mutants when the Double-Joint Fragment was cloned into the plasmid before transformation than when the linear Double-Joint Fragment was transformed into *F. verticillioidies*. As shown in Figure 4, there was a five-fold increase in successful transformation when the Double-Joint Fragment was cloned into a plasmid before transformation into wild type *F. verticillioides*.

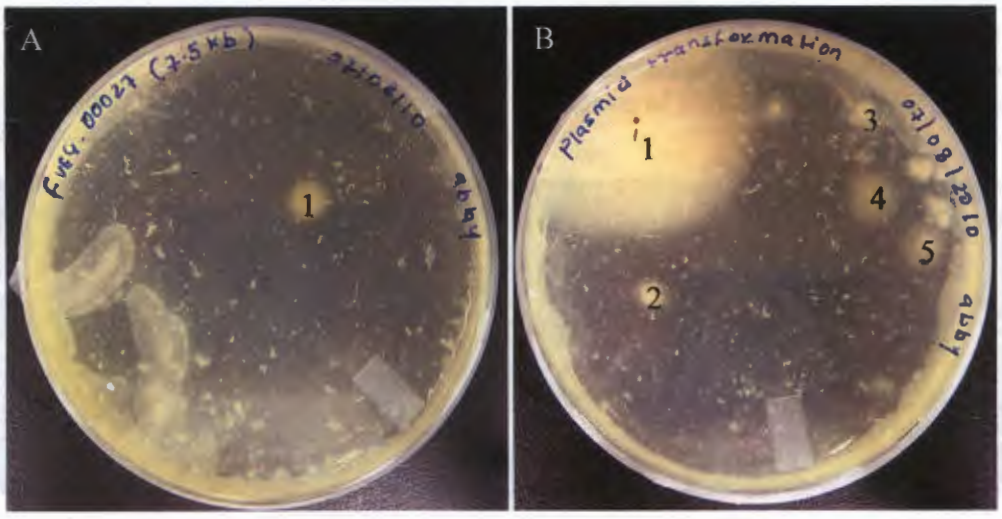

Figure 4: Pictures of fungal transformations after 12 days of incubation at room temperature. (A) Fv_00027 7.5 Kb Double-Joint 7/8/10. Pictured is the second attempt to transform the linear Double-Joint Fragment. (B) Fv_00027 11 Kb Plasmid 7/8/10. Pictured is the second attempt to transform the cloned plasmid.

The deletion mutants were checked using several different rounds of PCR. The rounds of PCR used were to amplify for the target gene, the Hygromycin Resistance Gene that was inserted into the genome, and the region where the insert meets the fungal genome. The gel pictures in

Figures 5 and 6 show that the deletion mutants were correct. There was correct amplification of the Hygromycin Resistance Gene and the fragment flanks, while there was no amplification of the target gene. All of these gel pictures are courtesy of Divya Chauhan's Master's Thesis Identification of Virulence Genes in the Corn Pathogen *Fusarium verticillioides*.

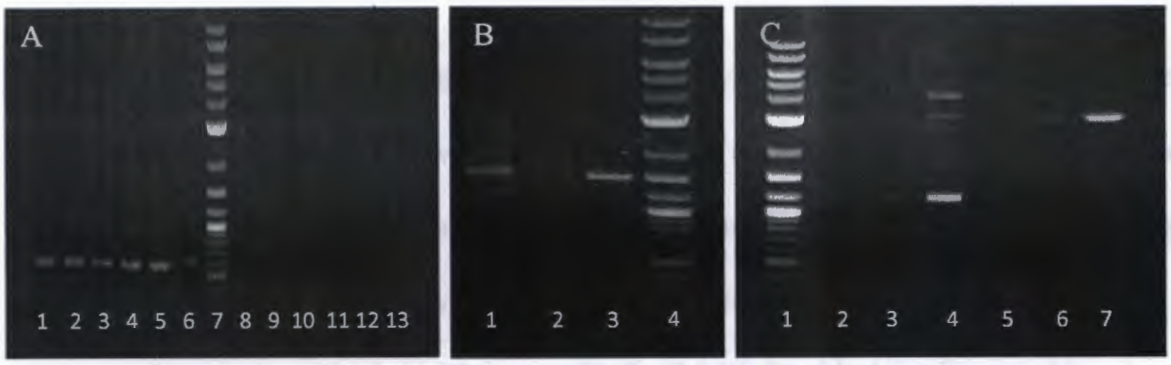

Figure 5: The results of the deletion mutant verification PCR of Fv_00027. (A) PCR for the amplification of ~500 bp of the Hygromycin Resistance Gene (lanes 1-6) and the target gene in deletion mutant at six different temperatues 48, 50, 52, 54, 56, and 60° C respectively (lanes 8-13). Lane 7 is the 2-log ladder. (B) PCR products from amplification of 5' flank along with HygB gene. Lane 1, 2-log ladder; lane 2-4, FVEG_00027_1 mutant at two different annealing temperatuers (56 and 60° C respectively). (C) Lane 1, 2-log ladder; lane 2-7, PCR products from amplification of 3' flank along with HygB gene at six different temperatures 60, 58, 55, 52, 50, 48° C respectively.

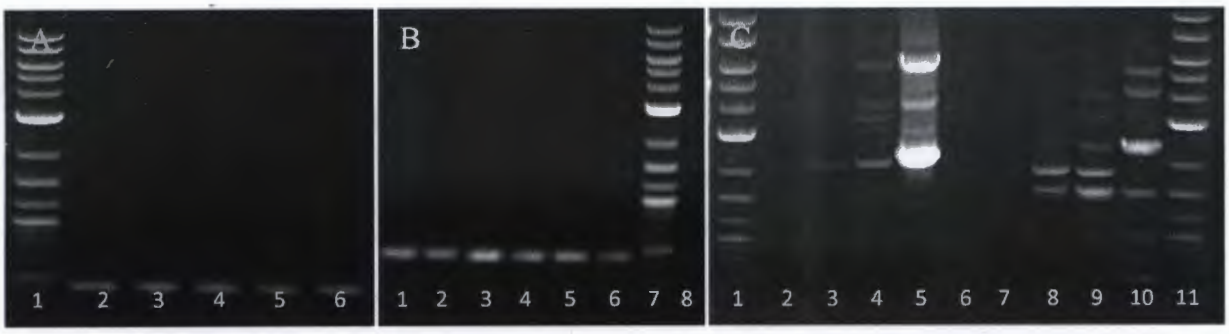

Figure 6: The results of the deletion mutant verification PCR of Fv_NPS6. (A) Lane 1, 2-log-ladder; lane 2-6, amplification of HygB gene from NPS6_1 to NPS6_5 mutant's respectively. (B) PCR products for target gene amplification. Lane 7, 2-log ladder; lanes 1-3, amplification from NPS6_4, Lanes 4-6 contains amplification from NPS6_5; lane 8 contains amplification from NPS6_1 at 48° C, which turned out to be the only correct deletion mutant. (C) PCR products from 5' and 3' verification for Fv_NPS6. Lanes 1 and11 2-log ladder; lanes 2-5,3' flank; lanes 7-10, 5' flank.

b. Sequencing of Plasmids

The plasmids used to create the deletion mutants were sent off to the Iowa State University Sequencing Facility for sequencing. Manual sequencing reactions were performed on

the LiCor instrument on campus at University of Northern Iowa, but these experiments yielded results that were not as reliable as the results from Iowa State University. The sequencing showed that the entire Double-Joint PCR product was successfully inserted into the plasmid vectors. The deletion mutants were already checked with PCR, and the sequencing results were further evidence that the deletion mutants were correct.

The sequencing of plasmids 14 and 8 was done to determine the accuracy of the Iowa State University sequencing, as well as to determine if the entire double-joint fragment was inserted into the vector. The expected sequence was compiled using the known vector sequence and the flanking sequences of Fv_00027 in both Double-Joint Fragments. The plasmids sequenced were plasmid 14, 11 Kb long, with the 7.5 Kb Double-Joint Fragment insert and plasmid 8 which was 15 Kb long and had a 12.5 Kb Double-Joint Fragment insert. The primers used in sequencing were m13 forward (U) and m13 reverse (R-1). BLAST was used to align the expected sequence, entered as the subject, and the Iowa State University sequence, entered as the query.

Results for Plasmid 14 reverse (R-1)

Query 83 CCGCCAGTGTGCTGGAATTCGCCCTTGCAATGACGCTAAGATAGGCGCCCTTGGTCAGGG 142

Sbjct 111 CCGCCAGTGTGCTGGAATTCGCCCTTGCAATGACGCTAAGATAGGCGCCCTTGGTCAGGG 170

The bases highlighted in red are the last five bases of the vector, and the five bases highlighted in green are the first five bases of the insert. As can be seen in the above sequence, there is no gap between the vector and insert sequences. The first five bases of the insert also agree with the expected sequence of GCAAT which shows that the insert was inserted properly and fully.

Results from Plasmid 14 forward (U)

Query 78 GAATTCGCCCTTAAAGATGTCAGCCACGTACCAGTCGACGAAGGGTACTCGACAGGCTCA 137

Sbjct 4779 GAATTCGCCCTTAAAGATGTCAGCCACGTACCAGTCGACGAAGGGTACTCGACAGGCTCA 4720

The above query is written 5'-3', while the subject sequence is written 3'-5'. The reverse complement of the expected sequence matches aligns with the sequence provided by Iowa State University. The vector sequence is in red, while the insert sequence is in green and there is no gap between the vector and insert. From this it can be inferred that the entire insert was inserted correctly into the vector.

Results from plasmid 8 reverse (R-1)

Query 93 GCTGGAATTCGCCCTTCCTTGCTGACCAATCTTTCGCAAGATACTCTCGGCTCTTGTAAT 152

Sbjct 121 GCTGGAATTCGCCCTTCCTTGCTGACCAATCTTTCGCAAGATACTCTCGGCTCTTGTAAT 180

The bases highlighted in red are the last five bases of the vector, and the five bases highlighted in green are the first five bases of the insert. As can be seen in the above sequence, there is no gap between the vector and insert sequences. The first five bases of the insert also agree with the expected sequence of CCTTG which shows that the insert was inserted properly and fully.

Results from plasmid 8 forward (U)

Query 25 TGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGC 84

Sbjct 10220 TGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGC 10161

Query 85 CCTTGCTTCAGTACAAGAATGCGGTAAGTCATTTATTGATACGGTATTGGAAGCGCGATA 144

Sbjct 10160 CCTTGCTTCAGTACAAGAATGCGGTAAGTCATTTATTGATACGGTATTGGAAGCGCGATA 10101

The above query is written 5'-3', while the subject sequence is written 3'-5'. The reverse complement of the expected sequence matches aligns with the sequence provided by Iowa State University. The vector sequence is in red, while the insert sequence is in green and there is no gap between the vector and insert. From this it can be inferred that the entire insert was inserted

into the vector.

Corn Assay Experiments

The hypothesis was that the mutant *F. verticillioides* would be less infective than the wild type *F. verticillioides*. The corn plants infected with the mutant *F. verticillioides* would be healthier than the corn plants infected with wild type *F. verticillioides*. The average weight of the corn seeds with each treatment would be statistically different for each treatment. The expected pattern of average total weights was negative control > mutant > positive control. Shoot weights were also recorded as another way to quantify infection of the corn seeds.

Table 6: Summary of Full Length Corn Assay Protocols. Even though the protocol had been previously optimized, the protocol needed to be optimized multiple times. This table summarizes any of the changes that were made in the protocol as a result of these protocol optimization experiments. Old* refers to seeds purchased in August 2010. New* refers to seeds purchased in August of 2011.

Experiment	Inoculation Time	Brand of Bleach	Age of Seed	Parafilm/Aluminum Foil	Deletion Mutant
	Day 1	Generic	Old*	Parafilm	Fv_00027
	Day 1	Clorox (50%)	New*	Parafilm	Fv_00027
	Day 3	Clorox (50%)	New*	Parafilm	Fv_00027
	Day 1	Clorox (50%)	Old*	Parafilm	Fv_00027
	Day 3	Clorox (50%)	Old*	Aluminum Foil	Fv_00027

c. Corn Assay Experiment 1

Table 7: Corn Assay Experiment 1 Results. This table includes the average masses for each condition at harvest time. Each treatment had 40 seeds. For full results, see Appendix II.

Negative Control		Mutant Fv_00027		Positive Control	
Total mass	Shoot mass	Total mass	Shoot mass	Total mass	Shoot mass
0.967 g	**0.309 g**	**0.463 g**	**0.004 g**	**0.363 g**	**0.0168 g**

The seeds used were Johnny's Selected Seeds Hybrid corn, white sweet silver queen F1 (with 85% germination). By looking at the average weights from each treatment, the Negative Control (no fungus) was healthiest (0.97g), the Positive Control (wild type *F. verticillioides*) was very sick with almost no growth (0.36g), and the mutant (Fv_00027) was also sick but less than the wild type (0.46g). These weights support the hypothesis because the average weight of the seeds with the mutant treatment was between the healthy negative control and the sick positive control. When the statistical analysis was done with the total weights though, there was not a significant statistical difference between the mutant and wild type. Statistical analysis was also performed with the shoot weights, and there was no statistically significant difference between the three treatments. The statistical analysis was done using SPSS and the results from the statistical analysis of both total weights and shoot weights are included in Appendix III. Specifically, the Tukey B and Scheffe tests both put the mutant and positive controls in the same subset, meaning that they are not statistically different from each other.

Approximately 25% of seeds germinated before being planted on the germination paper. The seeds used for this experiment were over a year old, so that may have been why the germination rates were so low for all the seeds. New seeds were ordered to increase germination, and only germinated seeds were placed in the rolls.

d. Corn Assay Experiment 2

Table 8: Corn Assay Experiment 2 Results. This table includes the average masses for each condition at harvest time. Each treatment had 40 seeds. For full results, see Appendix II.

Negative Control		Mutant Fv_00027		Positive Control	
Total mass	Shoot mass	Total mass	Shoot mass	Total mass	Shoot mass
0.526 g	**0.0588 g**	**0.303 g**	**0.011 g**	**0.338 g**	**0.000 g**

This experiment was done with newer seeds in order to optimize the protocol and get as high a yield of germinated seeds as possible. The seeds ordered were Johnny's Selected Seeds Hybrid corn, white sweet silver queen F1 with 94% germination. The rest of the protocol remained the same. The germination rates, however, were still low even with the new seeds, so barely any of the seeds grew under any treatment. One hypothesis was that since the thermometer for the water bath had stopped working, then the water bath for the 2 day incubation at 28°C was not actually at 28°C. If the seeds were not at 28°C then they would not have germinated as quickly. The statistical analysis was still done and showed that the assay was still valid because the positive and negative controls were different. Several of the rolls in the negative control though were also contaminated with a blackish/green bread mold. This contaminate may have already been on the germination paper, so in each experiment afterwards, germination paper was selected from the middle of the stack instead of from the top of the stack. The bread mold may have stunted growth of the negative control, but probably not as much as the low germination rates of the seeds. There were also very few shoots, which is most likely the result of low germination rates.

Statistical analysis was still done to ensure there was no difference between the Positive Control and the Fv_00027 treatments. In this case, a statistically significant difference would not support our hypothesis. Since in this experiment, the Positive Control had a heavier total weight a statistically significant difference would show that the Fv_00027 mutant was more virulent than the Positive Control, or wild type *F. verticillioides*. The results of the statistical analysis with the total average weights showed that there was a statistically significant difference between the Positive and Negative Controls, while there was no statistical difference between the Positive Control and the Fv_00027 mutant. The statistical analysis with the shoot weights also showed a statistically significant difference between the Positive and Negative Controls. The mutant

Fv_00027 treatment was however not statistically different from either of the controls. The full results from the statistical analysis are included in Appendix III.

e. Protocol Optimization 1: Bleach Germination Screens

Table 9: Germination Screen 1. This screen tested if the seeds were viable, or would germinate, with the new bleach.

	Percent Germination
Old Seeds (85%)	0%
New Seeds (94%)	0%

What followed next was a series of short experiments to determine the cause of low germination. The experiment consisted of germinating 40 old seeds from August 2010 and 40 new seeds from August 2011 by following the Corn Assay, but stopping the experiment before inoculating the seeds with the fungus. Then the seeds were examined to determine how many of the seeds germinated and how many did not. The older seeds (August 2010) were used as a control to compare the new seeds (August 2011) because the older seeds (August 2010) had germinated in the first experiment.

New bleach was purchased before Corn Assay Experiment 2 because the Hy-Vee bleach was used in Corn Assay Experiment 1 was gone. The new generic bleach, however, was more concentrated than the Hy-Vee bleach so there was no germination of any of the seeds. Germination Screen 1 used the new generic bleach and the results are in Table 9.

Table 10: Germination Screen 2. This table summarizes the variable that were tested and their results.

Conditions	Percent germination	
	Old Seeds (85%)	New Seeds (94%)
Clorox 10%	80.0%	65.0%
Generic Bleach 10%	47.5%	70.0%
No Heat Shock	75.0%	75.0%
Distilled Water	85.0%	85.0%

Germination Screen 2 was another screen experiment run, testing the generic bleach after dilution, Clorox bleach diluted to the same amount, and then one group of seeds was treated with bleach, but did not go through the heat shock step to rule out the heat shock as a potential step where the seeds were dying. One set of seeds did not go through the bleach treatment to determine if the seeds would germinate in distilled water to act as a control. The results of this screen, in Table 10, showed that the heat shock was not the cause of low germination, the 10% Clorox did not hinder germination as much as the generic bleach, and the newer seeds (August 2011) were viable, or able to germinate.

Table 11: Germination Screen 3 (Clorox Screen). This table has the results from t he screen testing multiple concentrations of Clorox bleach.

Conditions	Percent Germination
Clorox 10%	87.5%
Clorox 20%	95.0%
Clorox 40%	82.5%
Clorox 60%	77.5%

Germination Screen 3 was another experiment done to determine if a higher concentration of Clorox could be used without killing all the seeds. The seeds still had high

germination rates even when treated with up to 60% Clorox. Table 11 has the results of the final germination screen. It was determined that a 50% solution of Clorox bleach would ensure that the bleach was sterilizing the seed without killing the seed. In summary, after this round of germination screens, the changes made to the Corn Assay Protocol were using Clorox bleach and diluting the bleach to a 50% solution.

f. Corn Assay Experiment 3

Table 12: Corn Assay Experiment 3 Results. This table includes the average masses for each condition at harvest time. Each treatment had 40 seeds. For full results, see Appendix II.

Negative Control		Mutant Fv_00027		Positive Control	
Total mass	Shoot mass	Total mass	Shoot mass	Total mass	Shoot mass
0.567 g	**0.0235 g**	**0.408 g**	**0.0548 g**	**0.327 g**	**0.0213 g**

Table 13: Percent Germination of Each Condition. Germination rates were recorded at the planting step for each condition. The germination rate of each condition was recorded to ensure that at least 40 seeds had germinated before proceeding to the planting step. After the last round of optimization, there was an increase in germination.

	Percent Germination
Negative Control	87.3%
Mutant Fv_00027	80.8%
Positive Control	76.9%

Table 14: Total Mass Statistics ANOVA Results. Results from the ANOVA test run by SPSS with the average total masses from Corn Assay Experiment 3. Since the Significance value is < 0.05, at least one treatment is statistically different from another.

V2

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.191	2	.595	34.812	.000
Within Groups	2.001	117	.017		
Total	3.192	119			

Table 15: Total Mass Statistics Post-Hoc Tests Results. These are the results from the Tukey B and Scheffe tests.1=Negative control, 2=Fv_00027 mutant, 3=Positive control. Each condition was placed in a separate category showing that each treatment was statistically different from each other. This result supports our hypothesis. Means for groups in homogenous subsets are displayed. a. Uses Harmonic Mean Sample Size=40.000.

V2

V1		N	Subset for alpha=0.05		
			1	2	3
Tukey B[a]	3	40	.3272		
	2	40		.4078	
	1	40			.5670
Scheffe[a]	3	40	.3272		
	2	40		.4078	
	1	40			.5670
	Sig		1.000	1.000	1.000

After optimal germination conditions were determined, a third corn assay experiment was done. In the third experiment, the new seeds and Clorox bleach were used. Another change that was made to the protocol was that the inoculums (fungus) was not added to the seeds until after the two day incubation at 28°C and before placement in the fridge as opposed to immediately after the heat shock. This would ensure more even distribution of the inoculums.

The germination rate was 81.6%, which was encouraging. The average weights were as follows: negative 0.567 g, mutant 0.415 g, positive 0.327 g. This follows the pattern we would expect, and when the statistical analysis was done, it was determined that each condition was statistically different from each other. This supports the hypothesis that the mutant is less infective than the wild type *F. verticillioides.*

The Negative Control was contaminated with a yellowish slime, which prevented the kernels from growing much past germination. Unlike with *F. verticillioides* infection though, the kernels were still plump and yellow. When the seeds are killed by *F. verticillioides*, the kernels are usually shriveled, squishy and there is white/pink fuzz around the kernel. The seeds with the mutant treatment appeared to be the healthiest and several had shoots, but the majority of the seeds resembled the seeds treated with the positive control. Some of the yellow slime was present in the seeds treated with the mutant whereas none of the seeds treated with the positive control were contaminated with the slime. This slime may have been an opportunistic pathogen, which under normal circumstances may not fully infect the seed, but when the immune/defense system of the seed is compromised, the pathogen can take over. When the seeds are in the fridge, the seed defenses are down, which is exploited to let the fungus infect the seeds.

Despite the contamination, the statistical analysis was still done with the total weights and the shoot weights. The total average weights did have a statistically significant difference between each treatment, as seen in Table 15. This supports the proposed hypothesis that by deletion of gene Fv_00027 will decrease the virulence or the infectivity of *F. verticillioides*. When the statistics were done with the average shoot masses, none of the treatments were statistically different from one another and those results are included in Appendix III.

g. Protocol Optimization 2: Short Germination Experiment

Table 16: Short Germination Experiment Full Results. This table contains the results from the short germination experiment which was done to determine the effect of cutting down the incubation time in the fridge. By looking at the results, the *F. verticillioides* did not have time to fully infect the corn seeds.

Positive Control		Negative Control	
Total mass	Shoot mass	Total mass	Shoot mass
0.64	0	0.66	0.01
0.62	0	0.96	0.34
0.55	0	0.70	0.13
0.62	0	0.54	0
0.94	0.34	0.80	0.22
1.03	0.36	0.98	0.47
0.73	0.12	0.85	0.35
0.71	0.28	0.80	0.21
1.33	0.54	0.60	0.11
1.15	0.4	0.57	0.23
0.746	**0.207**	**0.832**	**0.204**

Table 17: Percent Germination of Each Condition. This table includes the germination rates of each condition before inoculation.

	Percent Germination
Negative Control	32.5%
Positive Control	100%

To prevent the occurrence of another experiment with slime contamination, a short germination screen was done. This experiment was done to test the effect of cutting down the time the inoculated seeds spend in the fridge. The time spent in the fridge was cut down from

two days to only one day, and only the positive and negative controls were grown. A 50% Clorox bleach solution was used for sterilization, germinated seeds were selected to infect with wild type *F. verticillioides* and were incubated in the fridge for one day. The seeds were then planted on germ paper. One roll/ten seeds of each treatment was planted and then harvested seven days later. The shorter harvest time was due to a lack of time in the semester. The results were that both controls grew much better. There was white fuzz on the negative control, and the positive control actually grew better than the negative control. The positive control had white fuzz with some red/pink. This indicates that there may still be some form of contamination in the negative control and the wild type *F. verticillioides* needs more than one day in the fridge to fully infect the seedlings. So that part of the protocol was not changed.

h. Corn Assay Experiment 4

Table 18: Corn Assay Experiment 4 Results. This table includes the average masses for each condition at harvest time. Each treatment had 40 seeds. For full results, see Appendix II.

Negative Control		Mutant Fv_00027		Positive Control	
Total Mass	Shoot Mass	Total Mass	Shoot Mass	Total Mass	Shoot Mass
1.179 g	**0.397 g**	**0.38 g**	**0.0213 g**	**0.395 g**	**0.0305 g**

Table 19: Percent Germination of Each Condition. Germination rates were recorded at the planting step for each condition. The germination rate of each condition was recorded to ensure that at least 40 seeds had germinated before proceeding to the planting step.

	Percent Germination
Negative Control	35.8%
Mutant Fv_00027	27.7%
Positive Control	41.0%

Figure 7: Pictures of the three treatments in the Corn Assay Experiment 4 at the time of harvest. Clearly, the Negative Control has several green shoots, which indicates the seedlings grew well without any fungus. In comparison, the Mutant and Positive Control do not have any green shoots out the top of the rolls, which indicated that the *F. verticillioides* hindered the growth of the corn seeds. (A) Negative Control with no *F. verticillioidies*. (B) Fv_00027 Mutant treatment. (C) Positive Control with wild type *F. verticillioides*.

Figure 8: The seedlings after the rolls were unwrapped at harvest time. (A) Negative Control with no *F. verticillioidies*. (B) Fv_00027 Mutant treatment. (C) Positive Control with wild type *F. verticillioidies*.

This experiment was done to replicate Corn Assay Experiment 1 with the original corn seeds from August 2010 to see if a greater number of germinated seeds would create a bigger difference between the mutant condition and the positive control. The newer seeds were bought to try and assist with the germination problem, but there were many problems with the newer seeds, so the approach taken was to try and use more seeds to get higher germination. In the first experiment, even though 40 seeds were planted, only about 20 germinated seeds were planted per condition whereas with this experiment there were no less 33 seeds that had germinated for each condition. The inoculation was done right after the heat shock so the fungal conidia were on the seeds a total of four days before planting as written in the original optimized protocol. This may account for why the average weights of the mutant and positive control are so low. The statistics were still done with both average total masses and average shoot masses. The result with the total weights was that there was a statistically significant difference between the

Negative Control and the Positive Control which showed that the assay is sound because both of the controls work. There was no difference between mutant and the Positive Control. The shoots followed the same pattern and those results can be found in Appendix III.

i. Protocol Optimization 2: Parafilm/Aluminum Foil Germination Screens

At the beginning of second semester, the protocol needed further optimization because there were such low germination rates among the corn seeds. Boi Lam Hong, an assistant in Dr. Nalin Goonesekere's lab, ran the following experiments. What follows are a series of germination screens to determine how best to get the corn seeds to germinate. The screens all explored whether or not the seeds were viable, if the enzyme phytase is critical to growth, the heat shock, and parafilm versus aluminum foil.

Table 20: New Germination Screen. This screen was used to test which factor was affecting germination.

Treatment	Percent Germination
50% Clorox + heat shock + Tween	37.5%
50% Clorox – heat shock + Tween	65.0%
dH2O + heat shock – Tween	20.0%
dH2O – heat shock – Tween	55.0%

A germination experiment, Table 20, was done by following the first part of the Corn Assay and stopping the experiment before the two day inoculation time in the fridge. After the two day incubation at 28°water bath, barely any of the seeds germinated (one flask had only three germinated seeds). Those seeds were put back in the water bath with 20 ml of fresh water to determine if the seeds would germinate after another day at 28°, or if they were dead. After recording the germination numbers the seeds were discarded and the planting was not done.

Table 21: Phytase Screen. This was a screen designed to determine if phytase was one of the germination issues and if the seeds in general were still viable.

Treatment	*Percent Germination*
50% Clorox + heat shock + Tween	7.5%
50% Clorox – heat shock + Tween	82.5%
dH2O + heat shock – Tween	2.5%
dH2O – heat shock – Tween	60.0%
50% Clorox + heat shock + Tween+ 45 degrees for 4 hours	0.0%
dH2O – heat shock – Tween + 45 degrees for 4 hours	37.5%

Phytase is an enzyme in the corn seed responsible for breaking down phytin to produce useable phosphates for energy during corn germination. This enzyme was determined to be most active at ~50° C (Chang, 1967) so incubating the corn seeds at a higher temperature would maybe activate the enzyme and cause an increase in germination due to an increase in the breakdown of phytin. Unfortunately, the higher incubation temperature had the opposite effect and most of the seeds were killed. From the results in Table 21 and Table 20, it was determined that the heat shock was killing off the seeds.

Table 22: Heat Shock Screen. This screen was designed to determine the effect of reducing the heat shock time in the sterilization step of the Corn Assay protocol. The other factor that was tested was how the germination rate differed when flasks were covered with parafilm versus aluminum foil.

0 min HS	1 min HS	2 min HS	4 min HS	A/P
85.0%	80.0%	50.0%	20.0%	Parafilm
95.0%	92.5%	80.0%	80.0%	Aluminum

With the results from Table 21 in mind, the length of time that the seeds were heat shocked was changed to determine the effect of reducing the time on the germination rate. After

further research, another factor that was found to be important in corn germination was the amount of oxygen available to the seed. In the original protocol, the flasks with the seeds were covered with parafilm the entire length of time before planting. The thought was that the parafilm was sealing off the flask and restricting oxygen to the corn seed; decreasing germination. Instead of parafilm, some of the flasks were covered in aluminum foil in this screen.

There was a significant difference between the flasks covered in aluminum foil versus the flasks covered with parafilm. As a result, Corn Assay Experiment 5 was conducted covering flasks with aluminum foil instead of parafilm as stated in the original protocol. The results also show that the heat shock time does play a factor in the germination rate because the seeds that received the shorter heat shock had a higher germination rate. Since the aluminum foil flasks still had ~75% germination, the heat shock was kept at the original four minutes. In summary, the protocol change from these experiments was covering the flasks with aluminum foil.

j. Corn Assay Experiment 5

Table 23: Corn Assay Experiment 5 Results. This table includes the average masses for each condition at harvest time. Each treatment had 40 seeds. For full results, see Appendix II. Total percent germination: 45.4%.

Negative Control		Mutant Fv_00027		Positive Control	
Total mass	Shoot mass	Total mass	Shoot mass	Total mass	Shoot mass
0.662 g	**0.108 g**	**0.425 g**	**0.0383 g**	**0.389 g**	**0.034 g**

At a first glance at the average total weights for each condition they seem to follow the pattern that the hypothesis lays out; the mutant condition average weight (0.425 g) falls between the Negative Control (0.662 g) and Positive Control (0.389 g) average weights. The statistical

analysis with the Tukey B and Scheffe tests, however, showed that the mutant condition was not statistically different from the Positive Control. This result is reasonable because there is only a difference of 0.04 g between the average weights of the mutant and positive control. The statistical tests did however show that the Negative and Positive controls are statistically different which shows that the assay is valid. The full statistical results are included in Appendix III.

6. Future Research Recommendations

One recommendation for future experiments with the Corn Assay is to add the *F. verticillioides* two days after germination, or on day three like Corn Assay Experiments 3 and 5. This is because adding the fungus after seeds have germinated ensures that the seeds infected have all germinated and that any seeds that do not grow any farther are due to added fungus. The infection is also more uniform when the all the seeds infected have germinated.

In order to say with confidence that Fv_00027 is a virulence gene, the results from Corn Assay Experiment 3 need to be replicated. These results followed the expected pattern Negative Control > Fv_00027> Positive Control, and each treatment was statistically different. More experiments need to be done with the Fv_NPS6 deletion mutant to determine if it has any effect on infectivity of the fungus.

As well as performing experiments with Fv_NPS6, it may be worthwhile to create mutants that lack both Fv_00027 and Fv_NPS6. Both genes may be virulent genes, but knocking out one gene may not be enough to show a statistical difference in infectivity. So by knocking out both genes and using those mutants in the Corn Assay, it may be possible to determine whether there is a statistical difference between the wild type *F. verticillioides* and the mutant.

Another recommendation would be to scale up the Corn Assay Experiments. Since a sample size of only 40 seeds per condition were used, it is possible that 40 is not enough to show that there is a statistical difference between any of the conditions. By scaling up the experiments, it would be easier to determine a statistical difference. The reason the Corn Assay Experiments had not been scaled up previously is because the resources available were not sufficient for scaling up the experiments. Aside from scaling up the Corn Assay Experiments, sequencing the deletion mutants would also be useful to definitively show that the deletion mutants are correct.

7. Expanded Discussion of Significance

This research is significant because corn is a major crop not only in Iowa, but it is also a staple food crop in several other parts of the world. Infection from *Fusarium verticillioides* causes a large economic burden every year resulting in millions of dollars in lost crops every year. Corn is a versatile crop used for multiple purposes including but not limited to human consumption, animal feed, and ethanol production. Since corn is such an important crop it is imperative to understand the interaction between corn and this dangerous pathogen.

Since *F. verticillioides* is also toxic to humans as well as many domesticated animals, it serves as a public health issue to understand whether or not this fungal pathogen has virulent genes that contribute to the infectivity of the pathogen. As mentioned in the introduction, toxins produced by *F. verticillioides* have been classified as potential carcinogens and have been linked to several diseases.

Fusarium verticillioides is not only a fungal pathogen of corn but several other crops as well including other cash crops such as potato and wheat. Understanding the interaction between *F. verticillioides* could serve as a model for how the pathogen infects other crops. Fully

characterizing the infection of corn by this fugal pathogen could also give insight on how to prevent infection by either engineering corn seeds to be resistant to the proteins produced by these genes, or engineering more efficient pesticides to destroy the fungus.

In summary, determining the genes responsible for *F. verticillioides* infection could have positive effects on the economy of Iowa and several other countries, improve public health, and aid in the prevention of infection of other important crops.

8. Conclusions

There were several limitations to this research specifically pertaining to the Corn Assays. These limitations include time constraints, uncontrolled variables, and non-reproducible results. The time it takes to run each experiment is two weeks. This restricted the number of Corn Assay experiments that could be performed in the allotted time. The protocol also had to be optimized several times due to contamination and low germination of the corn seeds. The protocol optimization experiments also restricted the time that was spent on running experiments with the deletion mutants.

The protocol optimization experiments were in part a result of the variability that could not be controlled in the experiments. One such example is the variability of the corn seeds. Even though the seeds all came from the same company and each packet of seeds came from the same lot, there were differences in germination. Some seeds would germinate well while other seeds would not germinate at all and so this step needed to be optimized several times. Another variable that could not be easily controlled was the temperature of the seeds during different stages of the corn assay protocol. For example, while the seeds were in the growth chamber, the exact temperature of the room could not be measured or controlled. Sterile technique was used

whenever possible to prevent contamination and the beginning of the corn assay was focused on sterilizing the seeds without killing them. However, there was still an instance when there was contamination quite possibly from the air.

Other limitations to this research pertain to the deletion mutants. The entire deletion mutants were not able to be sequenced, and even though the deletion mutants were checked with PCR, there is no other definitive evidence that the deletion mutants are correct. Not only was there not enough time to sequence the entire region of interest, but there was also an inherent limitation to sequencing. Sequencing reactions can only supply with certainty about 600-800 base pairs (bp) while the entire Double-Joint Fragment was well over one kilobase (Kb). To counter this, primers were designed to amplify the region where the Double-Joint Fragment and the *F. verticillioides* genome meet. Unfortunately, the primers did not work and this region was unable to be amplified. As a result, a region of ~500 bp in the Hygromycin Resistance Gene that was inserted into the *F. verticillioides* was amplified to be sequenced. Unfortunately, the sequencing results came back and the sequence was unable to be determined.

Only one of the experiments done with the Fv_00027 showed a difference between the mutant condition and Positive Control that was statistically significant. Since this result was not able to be replicated, it is hard to say one way or the other whether or not it is a virulent gene or not. The possibility of Fv_00027 being a virulent gene cannot be excluded, however, it cannot be said with certainty that it is a virulent gene. Looking at the other four experiments that were done, two followed the predicted pattern of the mutant condition average total weight falling between the Positive and Negative controls. The other two experiments however had results where the Positive Control had a greater average total weight than the mutant condition. By looking at all five of these experiments, it could be concluded that Fv_00027 does not affect the

infectivity of *F. verticillioides* alone. Since one experiment did show that there was a difference between the mutant and Positive Control, it cannot be ruled out the Fv_00027 is not involved in the infectivity of the fungal pathogen at all. No conclusion can be drawn about the gene Fv_NPS6 because no successful experiments were done with the Fv_NPS6 mutant due to time constraints.

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10. Appendix I: Sequence Alignment Results

Results for Plasmid 14 reverse (R-1)

Score = 1884 bits (1020), Expect = 0.0

Identities = 1053/1076 (98%), Gaps = 4/1076 (0%)

Strand=Plus/Plus

```
Query  24   CGTTAG-ATACTCAAGCTATGCATCAAGCTTGGTACCGAGCTCGGATCCACTAGTAACGG  82
Sbjct  51   CGTTAGAATACTCAAGCTATGCATCAAGCTTGGTACCGAGCTCGGATCCACTAGTAACGG  110

Query  83   CCGCCAGTGTGCTGGAATTCGCCCTTGCAATGACGCTAAGATAGGCGCCCTTGGTCAGGG  142
Sbjct  111  CCGCCAGTGTGCTGGAATTCGCCCTTGCAATGACGCTAAGATAGGCGCCCTTGGTCAGGG  170

Query  143  TGGTCCGACCACGAAAAGCTGCTCAGACAATTACGCAGCAACCAACAACCAACAACGACC  202
Sbjct  171  TGGTCCGACCACGAAAAGCTGCTCAGACAATTACGCAGCAACCAACAACCAACAACGACC  230

Query  203  AACGTATCATGTCTGTTCTCTTACGAGATTTATCCCGGGTCAGTCGGCATGTAGTTCCAA  262
Sbjct  231  AACGTATCATGTCTGTTCTCTTACGAGATTTATCCCGGGTCAGTCGGCATGTAATTCCAA  290

Query  263  CCTGTAAGGCAACCGGGAGGTCACA-TATTCAACTGTCTTTACGGCTAGAAACTAGAAAC  321
Sbjct  291  CCTGTAAGGCAACCGGGAGGTCACATTA-TCAACTGTCTTTACGGCTAGAAACTAGAAAC  349

Query  322  TGCTCATCCAACAACCAACAACTAACAGCAGCCATTGGCTATTCTTTACGAGTTTTGTCT  381
Sbjct  350  TGCTCAACCAACAACCAACAACTAACAGCAGCCATTGGCTATTCTTTACGAGTTTTGTCT  409

Query  382  CGGATCTATCGGCTGTAAGACAATCGTAGACCACATCCTCGTCGTTCTGTATGGCCGGTG  441
Sbjct  410  CGGATCTATCGGCTGTAAGACAATCGTAGACCACATCCTCGTCGTTCTGTATGGCCGGTG  469

Query  442  GATGAGACCAGTTCTTGGTCTCCCAGCAGAGAGCCGATTCAGGACATGTTTATAAAAGAG  501
Sbjct  470  GATGAGACCAGTTCTTGGTCTCCCAGCAGAGAGCCGATTCAGGACATGTTTATAAAAGAG  529
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Query 502 GCAGCACCCATCATGTCAAAGCACAAGCTTCTTACACAAGCTCATTTATATCAAACAACC 561

Sbjct 530 GCAGCACCCATCATGTCAAAGCACAAGCTTCTTACACAAGCTCATTTATATCAAACAACC 589

Query 562 CTTGGTAAGATACCCTGAGCTAGAATGTATCGCTGATTAACCTATCGTTAGAAACACTTT 621

Sbjct 590 CTTGGTAAGATACCCTGAGCTAGAATGTATCGCTGATTAACCTATCGTTAGAAACACTTT 649

Query 622 CTTCAACATGTTCGGCAATCGCGGTCCTCAGAATGTAATCCTCATACTCGCTATTCTAGG 681

Sbjct 650 CTTCAACATGTTCGGCAATCGCGGTCCTCAGAATGTAATCCTCATACTCGCTATTCTAGG 709

Query 682 GAGTATGCCCGGTAGCATGGTCGTCAGTTTGCCTGTTACTCCGGCTAAAGATAGCAAAGG 741

Sbjct 710 GAGTATGCCCGGTAGCATGGTCGTCAGTTTGCCTGTTACTCCGGCTAAAGATAGCAAAGG 769

Query 742 CAATGTCGACTCAGCAGATCAAATCGTCTACGCTGTTCGATGCCATGCCGATACCTACGT 801

Sbjct 770 CAATGTCGACTCAGCAGATCAAATCGTCTACGCTGTTCGATGCCATGCCGATATCTACGT 829

Query 802 TATAGCCCACCATTGTCAGGCTCACTGCAACGGAAGGGGTCACGTACTCTTCAATGAGAC 861

Sbjct 830 TATAGCCCACCATTGTCAGGCTCACTGCAACGGAAGGGGTCACGTACTCTTCAATGAGAC 889

Query 862 CAGATGCCCTGTCGGATCAGACAATGCTGACGATATCTTTTCGGATTGTTACTGCACTCC 921

Sbjct 890 CAGATGCCCTGTCGGATCAGACAATGCTGACGATATCTTTTCGGATTGTTACTGCACTCC 949

Query 922 ACAATGTGTGCCTTGTAANGAGGANGGCGAAGAACANGCTCAAGANGACGTGTCCGTTCC 981

Sbjct 950 ACAATGTGTGCCTTGTAAGGAGGAGGGCGAAGAACAGGCTCAAGAGGACGTGTCCGTTCC 1009

Query 982 TGAGTGAGTTGGATCTAACATATATCATCTCACAGGATCTGGNNCATCAATNNGACATAT 1041

Sbjct 1010 TGAGTGAGTTGGATCTAACATATATCATCTCACAGGATCTGGGCCATCAATAGGACATAT 1069

Query 1042 CTAGATGCTGAAGAATNNTCAAGTNCANAGGTGTGTCGTTCANNANCCNGCTGTAC 1097

Sbjct 1070 CTAGATGCTGAAAAATA-TCAAGTTCAGAGGTGTGTCGTTCAAGACCCTGCTGTAC 1124

Results from Plasmid 14 forward (U)

Score = 1921 bits (1040), Expect = 0.0

Identities = 1058/1069 (99%), Gaps = 3/1069 (0%)

Strand=Plus/Minus

```
Query 19   GGGCG-ATTGGGCCNTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCA 77
Sbjct 4839 GGGCGAATTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCA 4780

Query 78   GAATTCGCCCTTAAAGATGTCAGCCACGTACCAGTCGACGAAGGGTACTCGACAGGCTCA 137
Sbjct 4779 GAATTCGCCCTTAAAGATGTCAGCCACGTACCAGTCGACGAAGGGTACTCGACAGGCTCA 4720

Query 138  GACACCGCTTCCCTACACACTGACACTATGAGCAATTATCATAATGTCTTTGCTCCTCCT 197
Sbjct 4719 GACACCGCTTCCCTACACACTGACACTATGAGCAATTATCATAATGTCTTTGCTCCTCCT 4660

Query 198  GAAGCTGTGGATATCTACAAGATCGTGGATGGTTTCGAAGGTGCCCACCGTTTCGAACCT 257
Sbjct 4659 GAAGCTGTGGATATCTACAAGATCGTGGATGGTTTCGAAGGTGCCCACCGTTTCGAACCT 4600

Query 258  TCTGCGACTTGGGAATCCCAGGAAGAGGAGAAGCTGGTAAGACGGGTATGTAGCTTATGC 317
Sbjct 4599 TCTGCGACTTGGGAATCCCAGGAAGAGGAGAAGCTGGTAAGACGGGTATGTAGCTTATGC 4540

Query 318  GACAAGTTTTCATTGAATAGCGATAATAATGCCAACAGTAGCTTGATTGGTTTATCGCAC 377
Sbjct 4539 GACAAGTTTTCATTGAATAGCGATAATAATGCCAACGGTAGCTTGATTGGTTTATCGCAC 4480

Query 378  TTCCGGCCTGTATCATGTTCTTTGCTCTCCAGCTGGACCGTGGCAACATTGTCCAGGCTC 437
Sbjct 4479 TTCCGGCCTGTATCATGTTCTTTGCTCTCCAGCTGGACCGTGGCAACATTGTCCAGGCTC 4420

Query 438  TTTCAGATGGCATGCTAAGTGAGCTCACGGTCGAGAGTCGATTCTATGTGCTACTGGCGC 497
Sbjct 4419 TTTCAGATGGCATGCTAAGTGAGCTCACGGTCGAGAGTCGATTCTATGTGCTACTGGCGC 4360
```

Query 498 TAACTCTTCCAGGTGATCTCGGCCTCACAACAAATGACTACAACAATGGAATGACGATAT 557

Sbjct 4359 TAACTCTTCCAGGTGATCTCGGCCTCACAACAAATGACTACAACAATGGAATGACGATAT 4300

Query 558 TTTACTGTTCGTTCTTATTCGCAGAGCTCCCGTCACAAGTCATTGGCAAAAAACTTGGAC 617

Sbjct 4299 TTTACTGTTCGTTCTTATTCGCAGAGCTCCCGTCACAAGTCATTGGCAAAAAACTTGGAC 4240

Query 618 CAGACGTCTGGGTCCCTATCCAGATGGTGCTATGGAGTGTCGTAGCAATGTCACAAGCCG 677

Sbjct 4239 CAGACGTCTGGGTCCCTATCCAGATGGTGCTATGGAGTGTCGTAGCAATGTCACAAGCCG 4180

Query 678 CGCTTCAGGGAAGGACTAGCTTCTTTATCTGCCGCTGGTTACTTGGGATGCTTGAAGGAG 737

Sbjct 4179 CGCTTCAGGGAAGGACTAGCTTCTTTATCTGCCGCTGGTTACTTGGGATGCTTGAAGGAG 4120

Query 738 GTAATTATAAATCAACACAGGATGTCGCTCTTGCCCTGCTGACTGGCATACCTCTAGGCT 797

Sbjct 4119 GTAATTATAAATCAACACAGGATGTCGCTCTTGCCCTGCTGACTGGCATACCTCTAGGCT 4060

Query 798 TCATCCCTGACACTATTCTTTATCTTTCATATTTTTACAAAAATGTCGAGCTTCCCAAAC 857

Sbjct 4059 TCATCCCTGACACTATTCTTTATCTTTCATATTTTTACAAAAATGTCGAGCTTCCCAAAC 4000

Query 858 GCCTCAGTTGGTTCTGGACATCATATCAAGGCACGCAGATTATTGGTGCCTTTTTGGCAT 917

Sbjct 3999 GCCTCAGTTGGTTCTGGACATCATATCAAGGCACGCAGATTATTGGTGCCTTTTTGGCAT 3940

Query 918 ACGGTATTCTCCACCTCCGGGGCCATAGTGGGCTCCACGAAGGATGGAGATATCTGTTCG 977

Sbjct 3939 ACGGTATTCTCCACCTCCGGGGCCATAGTGGGCTCCACGAAGGGTGGAGATATCTGTTCG 3880

Query 978 TGATTGAGGGCTCATTTACTGCGCTGATCGGCATANTGACCTTCNTNCTACTTACCGCCA 1037

Sbjct 3879 TGATTGAGGGCTCATTTACTGCGCTGATCGGCATATTGACCTTCTT-CTACTTACCGCCA 3821

Query 1038 TCCCCTACGCAGACTTCTCGAACANGNCTGGAAAGGANTACTTCNTCCC 1086

Sbjct 3820 TCCCCTACGCAGACTTCTCGAACAGG-CTGGAAAGGATTACTTCGTCCC 3773

Results from plasmid 8 reverse (R-1).

Score = 1801 bits (975), Expect = 0.0

Identities = 1032/1068 (97%), Gaps = 6/1068 (1%)

Strand=Plus/Plus

Query 33 CTCNAGCTATGCATCAAGCTTGGTACCGAGCTCGGATCCACTAGTAACGGCCNNNNGTGT 92

Sbjct 61 CTCAAGCTATGCATCAAGCTTGGTACCGAGCTCGGATCCACTAGTAACGGCCGCCAGTGT 120

Query 93 GCTGGAATTCGCCCTTCCTTGCTGACCAATCTTTCGCAAGATACTCTCGGCTCTTGTAAT 152

Sbjct 121 GCTGGAATTCGCCCTTCCTTGCTGACCAATCTTTCGCAAGATACTCTCGGCTCTTGTAAT 180

Query 153 AAGCCCCTTCACATTGGAGAGATCCCTCCCTTCTACTGCCGCCTGATCTTCGCCCTTCAC 212

Sbjct 181 AAGCCCCTTCACATTGGAGAGATCCCTCCCTTCTACTGCCGCCTGATCTTCGCCCTTCAC 240

Query 213 CAACGTGTCTCCTTTGGGCGATATCTGTCGAATCTTACGTCGTAGCTCATTCCACGTTGA 272

Sbjct 241 CGACTTGTCTCCTTTGGGCGATATCTGTCGAATCTTACGTCGTAGCTCATTCCACGTTGA 300

Query 273 ATTGTGACAGTACGAAAATACCCGCTCGTTCACGAGTAAAGGGAGGGCTAATGCCGCCAC 332

Sbjct 301 ATTGTGACAGTACGAAAATACCCGCTCGTTCACGAGTGAAGGGAGGGCTAATGCCGCCAC 360

Query 333 CAAAAGACGTGTTGCATCCTGAACATCAGTGTAATATGCTACGAGATATGTCAGTTTATA 392

Sbjct 361 CAAAAGACGTGCTGCATCCTGAACATCAGTGTAATATGCTACGAGATATGTCAGTTTATA 420

Query 393 CTGCTAGTAGTAGCTGATGAGAGATGGAATGCCTACCAACTGAGCCAAAGACCTTGTCCC 452

Sbjct 421 CTGCTAGTAGTAGCTGATGAGAGATGGAATGCCTACCAACTGAGCCAAAGACCTTGTCCC 480

Query 453 ATTCACCCTCAGCCGCCGCTTTCAACATGCGGAAGGTAGAGGCAGTGTTTTCTGGATCCA 512

Sbjct 481 ATTCACCCTCAGCCGCCGCTTTCAACATGCGGAAGGTAGAGGTAGTGTTTTCTGGATCCA 540

Query 513 AGGCCCTTCCAAACTGGCCATCAGGTACAACACAGTTTGCAACAAAGGGAGGCTGGTTCG 572

Sbjct 541 AGGCCCTTCCAAACTGGCCATCAGGTACAACACAGTTTGCAACAAAGGGAGGCTGGTTCG 600

Query 573 TGCCAACCCATGACCAGAACGCCAATTCTGCCAAAGCCCGACTGGCGCTATAGACGTCCA 632

Sbjct 601 TGCCAACCCATGACCAGAACGCCAATTCTGCCAAAGCCCGACTGGCGCTATAGACGTCCA 660

Query 633 CAAATCTATCGATGCCATCGACATTCGGCTCACAACAGGCCTTCATGATGGCGTCGTAGT 692

Sbjct 661 CAAATCTATCGATGCCATCGACATTCGGCTCACAACAGGCCTTCATGATGGCGTCGTAGT 720

Query 693 TAAACATGGCTGAAGTGATATGGTGAGGGTGGTTATAATTGGTA-GATTCAACCGCTTTG 751

Sbjct 721 TAAACATGGCTGAAGTGATATGGTGAGGGTGGTTATAATTG-TAAGATTCAACTGCTTTG 779

Query 752 GAACTTGAACTGAGAACGTAGCGCTTCACTCCAGCGCGAGCTGCGGCTTCAAGACTGTTA 811

Sbjct 780 GAGCTTGAACTGAGAACGTAGCGCTTCACTCCAGCGCCAGCTGCGGCTTCAAGACTGTTA 839

Query 812 ATAGTCCCTTTTACCACAGGTTCTACCACCTTCACAGGGTCTGCGTTCAGTATGATCGGT 871

Sbjct 840 ATAGTCCCTTTTACCACAGGTTCTACCACCTTCACAGGGTCTGCGTTCAGTATGATCGGT 899

Query 872 GTTGAAACGTAGATTACACCCTTGGCCCCTGTGATACAGTGATTAAATTGAGTCATGGAA 931

Sbjct 900 GTTGAAACGTAGATTACACCCTTGGCCCCTGTGATACAGTGATTAAATTGAGTCATGGAA 959

Query 932 TAGAACACAGGGCGCCCTCCCAACTTTGAAGGCTTCGTCAAATGCGCCTTCAGCTTCAAA 991

Sbjct 960 TAGAACACAGGGCGCCCTCTCACCTTTGAAGGCTTCGTCAAATGCGCCTTCAGCTTCAAA 1019

Query 992 ATCAGACACCTGNNNCAGCTCAAACTTCTTTNGNNNCTCNGGTATCnaaanaaGTTGTGC 1051

Sbjct 1020 ATCAGACACCTGGACCAGCTCAAACTTCTTTGGCCACTC-GGTATC-AAACAAGTTATGC 1077

Query 1052 ATCCAATGNTGGNTTCNTNNTCCGCGTCTCTCANCGTTCCTCNGACCA 1099

Sbjct 1078 ATCCAATGTTGG-TTCTTC-TCCACGTCTCTCACCGTTCCTCGGACCA 1123

Results from plasmid 8 forward (U)

Score = 1873 bits (1014), Expect = 0.0

Identities = 1024/1029 (99%), Gaps = 2/1029 (0%)

Strand=Plus/Minus

Query 25 TGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGC 84

Sbjct 10220 TGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGC 10161

Query 85 CCTTGCTTCAGTACAAGAATGCGGTAAGTCATTTATTGATACGGTATTGGAAGCGCGATA 144

Sbjct 10160 CCTTGCTTCAGTACAAGAATGCGGTAAGTCATTTATTGATACGGTATTGGAAGCGCGATA 10101

Query 145 ATAATACCATTTCTAGTACGGAGCCAATTTGTATCGACGCGGTGAGATGAACGTTGTGAA 204

Sbjct 10100 ATAATACCATTTCTAGTACGGAGCCAATTTGTATCGACGCGGTGAGATGAACGTTGTGAA 10041

Query 205 GAGAATGGAGAACATCCTCAGCATGGGATCTCAACCCGATATCATCCAGCTGTTGACTTG 264

Sbjct 10040 GAGAATGGAGAACATCCTCAGCATGGGATCTCAACCCGATATCATCCAGCTGTTGACTTG 9981

Query 265 GGTCAGTGCCTCACATCGTAGCAAGGCTTCAGATCACTGACAATGCGTAGAATGATGGAC 324

Sbjct 9980 GGTCAGTGCCTCACATCGTAGCAAGGCTTCAGATCACTGACAATGCGTAGAATGATGGAC 9921

Query 325 CCGAGAGTCATCACTTCGGTAACCTCTGGCCCGAACAGAACACCGATGCCCAACCCAACC 384

Sbjct 9920 CCGAGAGTCATCACTTCGGTAACCTCTGGCCCGAACAGAACACCGATGCCCAACCCAACC 9861

Query 385 AATACGCCAGTCCCGACGGCTCCGATCATACCGCCCTTCAATCTCTATACTCGGCTTTCA 444

Sbjct 9860 AATACGCCAGTCCCGACGGCTCCGATCATACCGCCCTTCAATCTCTATACTCGGCTTTCA 9801

Query 445 TCCACGCTTGGAAGAACGGCGGTTTGATGGTCCCAGCATTCTCTAAGCGAGATGAAGCTA 504

Sbjct 9800 TCCACGCTTGGAAGAACGGCGGTTCGATGGTCCCAGCATTCTCTAAGAGAGATGAAGCTA 9741

Query 505 TACCTCAGGGTGCTTTATGGCATAAGAGCATCTTCCAGACCACTACTTGCCCTGGAGGTG 564

Sbjct 9740 TACCTCAGGGTGCTTTATGGCATAAGAGCATCTTCCAGACCACTACTTGCCCTGGAGGTG 9681

Query 565 ACTCTAGCGTCAAGTACTTTCAAGAACCAAATGGCACCGATGCTGGCCAAGACGCTCTTC 624

Sbjct 9680 ACTCTAGCGTCAAGTACTTTCAAGAACCAAATGGCACCGATGCTGGCCAAGACGCTCTTC 9621

Query 625 ACTGGGCCTTGGTAGTCCCTGCTCAGGCAGCTGGCTTTACCGTCAATGTGATGAGCAATG 684

Sbjct 9620 ACTGGGCCTTGGTAGTCCCTGCTCAGGCAGCTGGCTTTACCGTCAATGTGATGAGCAATG 9561

Query 685 GTAAGAGTATCAGCTCCAAGGTTCTCCAGGCCGGCCTCAACTACGATACTGTCGAGGACG 744

Sbjct 9560 GTAAGAGTATCAGCTCCAAGGTTCTCCAGGCCGGCCTCAACTACGATACTGTCGAGGACG 9501

Query 745 GAATTGAAGAAGGTACTCAGCGACTGGTGATCAAGAACGGCGACACCATCGTTGGTGGGA 804

Sbjct 9500 GAATTGAAGAAGGTACTCAGCGACTGGTGATCAAGAACGGCGACACCATCGTTGGTGGGA 9441

Query 805 CGGATCGAGGTCGATGCCTTGCTCGAGAATGCCACGATGGCATCTACAACTTCAACCCTG 864

Sbjct 9440 CGGATCGAGGTCGATGCCTTGCTCGAGAATGCCACGATGGCATCTACAACTTCAACCCTG 9381

Query 865 TCATCATGCCAGTCAAGGCGGTCTTTGACAATTCAGACTGTTGGCAAGTTGAGGGTGAGC 924

Sbjct 9380 TCATCATGCCAGTCAAGGCGGTCTTTGACAATTCAGACTGTTGGCAAGTTGAGGGTGAGC 9321

Query 925 CCATCTTANATTGGACAGGTGAAAAGGTAATGGGCACTACACTTGGCCCACGAGACGCTG 984

Sbjct 9320 CCATCTTAGATTGGACAGGTGAAAAGGTAATGGGCACTACACTTGGCCCACGAGACGCTG 9261

Query 985 ATCCTAGCCATAACCATGACTCGGCTCACAATTCCATCCNCGTTTGACACATTTCAAAGG 1044

Sbjct 9260 ATCCTAGCCATAACCATGACTCGGCTCACAATTCCATCC-CGTTTGACACATT-CAAAGG 9203

Query 1045 TTGTAGTaa 1053

Sbjct 9202 TTGTAGTAA 9194

11. Appendix II: Full Corn Assay Experiment Results

Table 24: Corn Assay Experiment 1 Full Results

Negative Control		Mutant Fv_00027		Positive Control	
Total mass	Shoot mass	Total mass	Shoot mass	Total mass	Shoot mass
1.43	0.47	0.28	0	0.41	0
1.42	0.44	0.52	0	0.52	0
1.10	0.48	0.46	0	0.36	0
0.36	0	0.37	0	0.38	0
1.17	0.54	0.53	0	0.35	0
1.16	0.92	0.49	0	0.44	0
1.09	0.43	0.38	0	0.42	0
0.60	0.24	0.39	0	0.39	0
1.62	0.65	0.41	0	0.24	0
1.68	0.62	0.61	0.13	0.27	0
0.57	0.05	0.40	0	0.32	0
1.22	0.51	0.41	0	0.36	0
1.02	0.20	0.40	0	0.35	0
1.81	0.73	0.86	0	0.26	0
1.04	0.37	0.53	0	0.26	0
0.84	0.27	0.46	0	0.41	0
1.38	0.61	0.35	0	0.50	0
1.61	0.75	0.30	0	0.42	0
1.27	0.46	0.40	0	0.75	0.20
0.86	0.29	0.48	0.01	0.76	0.47
0.45	0	0.53	0	0.22	0
0.55	0	0.38	0	0.57	0

0.44	0	0.40	0	0.32	0
0.25	0	0.51	0	0.27	0
0.23	0	0.53	0	0.22	0
0.80	0	0.37	0	0.40	0
1.01	0.65	0.42	0	0.26	0
1.99	0.88	0.55	0	0.32	0
1.23	0.43	0.54	0.01	0.61	0
1.28	0.42	0.47	0.01	0.37	0
0.47	0	0.62	0	0.22	0
0.60	0	0.63	0	0.24	0
0.57	0	0.38	0	0.23	0
0.51	0	0.39	0	0.25	0
0.59	0	0.35	0	0.13	0
1.02	0	0.55	0	0.42	0
0.47	0	0.53	0	0.20	0
1.07	0.44	0.42	0	0.37	0
0.87	0.11	0.50	0	0.33	0
1.04	0.41	0.42	0	0.40	0
0.967	**0.309**	**0.463**	**0.004**	**0.363**	**0.0168**

Table 25: Corn Assay Experiment 2 Full Results

Negative Control		Mutant Fv_00027		Positive Control	
Total mass	Shoot mass	Total mass	Shoot mass	Total mass	Shoot mass
0.43	0	0.46	0	0.28	0
0.50	0	0.28	0	0.26	0
0.49	0	0.21	0	0.46	0
0.34	0	0.27	0	0.35	0
0.51	0	0.15	0	0.43	0
0.51	0	0.22	0	0.29	0
0.66	0	0.31	0	0.32	0
0.45	0	0.31	0	0.32	0
0.37	0	0.23	0	0.23	0
1.42	0.66	0.28	0	0.34	0
0.49	0	0.24	0	0.16	0
0,56	0	0.34	0	0.16	0
0.32	0	0.26	0	0.37	0
0.51	0	0.28	0	0.33	0
0.44	0	0.28	0	0.33	0
0.39	0	0.28	0	0.38	0
0.74	0.33	0.27	0	0.30	0
1.37	0.74	0.23	0	0.23	0
0.87	0.28	0.29	0	0.30	0
0	0	0.82	0.44	0.23	0
0.54	0	0.27	0	0.35	0
0.44	0	0.31	0	0.42	0
0.56	0	0.35	0	0.43	0

0.37	0	0.31	0	0.35	0
0.39	0	0.29	0	0.34	0
0.46	0	0.29	0	0.40	0
0.50	0	0.38	0	0.33	0
0.46	0	0.21	0	0.32	0
0.58	0	0.30	0	0.37	0
0.58	0.34	0.27	0	0.27	0
0.43	0	0.21	0	0.44	0
0.38	0	0.30	0	0.40	0
0.48	0	0.25	0	0.41	0
0.44	0	0.38	0	0.26	0
0.54	0	0.31	0	0.57	0
0.53	0	0.36	0	0.35	0
0.50	0	0.35	0	0.38	0
0.56	0	0.27	0	0.36	0
0.49	0	0.38	0	0.33	0
0.43	0	0.33	0	0.35	0
0.52575	**0.05875**	**0.30325**	**0.011**	**0.3375**	**0**

Table 26: Corn Assay Experiment 3 Full Results

Negative Control		Mutant Fv_00027		Positive Control	
Total mass	Shoot mass	Total mass	Shoot mass	Total mass	Shoot mass
0.54	0	0.28	0	0.29	0
0.57	0	0.25	0	0.18	0
0.45	0	0.30	0	0.29	0
0.56	0	0.27	0	0.27	0
0.54	0	0.21	0	0.20	0
0.60	0	0.50	0.09	0.36	0.12
0.67	0.10	0.45	0.24	0.35	0
0.66	0.11	0.33	0.03	0.45	0
0.80	0.19	0.73	0.33	0.36	0
0.74	0.13	0.93	0.52	0.41	0.06
0.27	0	0.54	0	0.22	0
0.68	0	0.45	0	0.31	0
0.56	0	0.44	0	0.28	0
0.54	0	0.27	0	0.25	0
0.52	0	0.31	0	0.27	0
0.67	0	0.42	0	0.39	0
0.43	0	0.32	0	0.28	0.04
0.41	0	0.29	0	0.39	
0.45	0	0.52	0.06	0.26	0.04
0.62	0	0.58	0.18	0.78	0.24
0.72	0	0.45	0	0.25	0
0.44	0	0.39	0	0.33	0

0.67	0	0.32	0	0.26	0
0.52	0	0.40	0	0.35	0
0.55	0	0.46	0	0.23	0
0.57	0	0.30	0.01	0.35	0
0.53	0	0.43	0.01	0.28	0.09
0.48	0	0.45	0.05	0.25	0
0.56	0.09	0.53	0.09	0.37	0.011
0.94	0.32	0.87	0.41	0.58	0.23
0.54	0	0.28	0	0.39	0
0.61	0	0.23	0	0.26	0
0.52	0	0.22	0	0.49	0
0.54	0	0.25	0.05	0.25	0
0.51	0	0.40	0.02	0.24	0
0.49	0	0.36	0.01	0.35	0
0.48	0	0.31	0	0.32	0
0.54	0	0.47	0.06	0.38	0
0.68	0	0.33	0.03	0.28	0
0.51	0	0.47	0	0.29	0
0.567	**0.0235**	**0.408**	**0.0548**	**0.327**	**0.0213**

Table 27: Corn Assay Experiment 4 Full Results

Negative Control		Mutant Fv_00027		Positive Control	
Total Mass	Shoot Mass	Total Mass	Shoot Mass	Total Mass	Shoot Mass
1.15	0.45	0.38	0	0.31	0
1.60	0.67	0.19	0	0.39	0
1.17	0.46	0.24	0	0.38	0
0.85	0.21	0.42	0	0.27	0
1.19	0.48	0.40	0	0.28	0
0.90	0.44	0.18	0	0.20	0
1.55	0.55	0.41	0	0.57	0.02
1.01	0.45	0.45	0	0.40	0.02
0.81	0.03	0.58	0.17	0.78	0.32
1.02	0	0.41	0.05	0.70	0.24
1.69	0.68	0.37	0	0.32	0
1.22	0.59	0.35	0	0.11	0
0.83	0.27	0.57	0	0.49	0
1.23	0.51	0.25	0	0.44	0
1.16	0.48	0.32	0	0.38	0
1.98	0.72	0.21	0	0.49	0
0.80	0.16	0.25	0	0.36	0.06
0.88	0.21	0.23	0	0.62	0.01
0.87	0.03	0.47	0.10	0.39	0.01
0.62	0	0.38	0.04	0.40	0.01
1.16	0.49	0.30	0	0.27	0
1.63	0.61	0.42	0	0.29	0
1.51	0.61	0.45	0	0.15	0

1.51	0.63	0.38	0	0.38	0
1.03	0.36	0.37	0	0.42	0
0.87	0.15	0.36	0	0.38	0
1.31	0.61	0.37	0.01	0.35	0
1.29	0	0.58	0.12	0.35	0
0.60	0.03	0.47	0.02	0.45	0.04
0.61	0	0.45	0	0.28	0
1.90	0.85	0.33	0.01	0.26	0
1.82	0.82	0.30	0.01	0.39	0
1.28	0.58	0.36	0	0.33	0
1.63	0.58	0.35	0	0.24	0
1.49	0.76	0.27	0	0.41	0.05
1.12	0.32	0.35	0.01	0.36	0.02
1.77	0.93	0.55	0.06	0.64	0.07
0.94	0.10	0.39	0.03	0.32	0
0.52	0.01	0.65	0.18	0.32	0.02
0.62	0.03	0.44	0.04	0.91	0.33
1.1785	**0.3965**	**0.38**	**0.02125**	**0.3945**	**0.0305**

Table 28: Corn Assay Experiment 5 Full Results

Negative Control		Mutant Fv_00027		Positive Control	
Total mass	Shoot mass	Total mass	Shoot mass	Total mass	Shoot mass
0.64	0	0.27	0	0.33	0
0.47	0	0.38	0	0.37	0
0.59	0	0.59	0	0.38	0
0.67	0.15	0.66	0.26	0.42	0
0.58	0.03	0.37	0.01	0.3	0
0.54	0.05	0.43	0.04	0.4	0
1.37	0.58	0.55	0.12	0.3	0
1.31	0.62	0.51	0.08	0.48	0
1.33	0.62	0.5	0.17	0.48	0
0.56	0	0.81	0.37	0.51	0.03
0.51	0	0.38	0	0.32	0
0.53	0	0.29	0	0.27	0
0.56	0	0.29	0	0.48	0
0.46	0	0.33	0	0.36	0
0.54	0	0.31	0	0.39	0
0.45	0.01	0.24	0	0.32	0
0.44	0.01	0.45	0.03	0.32	0
1.04	0.28	0.46	0.02	0.27	0
0.79	0.28	0.57	0.09	0.39	0
0.93	0.36	0.43	0.04	0.45	0.07
0.6	0	0.45	0	0.22	0
0.6	0	0.41	0	0.3	0
0.6	0	0.46	0	0.27	0.01

0.5	0	0.26	0	0.51	0.05
0.43	0.05	0.48	0	0.41	0.14
0.56	0.08	0.42	0	0.51	0.11
0.44	0.01	0.45	0	0.35	0.06
0.49	0.02	0.7	0.11	0.65	0.17
0.69	0.2	0.36	0.01	0.79	0.27
0.77	0.26	0.4	0.03	0.57	0.1
0.53	0	0.24	0	0.35	0
0.54	0	0.35	0	0.47	0
0.57	0	0.38	0	0.26	0
0.59	0	0.39	0	0.26	0
0.51	0	0.35	0	0.36	0
0.5	0	0.36	0	0.26	0
0.51	0	0.33	0	0.25	0
0.59	0.06	0.4	0	0.31	0
0.88	0.27	0.39	0.02	0.36	0.01
1.26	0.38	0.61	0.13	0.56	0.34
0.662	**0.108**	**0.425**	**0.0383**	**0.389**	**0.034**

12. Appendix III: Statistical Tables for Corn Assay Experiments

Corn Assay Experiment 1

Table 29: Total Mass Statistics ANOVA Results. Results from the ANOVA test run by SPSS with the average total masses from Corn Assay Experiment 1. Since the Significance value was < 0.05, at least one treatment was statistically different from another.

V2 **ANOVA**

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	8.392	2	4.196	54.670	.000
Within Groups	8.980	117	.077		
Total	17.371	119			

Table 30: Total Mass Statistics Post-Hoc Test Results. These are the results from the Tukey B and Scheffe tests. Since the mutant and positive controls were placed in the same group, they are not statistically different. The Negative Control, no *F. verticillioidies*, though was placed in a different group, which demonstrates the assay was valid. 1=Negative control, 2=Fv_0027 mutant, 3=Positive control. Means for groups in homogenous subsets are displayed. a. Uses Harmonic Mean Sample Size=40.000.

V2

V1		N	Subset for alpha=0.05	
			1	2
Tukey B[a]	3	40	.3630	
	2	40	.4630	
	1	40		.9673
Scheffe[a]	3	40	.3630	
	2	40	.4630	
	1	40		.9673
	Sig		.276	1.000

Table 31: Shoot Mass Statistics ANOVA Results. Results from the ANOVA test run by SPSS with the average shoot masses from Corn Assay Experiment 1. Since the Significance value was < 0.05, at least one treatment was statistically different from another.

V2

ANOVA

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	2.385	2	1.193	40.089	.000
Within Groups	3.481	117	0.030		
Total	5.866	119			

Table 32: Shoot Mass Statistics Post-Hoc Tests Results. These are the results from the Tukey B and Scheffe tests and were identical to the total mass statistics (Table 25). Since the mutant and positive controls were placed in the same group, they are not statistically different. The Negative Control though was placed in a different group, which demonstrates the assay was valid.1=Negative control, 2=Fv_00027 mutant, 3=Positive control. Means for groups in homogenous subsets are displayed. a. Uses Harmonic Mean Sample Size=40.000.

V2

V1		N	Subset for alpha=0.05	
			1	2
Tukey B[a]	2	40	.0040	
	3	40	.0167	
	1	40		.3093
Scheffe[a]	2	40	.0040	
	3	40	.0167	
	1	40		.3093
	Sig		.947	1.000

Corn Assay Experiment 2

Table 33: Total Mass Statistics ANOVA Results. Results from the ANOVA test run by SPSS with the average total masses from Corn Assay Experiment 2. Since the Significance value was < 0.05, at least one treatment was statistically different from another.

V2

ANOVA

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	1.148	2	.574	23.256	.000
Within Groups	2.888	117	.025		
Total	4.037	119			

Table 34: Total Mass Statistics Post-Hoc Test Results. These are the results from the Tukey B and Scheffe tests and are identical to Corn Assay Experiment 1. Since the mutant and positive controls were placed in the same group, they are not statistically different. The Negative Control though was placed in a different group, which demonstrates the assay was valid.1=Negative control, 2=Fv_00027 mutant, 3=Positive control. Means for groups in homogenous subsets are displayed. a. Uses Harmonic Mean Sample Size=40.000.

V2

V1		N	Subset for alpha=0.05	
			1	2
Tukey B[a]	2	40	.303250	
	3	40	.337500	
	1	40		.525750
Scheffe[a]	2	40	.303250	
	3	40	.337500	
	1	40		.525750
	Sig		.623	1.000

Table 35: Shoot Mass Statistics ANOVA Results. Results from the ANOVA test run by SPSS with the average shoot masses from Corn Experiment 2. Since the Significance value was < 0.05, at least one treatment was statistically different from another.

V2

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.078	2	.039	3.415	.036
Within Groups	1.337	117	.011		
Total	1.415	119			

Table 36: Shoot Mass Statistics Post-Hoc Test Results. These are the results from the Tukey B and Scheffe tests. The Fv_00027 mutant was placed in both groups meaning that the positive and negative controls were different, but the mutant was the same as both.1=Negative control, 2=Fv_00027 mutant, 3=Positive control. Means for groups in homogenous subsets are displayed. a. Uses Harmonic Mean Sample Size = 40.000.

V2

V1		N	Subset for alpha=0.05	
			1	2
Tukey B[a]	3	40	.0000	
	2	40	.0110	.0110
	1	40		.0587
Scheffe[a]	3	40	.0000	
	2	40	.0110	
	1	40	.0587	
	Sig		.053	

Corn Assay Experiment 3

Table 37: Shoot Mass Statistics ANOVA Results. Results from the ANOVA test run by SPSS with the average shoot masses from Corn Assay Experiment 3. Since the Significance value was > 0.05, none of the treatments were statistically different from another.

V2 **ANOVA**

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	.028	2	.014	1.937	.149
Within Groups	.834	116	.007		
Total	.862	118			

Table 38: Shoot Mass Statistics Post-Hoc Tests Results. These are the results from the Tukey B and Scheffe tests. Since the mutant and positive controls were placed in the same group, they are not statistically different. The Negative Control though was placed in a different group, which demonstrates the assay was valid.1=Negative control, 2=Fv_00027 mutant, 3=Positive control. Means for groups in homogenous subsets are displayed. a. Uses Harmonic Mean Sample Size=39.661. b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type 1 error levels are not guaranteed.

V2

V1		N	Subset for alpha=0.05
			1
Tukey B[a,b]	3	39	.021308
	1	40	.023500
	2	40	.054750
Scheffe[a,b]	3	39	.021308
	1	40	.023500
	2	40	.054750
	Sig		.218

Corn Assay Experiment 4

Table 39: Total Mass Statistics ANOVA Results. Results from the ANOVA test run by SPSS with the average total masses from Corn Assay Experiment 4. Since the Significance value was < 0.05, at least one treatment was statistically different from another.

V2

ANOVA

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	16.700	2	8.350	130.950	.000
Within Groups	7.460	117	.064		
Total	24.160	119			

Table 40: Total Mass Statistics Post-Hoc Tests Results. These are the results from the Tukey B test. Since the mutant and positive controls were placed in the same group, they are not statistically different. The negative control though was placed in a different group which demonstrates the assay was valid.1=Negative control, 2=Fv_00027 mutant, 3=Positive control. Means for groups in homogenous subsets are displayed. a. Uses Harmonic Mean Sample Size=40.000.

V2

V1		N	Subset for alpha=0.05	
			1	2
Tukey B[a]	2	40	.3800	
	3	40	.3945	
	1	40		1.1785

Table 41: Shoot Mass Statistics ANOVA Results. Results from the ANOVA test run by SPSS with the average shoot masses from Corn Assay Experiment 4. Since the Significance value was < 0.05, at least one treatment was statistically different from another.

V2

ANOVA

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	3.665	2	1.832	63.689	.000
Within Groups	3.366	117	.029		
Total	7.031	119			

Table 42: Shoot Mass Statistics Post-Hoc Tests Results. These are the results from the Tukey B and Scheffe tests. Since the mutant and positive controls were placed in the same group, they are not statistically different. The Negative Control though was placed in a different group, which demonstrates the assay was valid.1=Negative control, 2=Fv_00027 mutant, 3=Positive control. Means for groups in homogenous subsets are displayed. a. Uses Harmonic Mean Sample Size=40.000.

V2

V1		N	Subset for alpha=0.05	
			1	2
Tukey B[a]	2	40	.0213	
	3	40	.0305	
	1	40		.3965
Scheffe[a]	2	40	.0213	
	3	40	.0305	
	1	40		.3965
	Sig		.971	1.000

Corn Assay Experiment 5

Table 43: Total Mass Statistics ANOVA Results. Results from the ANOVA test run by SPSS with the average total masses from Corn Assay Experiment 5. Since the Significance value was < 0.05, at least one treatment was statistically different from another.

V2

ANOVA

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	1.755	2	.878	27.170	.000
Within Groups	3.779	117	.032		
Total	5.534	119			

Table 44: Total Mass Statistics Post-Hoc Tests Results. These are the results from the Tukey B and Scheffe tests. Since the mutant and positive controls were placed in the same group, they are not statistically different. The Negative Control though was placed in a different group, which demonstrates the assay was valid.1=Negative control, 2=Fv_00027 mutant, 3=Positive control. Means for groups in homogenous subsets are displayed. a. Uses Harmonic Mean Sample Size=40.000.

V2

V1		N	Subset for alpha=0.05	
			1	2
Tukey B[a]	3	40	.3890	
	2	40	.4253	
	1	40		.6618
Scheffe[a]	3	40	.3890	
	2	40	.4253	
	1	40		.6618
	Sig		.667	1.000

Table 45: Shoot Mass Statistics ANOVA Results. Results from the ANOVA test run by SPSS with the average shoot masses from Corn Assay Experiment 5. Since the Significance value was < 0.05, at least one treatment was statistically different from another.

V2

ANOVA

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	.138	2	.069	4.610	.012
Within Groups	1.753	117	.015		
Total	1.891	119			

Table 46: Shoot Mass Statistics Post-Hoc Tests Results. These are the results from the Tukey B and Scheffe tests. Since the mutant and positive controls were placed in the same group, they are not statistically different. The Negative Control though was placed in a different group, which demonstrates the assay was valid.1=Negative control, 2=Fv_00027 mutant, 3=Positive control. Means for groups in homogenous subsets are displayed. a. Uses Harmonic Mean Sample Size=40.000.

V2

V1		N	Subset for alpha=0.05	
			1	2
Tukey B[a]	3	40	.0340	
	2	40	.0383	
	1	40		.1080
Scheffe[a]	3	40	.0340	
	2	40	.0383	
	1	40		.1080
	Sig		.988	1.00