ROLE OF THREE SMALL CYSTEINE-RICH PROTEINS FROM *TRICHODERMA VIRENS* AS ELICITORS OF PLANT RESISTANCE

An Undergraduate Research Scholars Thesis

by

RACHEL BEGER

Submitted to Honors and Undergraduate Research
Texas A&M University
In partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

Approved by
Research Advisor: Dr. Charles Kenerley

May 2013

Major: Microbiology

TABLE OF CONTENTS

Page

ABSTRACT

Role of Three Small Cysteine-Rich Proteins from *Trichoderma virens* as Elicitors of Plant Resistance. (May 2013)

Rachel Beger
Department of
Biology
Texas A&M University

Research Advisor: Dr. Charles Kenerley
Department of
Plant Pathology & Microbiology

Trichoderma virens, a symbiotic plant fungus has the characteristic of inducing systemic and local resistance in plants against plant pathogens and acting as a mycoparasite of pathogenic fungi. These traits have resulted in *T. virens* being used as a biocontrol agent for plant disease management. One mechanism of biocontrol is the direct attack of pathogen propagules. A second mechanism involves the induction of defense responses. The second mechanism is initiated by root colonization and secretion of small cysteine-rich proteins either on the root surface or upon ingress by *T. virens*. The plant then exhibits higher expression of defense responses and at a more rapid rate compared to untreated plants when inoculated with a pathogen. SM1 is one protein that has been determined to have elicitor activity.

This study will determine if three additional small, secreted proteins (similar to SM1) are capable of inducing plant systemic resistance to pathogens. Vectors have been constructed to disrupt and over-express the targeted genes and will be used to transform wild type Gv29-8. The mutant strains will be tested on their ability to protect the host against pathogen infection. Expression

analysis of the three selected genes will be performed by dual confrontation with fungal pathogens.

ACKNOLWEDGMENTS

I would like to thank Dr. Kenerley for allowing me to work in his lab and for all his mentorship, I am very grateful for the opportunity. I would also like to thank Dr. Maria E. Moran-Diez for guiding me and teaching me all along this process, her help and patience have been greatly appreciated and I would not have gained near what I have without her help. I would like to acknowledge my friends and family for all their support and time of listening to me talk through all the highs and lows of this research project.

CHAPTER I

INTRODUCTION

Trichoderma species are free-living fungi that are opportunistic, avirulent plant symbionts often antagonistic to plant pathogens including other fungi (7). *Trichoderma* species are of the ascomycete family and are found in soil and root ecosystems. Various species of *Trichoderma* invade the outer most layers roots systems of a wide variety of crop and woodland plants. However, the ingress into plant roots is limited to the outer cortical cells. Although colonization is in the roots, the resulting production of antimicrobial compounds is seen both in the roots and in the leaves (2). During the initial stages of root colonization and upon entry into the root, a number of compounds are secreted by *Trichoderma,* including proteins and secondary metabolites. These compounds may induce local or systemic resistance responses in the plant host against pathogens (7). Root colonization can also enhance root growth and development, crop productivity, resistance to abiotic stresses and the uptake and use of nutrients for dicots and monocots (9). *Trichoderma* species have been found to successfully protect against common soil-borne plant pathogens such as *Pythium ultimum* and *Rhizoctonia solani* (6).

Although these and other plant pathogens can be controlled by the application of pesticides, there is an increasing awareness that pesticides may have detrimental side effects such as development of pathogen resistance, contamination of the environment, and adverse health effects on applicators (6). One application of an effective strain of *Trichoderma* can control a range of soil-borne and foliar pathogens, either of bacteria or other fungi (7). Efforts throughout the world have been ongoing to understand how *Trichoderma* protects plants to use these fungi as agents in

a biocontrol strategy. Biocontrol is defined as the use of a living organism (e.g. fungi, bacteria, predatory insects) to control biotic pathogens of agricultural crops or noxious weeds (domestic or introduced) in the landscape (6). In the case of fungal agents, they may attack the plant pathogens through an antagonistic relationship involving the secretion of antibiotics/peptiabiotics and cell-wall degrading enzymes. Plant-fungal interactions often use small, secreted proteins as effecter molecules that are both recognized by receptors in the host to initiate innate immunity, leading to resistance to the eliciting pathogen (7). One agent that has been commercially developed for the management of plant disease, especially in greenhouses and nurseries is *T. virens* (8). In addition to directly attacking soil-borne pathogens, *T. virens* colonizes root systems of a plant host as a symbiont, and induce the plant's resistance mechanisms against bacterial and fungal pathogens by induced systemic resistance (ISR) and in some cases systemic acquired resistance (SAR) (2). This induced resistance is achieved by the action of an elicitor protein or peptaibol that initiates priming and sensitizing of the plant defense response. The plant then exhibits a higher expression of defense genes, and at a more rapid rate compared to untreated plants when inoculated with a pathogen. These defense responses include the pathogen-induced jasmonic acid-dependent pathways, salicylic acid-dependent pathway, and redox regulating proteins (1). *T. virens* also has the capability to degrade hazardous chemical compounds, such as pesticides, and to sequester heavy metals (JGI). The use of biocontrol agents could have a great impact on the management of pests in agriculture while promoting a more ecological solution than chemical applications.

The protein SM1 produced by *T. virens*, is the only elicitor to have been found in fungal biocontrol agents to date (10), although many other elicitors have been discovered from plant

pathogens that induce plant resistance. SM1 is a non-enzymatic, small, secreted protein that acts as a signaling molecule during the early stages of interactions between a host plant and *Trichoderma* (10). SM1 is part of the cerato-platanin protein family (12). This family of proteins is characterized by a signal peptide for secretion, cleavage of the signal peptide, four cysteine residues, formation of two disulfide bonds with the cysteine residues, and moderate hydrophobicity (12). Previous research has demonstrated that *T. virens* induces ISR in maize and that SM1 is required for this response (2). In cotton and in rice, SM1 induced SAR and in maize, the activity of SM1 induced ISR (1). When SM1 was applied to cotton, it increased the resistance of the cotton to the foliar pathogen *Colletotrichum* species (12). Maize leaves and roots treated with SM1 increased resistance to the pathogen *C. graminicola* (12). SM1 protective activity included an accumulation of reactive oxygen species and phenolic compounds, increased levels of transcription of defense genes regulated by salicylic acid and jasmonic acid, and increased transcription of genes for the biosynthesis of terpenoid phytoalexins (10). Deletion of the gene encoding SM1 resulted in loss of SM1 production and reduced disease protection when pathogens were challenged by these mutants. The results were comparable to the same level of disease with plants not treated with *T. virens* (2). The majority of the proteinaceous elicitors produced by fungi are characterized as small (300 amino acids or less), cysteine-rich (4 or more cysteine residues) and secreted (2). By bioinformatic analysis of the genome sequence of *T. virens*, 25 putative small secreted proteins were identified, including 79522, 93159, and 110650 (protein identification numbers as assigned to *T. virens* by the Joint Genome Institute) (5). The genes encoding these three proteins were experimentally shown to be expressed and the proteins secreted when *T. virens* was grown in the presence of maize seedlings in a hydroponic system (11). The effectiveness of these three proteins as elicitors for the induction of the host's defense

mechanism is not known beyond their sequence similarity to each other and SM1. The goal for this project was to determine if any of these proteins have elicitor activity after colonizing plant roots and confronted by a pathogen.

The research approach was to eliminate or replace, as well as over-express each gene and then test the ability of these generated mutants to protect maize against pathogen infection. The targeted genes were replaced by constructing vectors that contain a marker for mutant selection (hygromycin phosphotransferase) and flanking regions of the targeted gene for homologous integration. Wild type *T. virens* (Gv29-8) was the recipient strain for transformation using the knockout vectors by. Positive colonies were transferred sequentially on three rounds on PDA (potato dextrose agar) supplemented with hygromycin (PDAH), one round PDA (no selection) and finally on PDAH for further selection and characterization. Another approach was to over-express the genes to test whether an increase in expression results in enhanced production of the proteins and ISR. The over-expressing mutants were constructed by insertion of the gene into the vector pJMB1, under the activity of the constitutive promoter *gpd,* from *T. virens*. Expression studies of the three genes were conducted using Gv29-8 as a positive control for dual confrontation with fungal pathogens and growth analysis when grown in Vogel's minimal medium supplemented with different carbon sources.

CHAPTER II

MATERIALS & METHODS

Bacterial/fungal/plant materials

The wild-type strain of *Trichoderma virens* Gv29-8 was used in this study. The competent strain of *Escherichia coli*, XL-1 was used for the *E. coli* transformations. Mycoparasitism assays used the plant pathogens *Rhizoctonia solani, Pythium ultimum, Botrytis cinerea*. Expression assays used *P. ultimum, R. solani, B. cinerea,* colloidal chitin and maize roots (*Zea mays*) as supplemented carbon sources. Microarray analysis of gene expression was performed using tomato roots and maize roots.

Cultures and media

Strains, mutant and wild-type, were grown on PDA (Difco) and PDA supplemented with hygromycin to screen the transformants. To screen *E. coli* transformants, *E. coli* was grown up in LB (Luria broth) supplemented with ampicillin for antibiotic selection. Expression studies used Vogel's Medium supplemented with 1.5% sucrose, 1% lignin, 0.5% colloidal chitin or the cell walls from the pathogens *P. ultimum, B. cinerea, R. solani,* or maize as a carbon source. Single sporulation of the knockout mutants was performed using a water agar media supplemented with hygromycin for antibiotic selection. Maize seeds were grown hydroponically in chambers containing 0.5X Murashige and Skoog with Gamborg's Vitamins (MS), 0.5% sucrose.

Bioinformatics software

The restriction sites for vector design in the over-expression study were selected using the software GeneCoder. To ensure that the insertion of the construct was in-frame, the software FinchTV was used to view the sequence. Analysis of the *93159* gene and protein sequence was performed by a BLAST search for sequence homology, ClustalX for protein alignment, and Seaview for creating phylogenetic trees. Significant properties of the protein sequences were provided by the Joint Genome Institute website for *T. virens*.

Knock-Out construct design

Double-joint polymerase chain reaction (D-J PCR) was the method used to construct the gene deletion construct. This method used three sets of polymerase chain reactions: amplification of the left, right, and central flanks separately; fusion of the three flanks; and a nested PCR reaction to amplify the large amounts of the deletion construct. The left and right flanks amplified in the first step were the regions on the left and right side of the gene to be deleted from Gv29-8, and the central flank was the hygromycin resistance cassette. The primers designed over the left and right flanks amplified a fragment of approximately 0.8 kb of the gene to be deleted**.** The primers were also designed with segments that are homologous to the ends of the hygromycin cassette to join them together. A schematic design of the amplification step is presented in Figure 1.

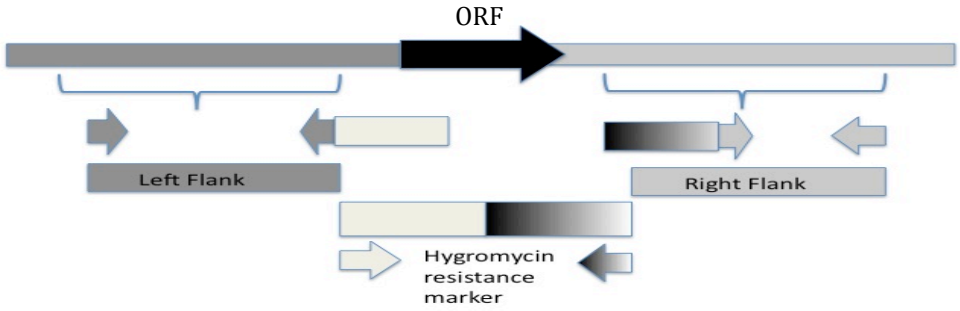

Figure 1. Diagram of the strategy for amplifying the flanking regions of each targeted gene for placement of selectable marker. The left flank, right flank, and hygromycin resistance marker were amplified separately. The primers for the left and right flanks anneal to the left and right regions of the ORF. The primers have "tails" that are homologous to the ends of the hygromycin resistance marker. The arrows represent the different sets of primers.

In the second step the three fragments were joined through the D-J PCR reaction that connected the "tails" of the primers to the ends of the hygromycin cassette. A schematic representation of the double-joint PCR is presented in Figure 2.

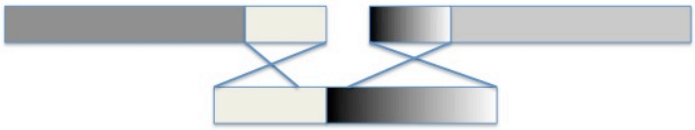

Figure 2. Double Joint PCR for Joining of Designed Primers to Selectable Marker. The three fragments were joined by a double-joint PCR reaction by the "tails" of the left and right primers anneal to the hygromycin resistance marker.

"Nested" primers were designed over the ends of the left and right flanks to amplify the major portion of the cassette for replacement of the chromosomal locus in the fungal genome. A representation of the "nested" reaction and product is presented in Figure 3.

Figure 3. Nested Reaction for Amplification of Selectable Marker. The "nested" reaction amplified a large portion of the gene replacement cassette. The arrows represent the "nested" primers.

In the fungal genome, the central region of the selected gene was replaced because the constructed fragment's flanking regions were homologous to the left and right regions of the gene. The hygromycin resistance marker replaced the ORF of the gene by a double crossover into the fungal chromosome. The primers designed are listed in Table 1.

Table 1. **Knock-Out Primer Design**

	93159	79522	110650
LF	CACTGATGAATCCTAAGTTCTCAC	CTCTCGTTATTCCAGCTCCCATC	CTCGTATTGCTTGGTGTCTCGCCA
LR	ATTGATGTGTTGACCTCCAC-CGACTGTGGTAGACTGTTGATG	ATTGATGTGTTGACCTCCAC-TGCAATGCGTGGAAGATGGTG	ATTGATGTGTTGACCTCCAC-CTTCCTTGATCTGTCAAGTGG
RF	TCTGGATATAAGATCGTTGGTCTC-GACTGTTGGAAATAGTGTGAAG	TCTGGATATAAGATCGTTGGTGTC-GATTGAATGAATTGGAATGTTG	TCTGGATATAAGATCGTTGGTGTC-CTTCCTTGATCTGTCAAGTGG
RR	CTTCGTCTCTCGAGGTGGCTATTG	AGTGGCAACCCTAGAGCACCGT	ACAACCCTCGCCACATTCATCGA
NTF	CTGATTATTCTGATTATGGAAAGC	CTCGCTATTCTAGACCGGGTGCT	CTCTTCGATTAGAATTGTGTGGCG
NTR	CTTCAAGCAAATCGAAGTCTATGC	GAGCACCGTTTTGGATTCTTCTTA	CAGGAAACCATCGAGAAGATGGC

Primer Design for DJ-PCR: LF= Left Flank Forward; LR= Left Flank Reverse; RF= Right Flank Forward; RR= Right Flank Reverse; NTF= Nested Forward; NTR= Nested Reverse

For the amplification of the flanking regions the reaction consisted of priming at 96° C for 3 minutes, and 35 cycles of: 94° C strand separation for 30 seconds, 52° C annealing for 30 seconds, 72° C elongation for 1 minute; then a holding period of 72° C for 10 minutes and a final hold at 4° C. The reaction consisted of the components in Table 2. The template for the left and right flanking regions was Gv29-8 genomic DNA, and the vector containing the hygromycin cassette, pCSN43.

Table 2. **Components of Amplification Reaction**

	Volume (µL)
10X ThermoPol Buffer	5.0
2.5 mM dNTP mix	4.0
Taq DNA Polymerase	0.5
10 nM Primers (Forward and Reverse)	1.0
DNA Template	1.0
Distilled Water	37.5
Total Reaction	50.0

Double-joint PCR (D-J PCR) joins the three fragments, left flank, right flank, and hygromycin cassette, together. D-J PCR does not require primers because the "tails" of the left and right fragments are homologous to regions of the hygromycin cassette to act as "glue" to bring the fragments together. The reaction consisted the components in Table 3. The parameters of the reaction were: 94° C priming for 2 minutes; then 15 cycles of 94° C strand separation for 30 seconds, 55° C annealing for 20 minutes, 72° C elongation for 7 minutes; followed by a hold for 10 minutes at 72° C and a final hold at 4° C. The product of the D-J PCR reaction served as the template for the nested PCR reaction.

Table 3. Components of DJ-PCR Reaction

	Volume (μL)
10X ThermoPol Buffer	2.5
2.5 mM dNTPs mix	2.0
Taq DNA Polymerase	0.25
Left Flank	2.5
Right Flank	2.3
Hygromycin cassette	7.5
Distilled Water	7.75
Total Reaction	25.0

The nested PCR reaction uses the primers that are homologous to fragments just inside the left and right flanks to amplify large amounts of fragment containing the hygromycin cassette to replace the gene. The reaction consisted of the components listed in Table 4. The parameters of the reaction were: 96° C priming for 3 minutes then 35 cycles of 94° C strand separation for 30 seconds, 57° C annealing for 30 seconds, and 72° C elongation for 4 minutes, followed by a holding period at 72° C for 10 minutes and a final hold at 4° C.

Table 4. Components of "nested" reaction

	Volume (μL)
10X ThermoPol Buffer	5.0
2.5 dNTPs mix	2.0
Taq DNA Polymerase	0.5
10 nM Primers (Forward and Reverse)	2.0
DNA Template	1.0
Distilled Water	37.5
Total Reaction	50.0

Over-expression vector design

The vector pJMBI was used for over-expression studies of 93159. Primers were designed with a C-terminal His-tag of six histidine residues, and cut sites for 2 restriction enzymes found on

pJMB1 for incorporation into the vector by creation of "sticky ends". A display of the vector design using *Sma*I and *Hind*III is presented in Figure 4. The primers were designed for insertion of the ORF into the vector under the regulation of the constitutive promoter Pgpd. The vector also included ampicillin resistance used for selection of *E coli* positive transformants. A scheme of the PCR-fragment cloned into pJMB1 is represented in Figure 5.

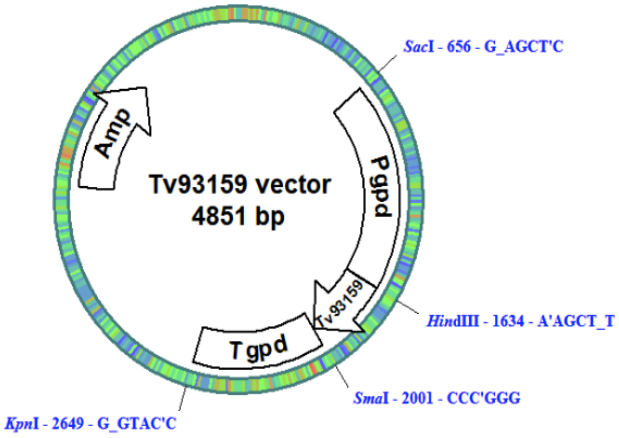

Figure 4. Vector Design for Transformation of *E. coli*. The vector design for over-expression of 93159 under the regulation of the constitutive promoter Pgpd, using *Sma*I and *Hind*III for incorporation.

Figure 5. Construct Design For Insertion into pJMB1. The design for the PCR fragment that was cloned into pJMB1 using *Hind*III and *Sma*I.

T. virens transformation

To transform *T. virens* 29-8 strain , "naked cell" or protoplast must be obtained from the conidia of the fungus. The DNA will be able to ender the naked protoplast and disrupt the gene with the hygromycin cassette. To form the protoplasts, 100 mL of Difco PDB was inoculated with a

conidial suspension to get a final concentration of 10^6 to 10^8 conidia/mL. The solution was incubated at 27° C for 14-16 hours, shaking at 150 rpm. To prepare the enzyme, NovoEnzyme was used, 24 mg were weighed out and added to a 15 mL Falcon tube and then 2.4 mL of mannitol osmoticum were added. The solution was vortexed to mix and then divided into 3 microcentrifuge tubes with 800 µLs in each tube. The tubes were centrifuged for 25 seconds at full speed. The solution was filtered through a sterile 0.22 micrometer membrane filter into a 25 mL sterile flask using a 5 mL syringe. The fungal material was harvested using a 100 micron nylon filter and rinsed with 250 mL of sterile distilled water. To the enzyme solution, 0.6 to 0.7 grams of the fresh fungal material was added and homogenized with a wide bore pipette. The solution was shook at room temperature at 225 rpm for 25 minutes then checked under the microscope for formation of protoplasts. The desired number of protoplasts is 10^7 protoplasts/mL. The protoplast mixture was filtered through a 10 micron nylon filter in a Swinnex filter apparatus attached to a glass 10 mL syringe into a 15 mL centrifuge tube. The filters were rinsed with 5 mL of osmoticum. The protoplasts were centrifuged at 5500 rpm for 10 minutes. The osmoticum supernatant was decanted and protoplast pellet resuspended in 300 µL of fresh osmoticum. For the transformation, 240 µL of protoplasts and 20 µL of DNA were added to a microcentrifuge tube. For the controls, no DNA was added. The protoplasts were incubated on ice for 20 minutes after adding the DNA. After the 20 minutes, the tubes were brought back to room temperature before 130 µL PEG solution (40% PEG 8000 in osmoticum) were added and mixed by inversion. To the solution, 130 µL were added again and mixed by inversion. The tubes were incubated at room temperature for 30 minutes. The treatments were added to the top agar of PDA sucrose supplemented with hygromycin before being poured on top of the bottom agar of PDA. The top agar was in a water bath at 42° C. The positive control

consisted of top agar of PDA sucrose without hygromycin and without DNA and the negative control consisted of top agar PDA sucrose + hygromycin and no DNA. Osmoticum: 50 mM CaCl2, 0.5 M Mannitol, 50 mM MES, adjust pH to 5.5 with KOH. PDA sucrose: 3.9 g PDA + 17.12 g Sucrose per 100 mL; Hygromycin final 250 mg/L.

Digestion and ligation for over-expressors

A double digestion of the gene construct and of the pJMB1 vector was conducted using the same enzymes to create "sticky ends" for integration. A 50 µL reaction for a double digestion of the gene construct consisted of 9 µg of the DNA template, 1 µL of each chosen restriction enzyme, 5 µL of the corresponding buffer, 5 µL BSA if needed, and up to 50 µL distilled water. A 50 µL reaction for digestion of the pJMB1 vector consisted of 3 µg of the vector, 1 µL of each of the same restriction enzymes, 5 µL of the corresponding buffer, 5 µL BSA if needed, and up to 50 µL distilled water. The digestions were incubated at 37° C overnight. For insertion of the construct into the vector, a ligation reaction was set up consisting of a 3:1 molar ratio of insertion to vector. The reaction consisted of 1 µL T4 DNA ligase, 5 µL 2X DNA Ligase Buffer, 3:1 molar ratio of insert to pJMB1, and up to 10 µL distilled water. The reaction was incubated at 16° C overnight. The ligated vector was then transformed into *E.coli.*

***E. coli* transformation**

The vector for the over-expressing mutants was transformed into *E.coli*. The strain of *E. coli* used was the XL-1 strain of competent cells. The method of transformation of the competent cells was heat shock. The transformation reaction consisted of 10 µL ligation and 90 µL

competent cells, mixed slowly twice with a pipette. The solution was on ice for 20 minutes, then 45 seconds at 45° C, and then 2 minutes on ice. To each tube, 1 mL of LB is added then the tubes are incubated at 37° C for 1 hour with shaking. Prepare 2 plates of LB + Ampicillin per each sample. For each sample, pour 35 mL melted LB agar into a falcon tube then add 35 µL ampicillin and invert to mix. Half the solution was poured into each plate. 500 µL of the supernatant was removed then the pellet was partially resuspended and100 µL was spread on 1 plate to be less concentrated. The pellet was fully resuspended and 100 µL spread on the other plate. Plates were incubated at 37° C overnight.

Southern blot analysis

Genomic DNA from wild type and positive transformants was digested with a chosen restriction enzyme. 10 µg of extracted DNA were digested with 1.5 µL restriction enzyme and appropriate buffer and/or BSA to be used in the Southern blot. The DNA was digested overnight at 37° C. The DNA was precipitated using 2.5X total volume of ethanol (750 µL) and 0.1X total volume of salts (30 µL). The solution was inverted to mix and incubated at -20° C. The DNA was then centrifuged at 12000 rpm for 10 minutes at 4° C, decanted and the pellet washed with 600 µL 70% ethanol. The DNA was then centrifuged again at 12000 rpm for 5 minutes at 4° C, dried and resuspended in 20 µL of distilled water. The digested DNA was run out on a gel of 0.8% agarose by electrophoresis. After electrophoresis, the DNA is denatured by soaking for 30 minutes in a solution of 1.5 M NaCl and 0.5 M NaOH. The gel is then rinsed with distilled water and then neutralized in a solution of 1 M Tris-HCl, 1.5 M NaCl for 30 minutes. Two pieces of 3 MM paper soaked in 10X SSC are placed over a support in a container with more 10X SSC. The bottom paper should be long enough to touch on either side to act as a capillary action. The gel is

placed on top of the papers and then the membrane soaked in 10X SSC on top of the gel. Two more pieces of 3 MM paper soaked in 10X SSC are placed on top of the membrane. Pour in more 10X SSC buffer in the container. A large stack of paper towels are placed on top and covered with saran wrap to limit evaporation. A weight is added on top overnight. The buffer will flow through the papers and gel and the membrane to the paper towels on top by capillary action. After the transfer is complete, the DNA is crosslinked to the membrane using Stratalinker to facilitate hybridization. Before adding the P32-dCTP labeled probe, the blot is incubated at 42 C rotating for 30 minutes with buffer to block unwanted DNA. The probe is prepared following Takara Random Primer DNA Labeling protocol. Add the probe to the tube with the blot and incubate overnight rotating at 42 C. The blot was washed of any excess probe and then viewed by autoradiography.

Northern blot analysis

Wild-type and mutant RNA was extracted after grown in VMS (1.5% sucrose) for 3 days with shaking at 150 rpm at room temperature and assayed by a northern blot analysis. The northern gel consisted of 1% agarose (0.3 g), 25.25 mL DEPC water, 3 mL 10X MOPS, and 1.75 mL formaldehyde. The samples were added to a master mix consisting of 2.5 µL 10X MOPS, 4.58 µL formaldehyde, and 12.5 µL formamide; 5 µL of RNA added to 20 µL master mix and incubated at 65 C for 10 minutes then on ice for 2 minutes. The gel was run by electrophoresis in a 1X MOPS buffer and then blotted onto a membrane by the same blot process order as mentioned in the Southern Blot protocols except the buffer solution was 20X SSC MOPS. After the transfer of the RNA to the membrane, the membrane is auto-crosslinked to facilitate hybridization so the probe can label the RNA. The protocols for the Takara Random Primer

DNA Labeling are followed except using the DNA probe for the 93159 and a histone probe (H3) as a control.

Screening of mutants by colony PCR

Screening of colonies for the ectopic copy of the gene was performed by colony PCR. The reaction consisted of: 2.5 µL 10X ThermoPol Buffer; 0.5 µL 2 mM dNTPs; 0.4 µL T7 primer (forward); 0.4 µL left flank reverse primer; 0.25 taq DNA polymerase; and up to 25 µL distilled water. Selected colonies were picked and mixed in the PCR master mix. The parameters of the reaction were: 94° C priming for 2 minutes; then 35 cycles of 94° C strand separation for 30 seconds, 52° C annealing for 30 seconds, 72° C elongation for 1 minute; then a hold at 72° C for 10 minutes and a final hold at 4° C. The PCR samples were run through a gel by electrophoresis and check for the correct sized of insertion. The transformed colonies were grown in LB medium containing ampicillin for antibiotic selection overnight at 37° C. Purification of the plasmid was performed using the Promega Miniprep system kit according to the manufacture's instructions and streaked on LB supplemented with ampicillin for further selection.

Sequencing

Positive transformants were sequenced to check that the insertion was in the right place. To prepare the samples for sequencing, a sequencing PCR was performed. The reaction consisted of 2 µL Big Dye, up to 200 ng of colony PCR template, 1.5 µL of each primer (forward and reverse), up to 6 µL of distilled water. The parameters of the reaction were 96°C for 2 minutes;

30 cycles of 96°C for 30 seconds, 54°C for 15 seconds, 60°C for 4 minutes; and a final hold at 4°C.

Protein precipitation

To obtain the culture filtrate, 1 X 10^6 cells/mL of *T. virens* wild-type and over-expression mutants were grown in 50 mL GYEC for 3 days with shaking at 150 rpm. Samples were collected and precipitated with 80% ammonium sulfate at 4°C. The protein was equilibrated for 10 minutest at 4°C. The proteins were centrifuged at 10,000 rpm for 10 minutes at 4°C and the pellet resuspended in 1 mL 20 mM ammonium bicarbonate. The samples were dialyzed against the same 20 mM ammonium bicarbonate buffer for 2 days.

SDS-PAGE and Western blot analysis

Fungal tissue and culture filtrate of wild-type and mutants were obtained by inoculating 50 mL VMS with a conididal suspension of 10^6 conidia/mL and shook at 150 rpm at room temperature for 3 days. The proteins in the culture filtrate were filtered from the tissue and precipitated as mentioned above The resuspended pellet was dialyzed against the same buffer of NH_4HCO_3. The extracted proteins were analyzed by SDS-PAGE following the BioRad Manual and stained with Coomassie Brilliant Blue for protein visualization. The 8% resolving gel consisted of 3.8 mL Tris (pH 8.8), 2.7 mL 30% Acrylamide, 3.3 mL distilled water, 100 µL 10% SDS, 100 µL 10% APS, 3 µL TEMED. The stacking gel consisted of 500 µL Tris (pH 6.8), 600 µL 30% Acrylamide, 2.7 mL distilled water, 40 µL 10% SDS, 100 µL 10% APS, and 3.2 µL TEMED. The gel was run in a 1X Running Buffer consisting of 250 mL of 4X SDS-PAGE Running

Buffer, 750 mL distilled water, and 10 mL 10% SDS. One gel was stained with Coomassie Blue overnight then washed with destaining solution and distilled water. For the western blot, one resolving gel was washed to remove salts and SDS. The buffer was a 1X working electrolyte solution. The blot was run at 260 amps for 1 hour. The membrane was placed in a container with dry milk + TBS-T as the blocking reagent and the primary antibody, anti-His (in rabbit) and incubated at 4°C overnight. The blot was washed with 20 mL TBS-t twice for 15 minutes. The secondary antibody (goat anti-rabbit) was added to the blot for 1 hour then the blot was washed twice with 12 mL of TBS-t. The protocols in the Thermo Scientific Chemoimmunoiluminescent kit were followed for chemiluminescent detection for visualization.

Expression assay

Expression assays of the three genes were analyzed when *T. virens* 29-8 was grown in Vogel's minimum media (VM) supplemented with different carbon sources. Conidial suspensions inoculated 8 flasks 50 mL of VM with 1% lignin, and cell walls of 0.5% *R. solani*, 0.5% *P. ultimum*, 0.5% *B. cinerea*, 1% colloidal chitin, 0.2% maize roots, 1.5% sucrose or no supplement. The cultures were grown for 48 hours with shaking at 150 rpm at room temperature before the tissue was collected for expression analysis.

Dual confrontation

T. virens 29-8 was grown opposite pathogens *B. cinerea, P. ultimum,, R. solani* on VMS agar plates overlaid with cellophane. Plugs from an actively growing colony on VMS were placed on one edge of a fresh VMS plate and allowed to grow for 24 hours. The growing culture was

confronted with a plug from one of the plant pathogens and allowed to grow for 3 days. Expression analysis was performed after the 3 days by collecting the tissue in contact with the pathogen for RNA extraction and analysis by RT-PCR. *T. virens* 29-8 genomic DNA was used as a positive control and histone H3 was used as a loading control.

Expression analysis by microarrays

Expression of the three genes in the presence of roots was assayed by growing *T. virens* hydroponically with tomato roots, maize roots, or no roots. Maize and tomato seeds were surface disinfected with 70% ethanol for 5 minutes, then 2 hours in 10% hydrogen peroxide. The disinfected seeds were incubated in a petri dish on a damp paper towel for 3 days for the seeds to germinate. The seeds were placed in a hydroponic chamber containing 0.5X Murashige and Skoog with Gamborg's Vitamins (MS), 0.5% sucrose and grown for 3 days. Fungal tissue was collected after being grown in 100 mL VMS with $3X10^7$ conidia/mL with shaking at 135 rpm for 16 hours. The seeds were then grown for 2 days with shaking at 25 rpm after inoculating the hydropnic chambers with 1 gram of fungal tissue. The roots and associated fungal hyphae were washed with distilled water and then ground up in liquid nitrogen for RNA extraction and analysis of expression.

CHAPTER III

RESULTS AND DISCUSSION

Wild-type gene analysis

Analysis of selected proteins by BLAST search to include significant properties was performed. All three proteins contain a N-terminal signal peptide that is cleaved. The properties are shown in Table 5. The properties actually presented the differences in the sizes of ORFs, isoelectric points, molecular weights and number of exons; while the signal peptide is around the same size.

Table 5. Significant Gene Properties

Protein ID	Gene/ORF	Signal Peptide	M.W. (D)	# of Exons	pI
93159	342 bp	Cleaves between 16/17	11830.52	1	7.48
79522	537 bp	Cleaves between 16/17	17970.76	3	4.53
110650	600 bp	Cleaves between 17/18	22414.26	2	6.10

Sequence analysis

Protein Alignment:

Trichoderma virens wild type protein sequences for 93159, 79522, and 110650 were compared to sequences in other species that produced significant alignments. From the BLAST search, species with percentages of max identification of 60% or greater were used in the alignment. The alignments were made using ClustalX software. The protein alignment shows the greatest homology to other *Trichoderma* species' proteins. Among the *Trichoderma* species, there are

many conserved regions within the sequence. The protein alignment for 93159 is in Figure 3, the protein alignment for 79522 is in Figure 4, and the protein alignment for 110650 is in Figure 5.

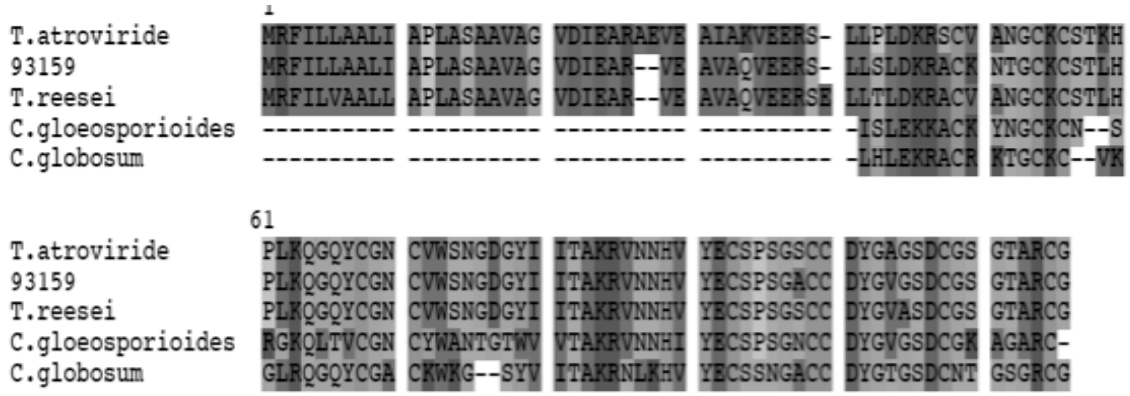

Figure 6. Protein Alignment for *93159*. The protein sequence for 93159 was compared to other sequences in *Trichoderma* species and the two plant pathogens of *C. gloeosporioides* and *C. globosum* to observe conserved regions across species.

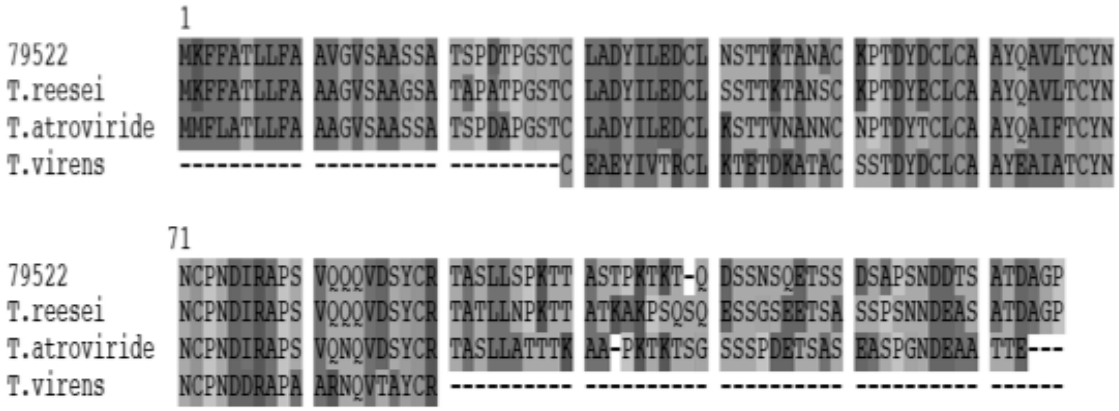

Figure 7. Protein Alignments for *79522*. The protein sequence for 79522 was compared to other sequences in *Trichoderma* species to observe conserved regions.

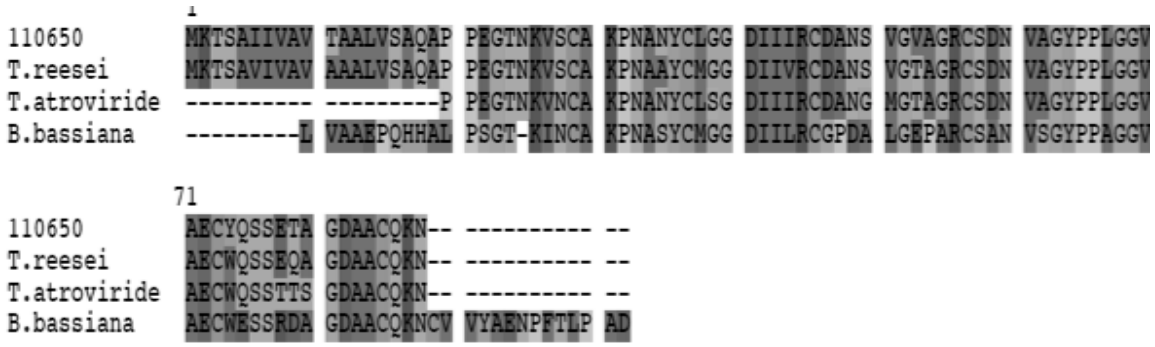

Figure 8. Protein Alignment for *110650*. The protein sequence for 110650 was compared to other sequences in *Trichoderma* species and the insect/arthropod pathogen *B. bassiana* to observe conserved regions.

Protein Phylogeny:

A BLAST search was conducted to find sequences with similar homology to the protein sequences of 79522, 93159, and 110650 among other species of ascomycetes. The protein alignments in Figures 6-8 were used to construct phylogenetic trees.

The phylogenetic trees depict the evolutionary relationship between the protein sequences across the different species. The protein 79522 was compared to the other genes in the ascomycetes: *C. higginsianum, G. graminicola, N. haematococca, F. oxysporum, G. zeae, B. bassiana, C. militaris, T. virens 48810, M. anisopliae, T. atroviride, and T. reesei* in Figure 9. The gene for 79522 was closest to other *Trichoderma* species with the greatest homology to *T. reesei.* The gene *79522* showed the least homology to the plant pathogens group containing *C. higginsianum* through *G. zeae*.

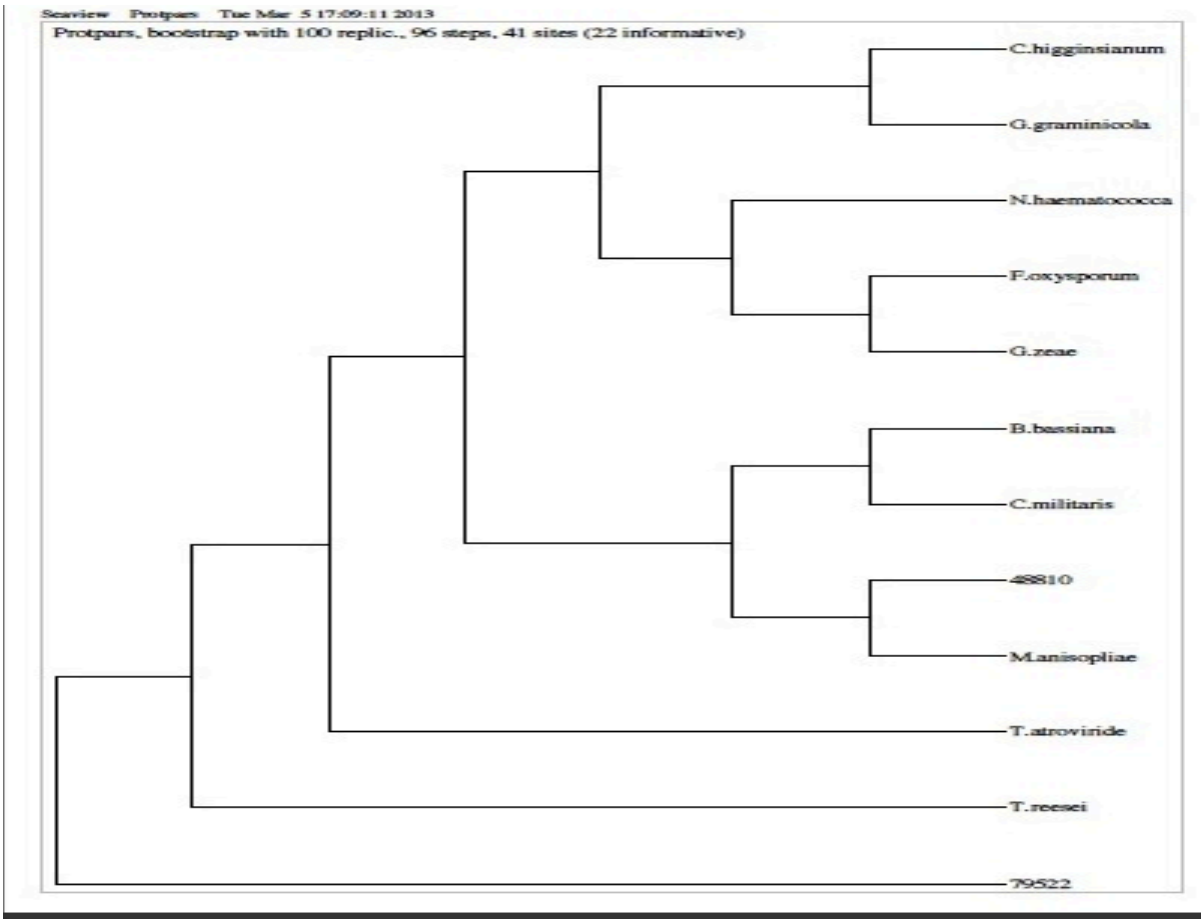

Figure 9. Parsimonious Tree for *79522*. A phylogenetic tree depicts the evolutionary relationship between the gene *79522* to genes in other fungal species.

The gene *93159* was compared to other genes in the ascomycetes: *C. higginsianum, G. graminicola, C. gloeosporioides, V. albo-atrum, V. dahliae, C. sativus, C. globosum, T. atroviride,* and *T. reesei* in Figure 10. The gene showed is evolutionarily closer to other *Trichoderma species* and evolutionarily further from the plant pathogens group containing *C. higginsianum* through *C. sativus*. Interestingly, the gene *93159* is closer to a sequence in C. *globosum,* a pathogen to humans, than to the sequence found in another *Trichoderma* species, *Trichoderma reesei*.

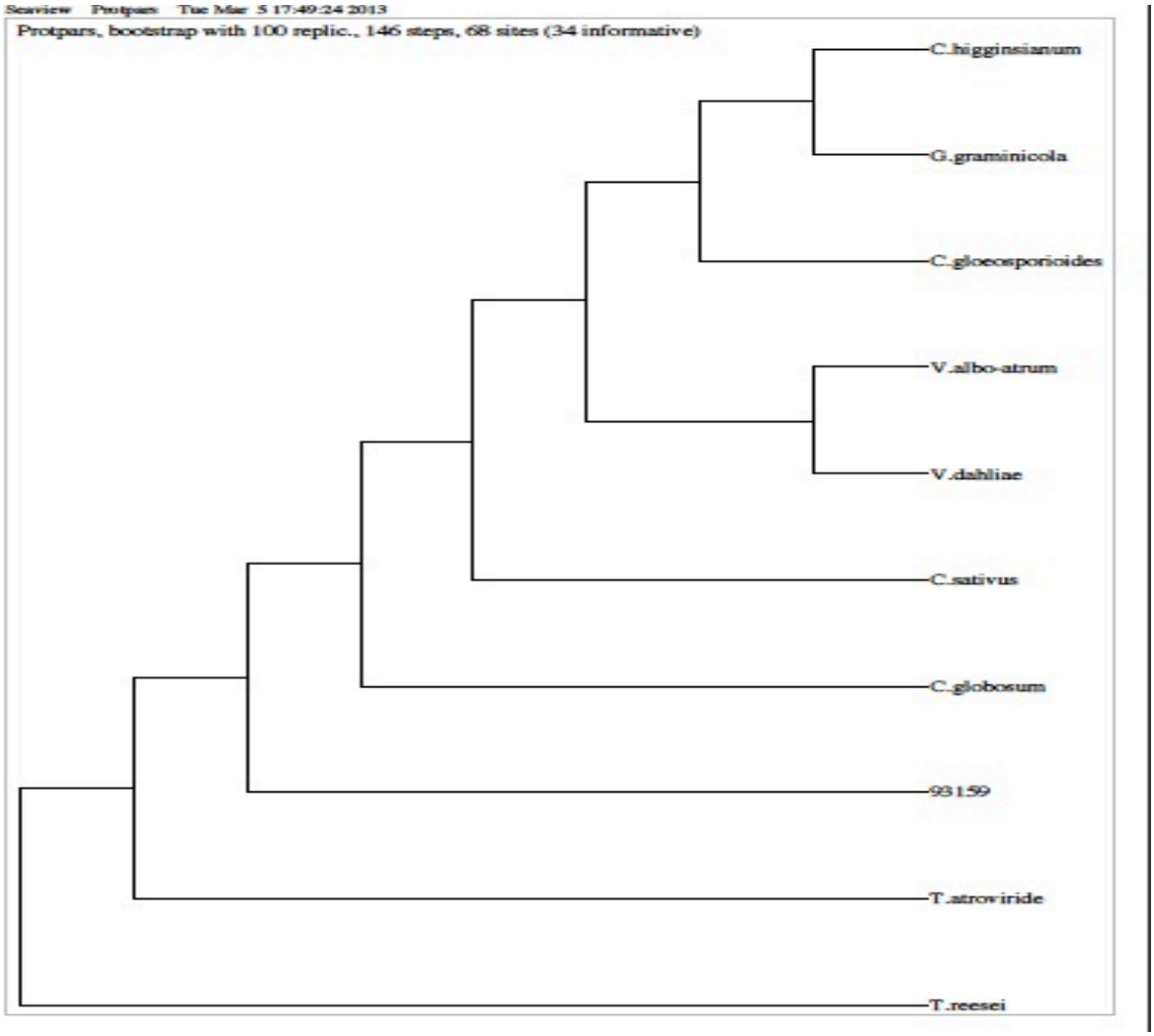

Figure 10. Parsimonious Tree for *93159*. A phylogenetic tree depicting the evolutionary relationship between the gene *93159* and genes in other fungal species.

The gene *110650* was compared to other genes in the ascomycetes: *T. reesei, T. atroviride, B. bassiana, C. militaris, M. acridum, G. clavigera, T. terrestris, G. graminicola, C. higginsianum, V. dahliae,* and *V. albo-atrum* in Figure 11. Protein *110650* is the least evolutionarily related to the plant pathogens group of *G. graminicola* through *V. albo-atrum* and is closely related to the other *Trichoderma* species. *B. bassiana, C. militaris, and M. acridum* are all pathogens to insects and other arthropods, and *B. bassiana* is even used as an insecticide.

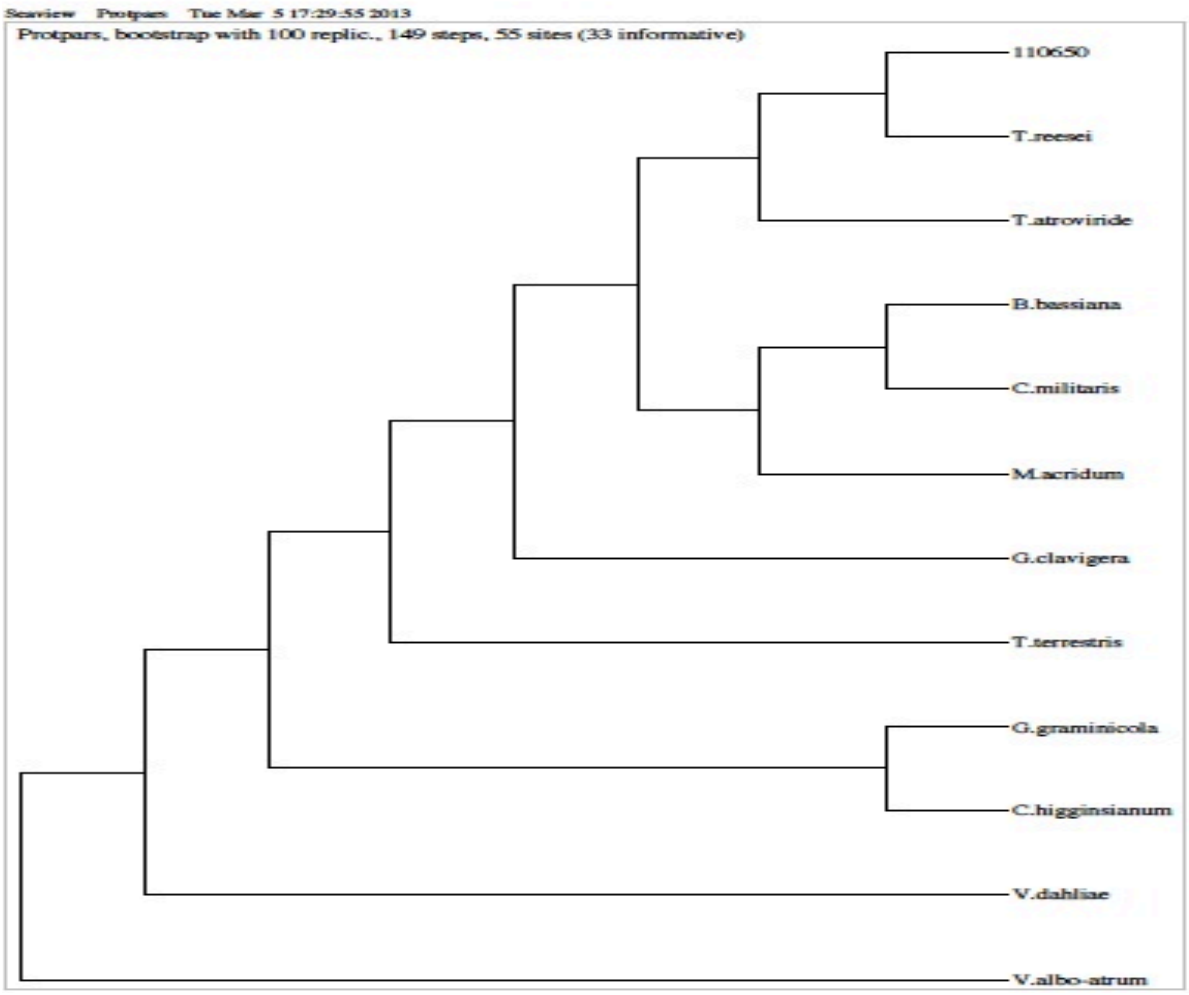

Figure 11. Parsimonious Tree for *110650*. A phylogenetic tree displaying the evolutionary relationship between the gene *110650* and genes in other fungal species.

Expression of genes in the presence of roots

Up regulated expression of the three genes was detected by microarray analysis when grown hydroponically in the presence of tomato roots or maize roots, compared to grown without roots; three biological replicates were used for each condition. The increase in regulation of the three genes in the presence of roots is shown in Table 6.

Table 6. Up Regulation of Genes in Presence of Roots

Protein ID	T1	T2	T3	M1	M2	M3	Tv1	Tv2	Tv3
93159	59920.61	53608.85	13653.09	18022.93	15106.21	13557.88	1801.973	1650.346	17057.12
79522	66109.89	69809	15791.6	17586.78	23502.14	18928.97	43962.07	38963.24	6859.063
110650	26770.83	29113.35	85342.84	79796.12	86615.81	90726.75	107470.9	115275.9	81944.23

Microarray analysis of gene expression in the presence of tomato roots (T), maize roots (M) or no roots (Tv) when wild type *T.* virens was grown hydroponically.

Expression of genes in wild-type 29-8

Expression analysis was performed on the three genes by RT-PCR after grown in VM supplemented with different carbon sources: lignin (1), 0.5% *R. solani* (2), 0.5% *P. ultimum* (3), 0.5% *B. cinerea* (4), 1% colloidal chitin (5), 0.2% maize roots (6), no supplement to VM (7), 1.5% sucrose (8). Wild type gDNA was used as a positive control (9) and H3 was used as a loading control in Figure 12. The genes for 93159 and 110650 were expressed in response to the presence of lignin, plant pathogens (2-4) and the different carbon sources. Expression of the three genes during dual confrontation was analyzed in *T. virens* 29-8 against *T. virens* 29-8 (1), *B. cinerea* (2), *P. ultimum* (3), *R. solani* (4) by RT-PCR. *T. virens* 29-8 gDNA was used as a positive control (5) and H3 was used as a loading control in Figure 13. Expression was observed in 110650 against all the pathogens and expression was observed for most of the pathogens in 79522. The expression in response to plant pathogens is like that of the expression of Sm1, the known elicitor or ISR, in response to plant pathogens.

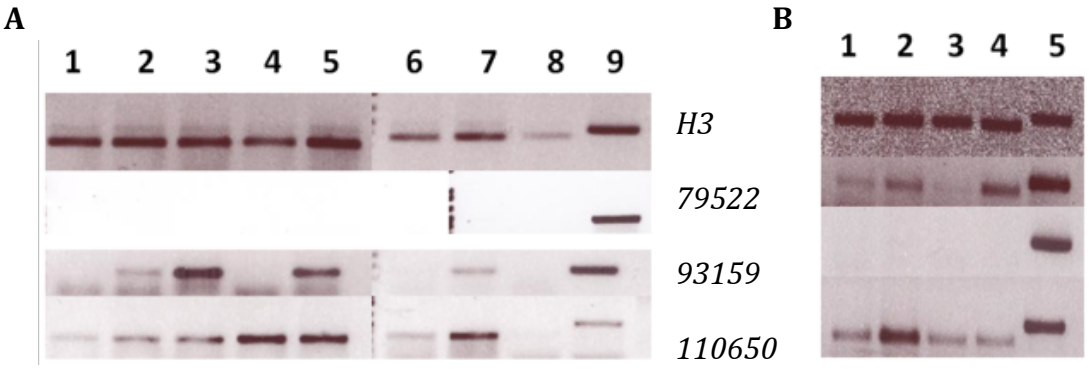

Figure 12. & Figure 13. Expression Analysis. A) Microarray analysis of expression of the three genes in response to different carbon sources: 1)lignin, 2)0.5% *R. solani*, 3)0.5% *P. ultimum*, 4)0.5% *B. cinerea*, 5)1% colloidal chitin, 6)0.2% maize roots, 7)no supplement, 8)1.5% sucrose, 9)*T. virens* 29-8 gDNA was used as a control **B)** Expression of the three genes during dual confrontation of *T. virens* 29-8 against: 1)*T. virens* 29-8. 2)*B/ cinerea*, 3)*P. ultimum*, 4)*R. solani, 5)T. virens* 29-8 gDNA was used as control

Screening of OE mutants by PCR, Southern, Northern, and SDS-PAGE

Screening by PCR

Potential transformants were screened by PCR for the presence of the vector using Gpd forward primer and 93159 reverse primer. Positive transformants were identified as having the ectopic copy of the gene. Transformants 16, 17, 18, 19, 25, 30, 35, 36, 37, 39, 40, 47, and 50 were all positive.

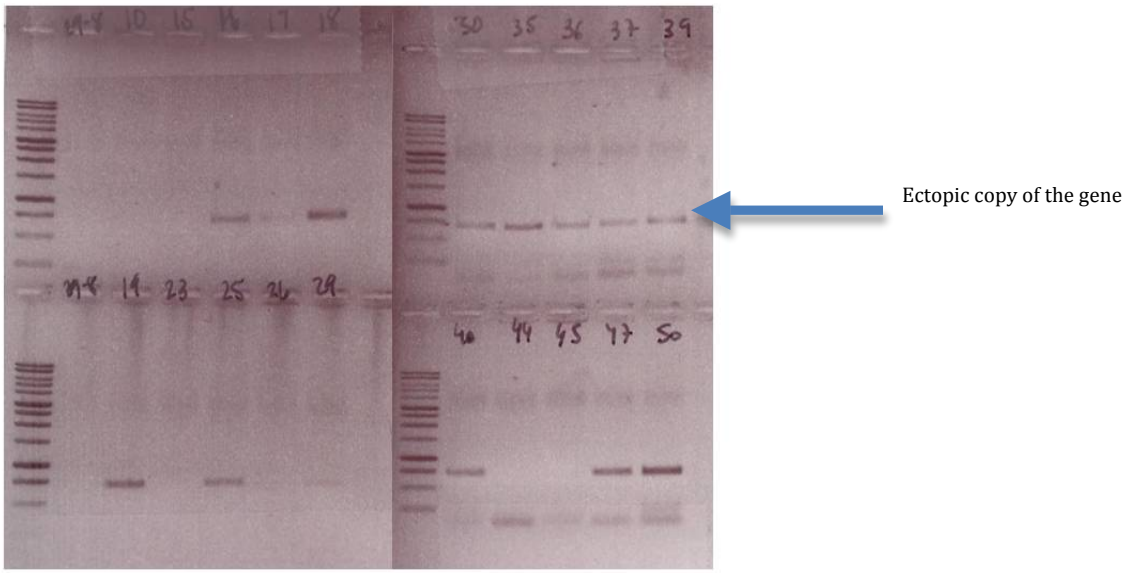

Figure 14. PCR Screening of OE Mutants. Positive transformants confirmed by the presence of a band for the ectopic copy of the gene

Southern Blot:

A Southern Blot assay was performed to screen for the insertion of the ectopic gene by digestion of extracted 93159 OE DNA with PvuII. Of the seven potential over-expressing mutants assayed, number 9 and number 25 showed over-expression of the gene *93159*. The ectopic gene in vector pJMB1 is under control of the constitutive promoter for gpd so the gene for 93159 is constitutively transcribed when the ectopic copy is present making it over-expressed.

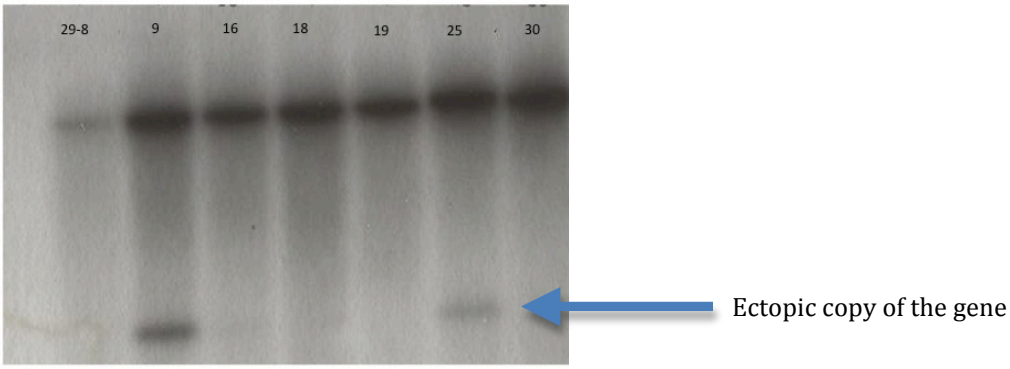

Figure 15. Southern of OE Mutants. Southern blot analysis of transformants for ectopic gene *93159*

Northern Blot:

A Northern Blot was performed for the same over-expressing mutants for 93159 RNA. The Northern Blot assay confirmed the over-expression of the mRNA in numbers 9 and 25. The bands corresponding to numbers 9 and 25 show greater strength than wild type, depicting higher expression of the RNA.

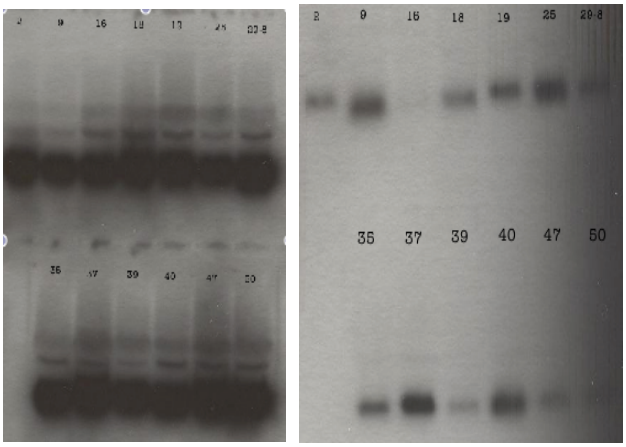

Figure 16. Northern of OE Mutants. The left blot was labeled with the H3 probe; the right blot was labeled with 93159 probe. The Northern confirms the over-expression of 93159 because the RNA was over-expressed in samples 9, 25, 37, and 40.

SDS-PAGE & Western Blot:
The SDS-PAGE was performed on extracellular proteins to assay for the presence and over-expression of 93159. The SDS-PAGE and Western blot analysis were negative under these conditions for the presence of 93159. Figure 17 displays the SDS-PAGE for the extracellular proteins.

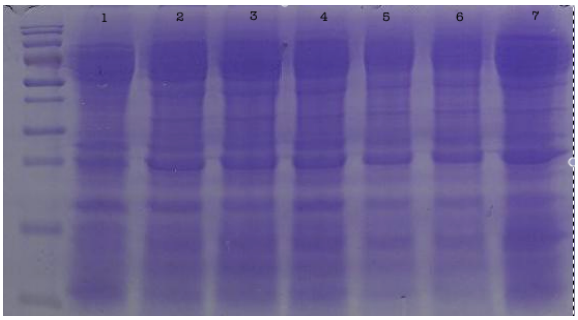

Figure 17. Western Blot of Extracellular Proteins
1)wild-type 2-7)OE mutants extracellular proteins

Knock-out expression

Potential knock out mutants were screened by PCR using the designed left-forward primer and the terminator of the gene to screen for wild-type. The expected size of the band for wild-type should be around 1.2 kb and no band for mutants. The results of the PCR screening, in Figure 18, show wild-type expression in most of the mutants, so further selection will have to be done

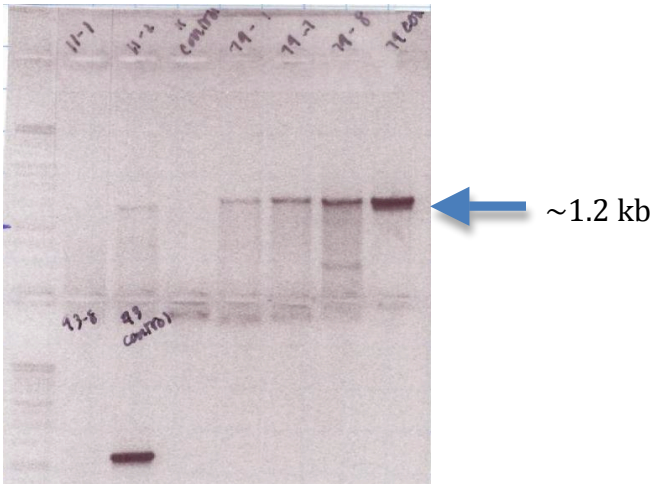

Figure 18. PCR Screening using LF and Terminator of Gene. PCR screening of KO and wild-type of each gene yielded wild-type expression in KO 11-2, 79-1, 79-7, and 79-8

RNA was extracted from wild-type and knock out tissues grown in PDB AND VMS and checked for quality before being used in RT-PCR. The gel for checking the quality of the RNA is in Figure 19, and the screening by PCR is in Figures 20 and 21. The quality of the RNA is good because you can see both the 28s and 18s subunits of the RNA.

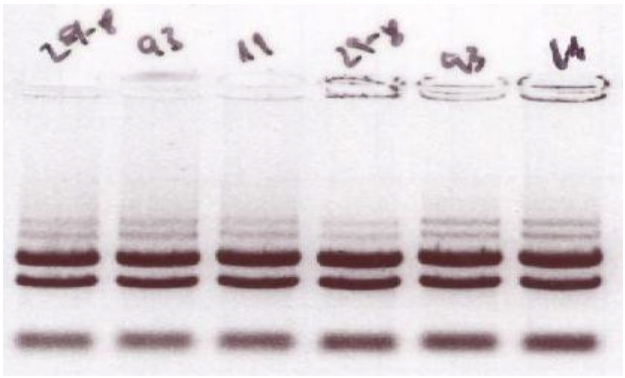

Figure 19. Extracted RNA from KO and Wild-type tissues grown in PDB and VMS
1-3)RNA extracted from tissue grown in PDB
4-6)RNA extracted from tissue grown in VMS

The quality RNA was used for a RT-PCR reaction to generate cDNA. The cDNA was used for screening by PCR using the forward and reverse primers of genes *93159* and *110650.* A histone (H3) was and wild-type gDNA were used as controls. Figure 20 is the results of the PCR reaction using H3. Figure 21 is the results of screening using the forward and reverse primers of each gene. The screening yielded wild-type represented by the presence of a band, so further selection and screening will be needed.

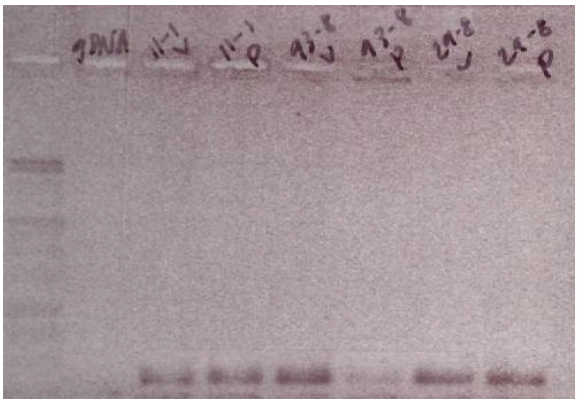

Figure 20. Screening of KO by PCR with H3.
H3 was used as a positive control.

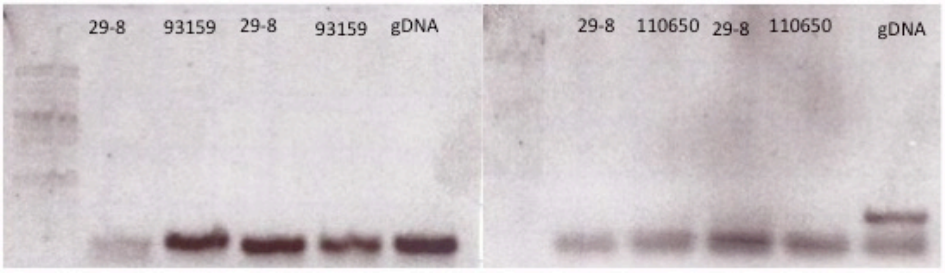

Figure 21. Screening of KO by PCR with Gene Primers
PCR screening of *93159* and *110650* in VMS (2 & 3) and PDB (4 & 5) for each gene.

CHAPTER IV

CONCLUSION

The knock out mutants still show expression of wild-type and will have to undergo further selection on water agar supplemented with hygromycin before further experiments can be performed to test the elicitor capabilities of the knock out mutants. The over-expressing mutants were confirmed by the PCR screening, Southern blot, and Northern blot but the protein 93159 could not be found in the Western of extracellular proteins in the conditions used. The intracellular proteins are undergoing screening for confirmation of the protein. The protein's presence will be confirmed before continuing to test the elicitor capabilities of the over-expressing mutants.

REFERENCES

1. Buensanteai, Natthiya, Prasun K. Mukherjee, Benjamin A. Horwitz, Cheng Cheng, Lawrence J. Dangott, and Charles M. Kenerley. "Expression and Purification of Biologically Active Trichoderma Virens Proteinaceous Elicitor Sm1 in Pichia Pastoris." *Protein Expression and Purification* 72.1 (2010): 131-38. Print.

2. Djonovic, S., W. A. Vargas, M. V. Kolomiets, M. Horndeski, A. Wiest, and C. M. Kenerley. "A Proteinaceous Elicitor Sm1 from the Beneficial Fungus Trichoderma Virens Is Required for Induced Systemic Resistance in Maize." *Plant Physiology* 145.3 (2007): 875-89. Print.

3. Yu, Jae-Hyuk, Zsuzsanna Hamari, Kap-Hoon Han, Jeong-Ah Seo, Yazmid Reyes-Domínguez, and Claudio Scazzocchio. "Double-joint PCR: A PCR-based Molecular Tool for Gene Manipulations in Filamentous Fungi." *Fungal Genetics and Biology* 41.11 (2004): 973-81. Print.

4. Invitrogen. 2010. *Multi-Copy Pichia Expression Kit: For the Isolation and Expression of Recombinant Proteins from Pichia pastoris Strains Containing Multiple Copies of a Particular Gene.* Invitrogen Corporation, Carlsbad, CA.

5. The Genome Portal of the Department of Energy Joint Genome Institute I. V. Grigoriev, H. Nordberg, I. Shabalov, A. Aerts, M. Cantor, D. Goodstein, A. Kuo, S. Minovitsky, R. Nikitin, R. A. Ohm, R. Otillar, A. Poliakov, I. Ratnere, R. Riley, T. Smirnova, D. Rokhsar, and I. Dubchak. **Nucleic Acids Res** 2011 0: gkr947v1-gkr947

6. Vittone, Gloria. *Genetic and Functional Analysis of Siderophores in Trichoderma Virens*. Thesis. Texas A&M University, 2008. College Station: Texas A&M University, 2008. Print.

7. Harman GE, Howell CR, Viterbo A, Chet I, Lorito M (2004a) *Trichoderma* species—opportunistic, avirulent plant symbionts. Nat Rev Microbiol **2:**43-56

8. Wilhite, S. E., R. D. Lumsden, and D. C. Straney. "Peptide Synthetase Gene in Trichoderma Virens." *Applied and Environmental Microbiology* 67.11 (2001): 5055-062. Print.

9. Harman, G.E. 2011. *Trichoderma*—not just for biocontrol anymore. Phytoparasitica 39:103-108.

10. Djonovic S, Pozo MJ, Dangott LJ, Howell CR, Kenerley CM (2006a) Sm1, a proteinaceous elicitor secreted by the biocontrol fungus *Trichoderma virens* induces plant defense responses and systemic resistance. Mol Plant Microbe Interact 19: 838-853

11. Idit Kosti, Prasun K. Mukherjee, Mala Mukherjee, Fabian Glaser, Naomi Trushina, Yael Mandel-Gutfreund, Benjamin A Horwitz, Charles M. Kenerley. *Small secreted proteins of three Trichoderma species: candidate effectors for interaction in the rhizosphere.*

12. Crutcher, Frankie (2011). An Investigation of Two Modes of Plant Protection by the Biocontrol Agent Trichoderma virens.Doctoral dissertation, Texas A&M University. Available electronically from http : / /hdl .handle .net /1969 .1 /ETD -TAMU -2011 -12-10535.