FUNCTIONAL AND PHYLOGENETIC ANALYSIS OF EXO-1,3-BETA GLUCANASE GENE (PinsEXO1) FROM THE PATHOGENIC OOMYCETE *PYTHIUM INSIDIOSUM*

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ABSTRACT

Vipaporn Phuntumart, Advisor

Pythium insidiosum is an animal pathogenic oomycete. It is the causative agent of pythiosis, a disease affecting mammals, including humans. An exo-1,3-beta glucanase gene (*PinsEXO1*), encoding a 74-kDa immunoreactive protein recognized by serum samples from patients with pythiosis was identified in all strains of *P. insidiosum* tested. *PinsEXO1* is highly expressed during infection, and is thought to be involved in cell wall remodeling of the oomycete. The gene for *PinsEXO1* was cloned into the pRSET-B plasmid vector and expressed in *E. coli*. An *in vivo* functional analysis of *PinsEXO1* was developed and it was determined through two functional analyses that *PinsEXO1* is capable of hydrolyzing laminarin and beta-glucan. An *in silico* protein domain analysis using the Pfam database identified three domains in *PinsEXO1* and its homologs; BG1C domain, cellulase domain, and X8 domain. In addition, three phylogenetic trees were constructed to investigate the evolutionary relationships of *PinsEXO1* with glucanase enzymes in plant and animal pathogens. One tree was built using the full-length protein sequences, the second was built using the cellulase domain sequences, and the third was built using the BG1C domain sequences. The resulting trees were highly similar to one another. Based on the phylogenetic analysis, *PinsEXO1* and its homologs are highly conserved among oomycetes. Finally, an *in silico* expression analysis using existing RNAseq data was performed on homologs of *PinsEXO1* andshowed that the expression was more highly expressed during mycelial growth as compared to zoospores.

ACKNOWLEDGMENTS

Firstly I would like to thank my advisor, Dr. Vipa Phuntumart, for her mentorship and support while working on this project. Her guidance made the success of this project possible. I would also like to thank my committee members Dr. Paul Morris and Dr. Theerapong Krajaejun for their time, help and advice. A special thanks to Dr. Krajaejun's lab as well, for collaborating with our lab and sharing the *PinsEXO1* gene with us. His student, Angsana Keeratijarut, worked with me early on. I want to thank her for her time, patience and collaboration.

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CHAPTER I: BACKGROUND

Introduction

Pythium insidiosum is a pathogenic organism capable of infecting a variety of mammals including dogs, horses, cattle, cats, and humans. Infection by *P. insidiosum* results in pythiosis, an emerging disease endemic to South Asia (Krajaejun, et al, 2006). The disease can present in a variety of ways including subcutaneous infection, vascular infection, ocular infection, and systemic infection (Vanittanakom, et al, 2004)(Krajaejun, et al, 2006). Vascular infection is the most common form, as well as the second most deadly (Vanittanakom, et al, 2004). In the majority of cases, the only effective treatment is amputation of the infected area. Pythiosis is a rare disease with a high mortality rate, in part due to ineffective treatments and a lack of rapid diagnostic tools.

P. insidiosum is an oomycete; organisms that are morphologically similar to fungi, but are more closely related to diatom algae (Gaastra, et al, 2010). Fungi and oomycetes are similar in that they both develop mycelium and hyphae, and they both contain beta glucans in their cell walls. However, unlike fungi, oomycete cell walls contain very little to no chitin, and they produce biflagellate zoospores, which are crucial for infection of plant and animal tissue (Gaastra, et al, 2010). Zoospores are motile, single cells that engage in chemotaxis and seek out injured tissue to infect. *P. insidiosum* has been shown to be attracted to injured mammalian tissue and hair (Gaastra, et al, 2010).

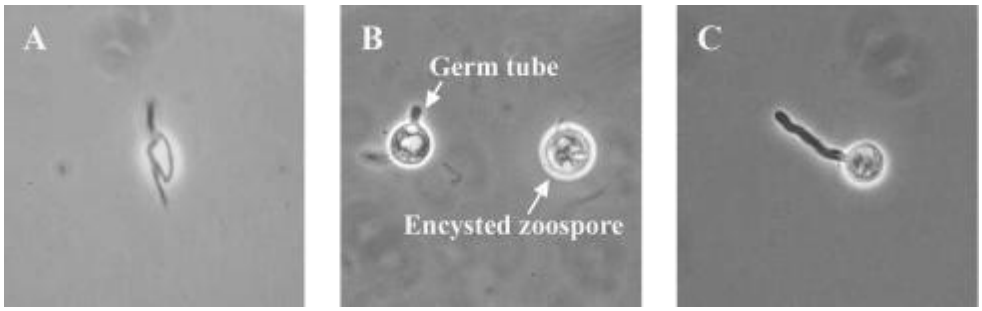


Figure 1: Zoospores of *Pythium insidiosum*. A) A biflagellate zoospore. B) Encysted *P. insidiosum* zoospores. C) A germinating *P. insidiosum* zoospore. (Vanittanakom, et al. 2004).

Infection occurs when a zoospore of *P. insidiosum* invades injured animal tissue, such as an open sore or cut (Gaastra, et al. 2010). Once the zoospore has attached to the tissue, it develops hyphae which further invade the host (Krajaejun, et al, 2006). Treatment options are extremely limited, as traditional antifungals have shown to be inadequate due to the biochemical differences between true fungi and oomycetes (Krajaejun, et al, 2010). The vast majority of patients with vascular, subcutaneous or ocular pythiosis require amputation or removal of the infection site. Systemic pythiosis is always fatal (Krajaejun, et al, 2006). In addition, due to the rarity of the disease, diagnoses are often delayed. Lack of effective treatment and underdiagnosis contribute to the high mortality rate of pythiosis. The development of new treatments and more rapid diagnostic tests are needed to better combat this disease.

When a zoospore of *P. insidiosum* attaches to host tissue, a number of proteins are expressed to aid the pathogen in the invasion of the tissue. It first produces a glycoprotein that allows it to adhere to the tissue, before developing hyphae that further invades the host (Gaastra, et al, 2010). *P. insidiosum* also produces a number of serine proteases during infection, which are hypothesized to weaken tissue strength to allow hyphae to break through (Davis, et al, 2006)(Gaastra, et al, 2010). The host immune system responds to infection by inciting an inflammatory response via the activation of leukocytes such as macrophages and eosinophils (Gaasta, et al, 2010). Antibodies are produced against *P. insidiosum* antigens, which may vary

from host to host (Chindamporn, et al, 2009). Unfortunately, the immune response not only fails to clear the infection, but in fact causes the host's condition to worsen due to damage to surrounding tissue caused by inflammation (Gaasta, et al, 2010).

A team of researchers at Mahidol University in Thailand discovered a previously unknown antigen in the extracts of *P. insidiosum* human isolates (Krajaejun, et al, 2010). The research team that made this discovery identified the antigen as a putative 1,3-exo-B-glucanase and named it *PinsEXO1*. Glucanases, which are classified as glycosyl hydrolases, are enzymes capable of hydrolyzing glucans, polymers of glucose. Further analysis showed that *PinsEXO1* was highly immunoreactive, making it a candidate as a potential vaccine target, anti-microbial drug target, and diagnostic marker (Krajaejun, et al, 2010). Serum tests showed that sera from those infected with pythiosis reacted to *PinsEXO1*, compared to healthy sera that did not recognize the antigen (Krajaejun, et al, 2010). It was also discovered that *PinsEXO1* is expressed near temperatures of 40°C, suggesting that the mammalian body temperature induces expression its expression during infection (Krajaejun, et al, 2010). It remains unclear precisely what role this enzyme plays during infection.

In other oomycetes, glucanase genes are also expressed during infection. In plant pathogens such as *P. infestans*, glucanase is expressed during infection and is thought to aid in the breakdown of plant cell walls, which also contain beta glucans, during tissue invasion (Latijnhouwers, et al, 2003)(McLeod, et al, 2003). It has also been hypothesized that glucanases play a role in altering the morphology of the oomycete cell wall (Krajaejun, et al, 2010). Understanding the exact functions of *PinsEXO1* and why it is expressed during infection of an animal will further research into new, more effective treatments and could support the candidacy of *PinsEXO1* as a potential drug target.

Many oomycete genomes have been sequenced and analyzed, including: plant pathogens *Phytophthora sojae* (Tyler, et al, 2006)*, Phythopthora ramorum* (Tyler, et al, 2006), *Phytophthora infestans* (Haas, et al, 2009), *Pythium ultimum* (Lévesque, et al, 2010), and *Hyaloperonospora arabidopsidis* (Baxter, et al, 2010); and animal pathogen *Saprolegnia parasitica* (Jiang, et al, 2013). *P. sojae*, *P. ramorum* and *P. infestans* are reported to each have a similar, relatively large amount of genes that can be classified as glycosyl hydrolases (Tyler, et al, 2006)(Jiang, et al, 2013). *P. ultimum* also possesses a large number of genes involved in carbohydrate metabolism. In addition, the *P. ultimum* genome contains a large number of genes for serine proteases compared to *Phytophthora spp.* (Lévesque, et al, 2010). *H. arabidopsidis*, on the other hand, contains relatively few glycosyl hydrolases and proteases. This may be attributed to *H. arabidopsidis*' role as an obligate biotroph (Baxter, et al, 2010).

S. parasitica, a pathogen of fish and amphibians, contains fewer glycosyl hydrolase genes compared to plant pathogens, and significantly more serine proteases (Jiang, et al, 2013). Interestingly, although *Pythium* and *Phytophthora* are closely related to one another, *P. ultimum* shares a trait with *Saprolegnia parasitica* in that they both have very few RXLR proteins, which are abundant in *Phytophthora spp.* and heavily expressed during plant infection (Lévesque, et al, 2010)(Jiang, et al, 2013). The differences in the genomes between plant pathogens and *S. parasitica* suggest that, as an animal pathogen, *S. parasitica* has evolved to become more adapted to an animal host by losing genes involved in plant tissue invasion (Jiang, et al, 2013). Whether or not the same is true for *P. insidiosum* is not yet certain.

The Mahidol University researchers were able to clone the *PinsEXO1* gene into the pRSET-B plasmid (Invitrogen, CA) and transformed it intothe JM109 strain of *E. coli* and shared it with Dr. Vipaporn Phuntumart's lab as a collaborative effort to perform a functional analysis.

Attempts to purify *PinsEXO1* resulted in low yield of the enzyme. In this study the development of functional analyses without purifying *PinsEXO1* from the cell cultures was explored. Understanding the function of this enzyme may explain why it is expressed during infection, and what this means for the development of new treatment options.

There are a number of methods available to measuring the hydrolytic capability of glucanase. These include: Spectrophotometric methods, which utilize spectrophotometry to measure the amount of reducing sugars resulting from hydrolysis of the glucan; viscosity methods, which measure the viscosity of the substrate as hydrolysis occurs, and; staining methods, in which the substrate is stained and hydrolytic activity is determined via change in color (Wood 2003).

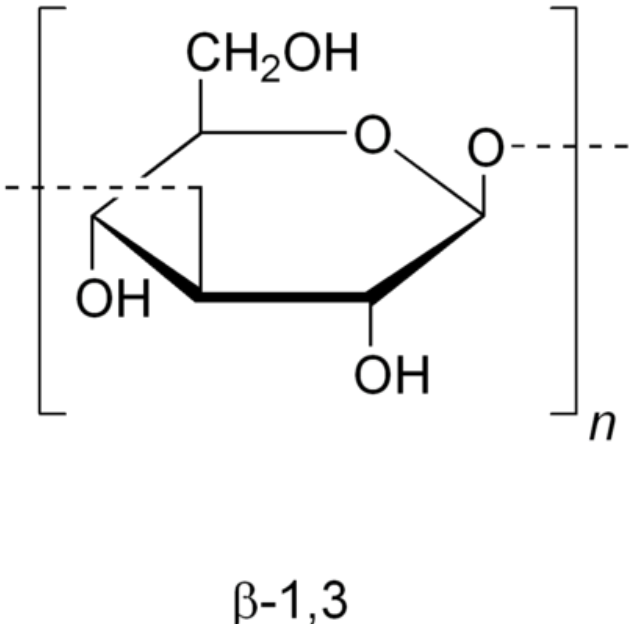


Figure 2: An image of a 1,3 beta glucan. A beta-1,3-glucanase would break the glycosidic bonds at the 1,3 position, resulting in the release of the glucose subunits.

This project used two methods: Spectrophotometric and agar plate staining assays. For the spectrophotometric method, a dinitrosalicylic acid (DNS) assay was performed. Hydrolysis of beta-1,3-glucans by beta-1,3-glucanase results in the production of glucose, which can be bound by dinitrosalicylic acid reagent. This results in the production of a red color that absorbs light strongly at 540-590nm. By measuring the optical density, which would increase as glucans are broken down, the activity of glucanase can be quantified (Sun Wang 2007).

For the staining method, agar plate assays were performed using Congo red dye that binds to long polysaccharide chains in agar media. Polysaccharides that have been hydrolyzed into smaller sugars (such as glucose) are not stained, leaving yellow halos or “clear zones” in areas where enzyme activity has occurred (Wood 2003).

CHAPTER II: EXPERIMENTAL DESIGN AND RESULTS

Hypothesis

PinsEXO1 is capable of hydrolyzing beta-glucans and there is homology between *PinsEXO1* and glucanase genes from plant and animal pathogenic oomycetes.

Goals

This project seeks to achieve the following: 1) Develop an in vivofunctional analysis that can be performed without purifying recombinant glucanase from the *E. coli* cell culture; 2) determine the substrate specificity of *PinsEXO1;* and 3) identify the homologousgenes between *PinsEXO1* and other oomycetes using a phylogenetic analysis.

Materials and Methods

Biological Materials and Growth Conditions

The *Pythium insidiosum* strain (P6) used in this study was isolated from a patient in Nan, Thailand suffering from vascular pythiosis (Krajaejun, et al. 2006). The partial sequence of *PinsEXO1* is listed under the accession number GU994093.1 and NCBI gene ID number 298567133. The full-length protein sequence can be found on Figure 7c.

In order to express the protein in *E. coli* strain JM109, the full-length sequence of *PinsEXO1* was cloned into the pRSET-B plasmid expression vector (Invitrogen, CA)[Figure 3] by the Mahidol researchers. A separate JM109 culture was transformed with the pUC19 plasmid vector (Invitrogen, CA)[Figure 3] in order to act as a negative control in all lab experiments. Cellulase (Sigma-Aldrich, MO) at a concentration of 0.5% was used as a positive control in all experiments.

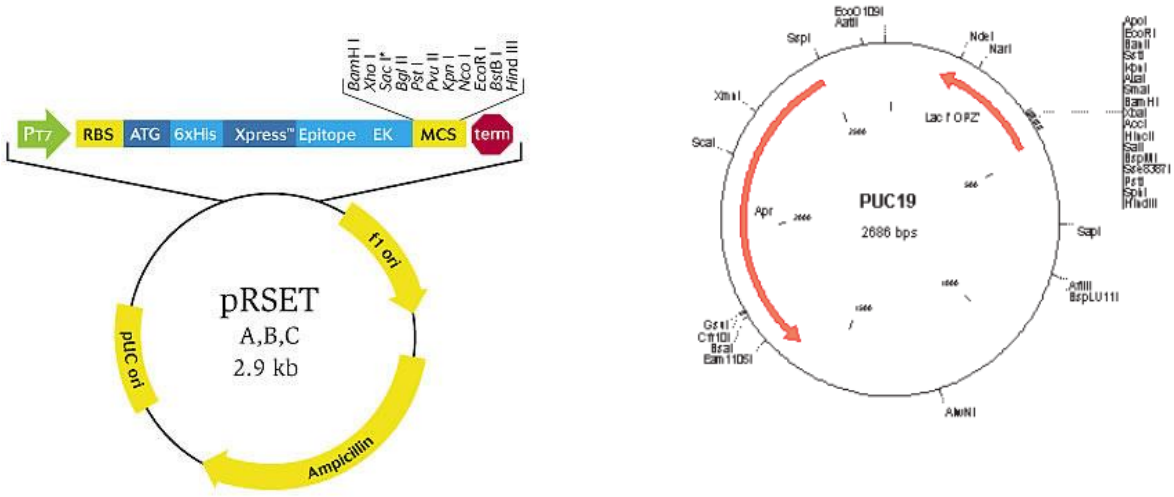


Figure 3: The maps of pRSET (derived from pUC19) and pUC19 plasmid vectors. Both vectors contain the ampicilin resistance gene and the Lac operon, which is inducible by IPTG. pRSET-B contains the *PinsEXO1* gene insert, whereas pUC19 without a gene insertwas used as anemptyvector for negative control (Images by Invitrogen, CA).

JM109 colonies were grown overnight on LB agar plates. A single colony was then inoculated in 5mL of liquid LB media and incubated overnight with shaking at 37°C and 200RPM. Following this, 1mL of the liquid culture was transferred to a flask containing 25mL of LB media and incubated at 37°C and 200RPM until the culture reached an optical density (OD600) of 0.6-0.8. The culture was then induced at that point with 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated for another 3-4 hours until the OD600 reached 2.0.

Functional Analysis

Agar Plate Assay

Unless otherwise stated, all media used in experiments contained ampicilin at a concentration of 100 ug/mL. Five mL of liquid culture of the JM109 culture was centrifuged at

8000RPM for 2 minutes. After disregarding the supernatant, the pellet was resuspended in 1mL fresh media containing 1mM IPTG, bringing the concentration of cells to approximately 7.75x10^7 CFU/mL. Paper discs were then applied with 40uL of the resuspended culture and subsequently placed on 1) LB agar plates containing laminarin (Sigma-Aldrich, MO) at a final concentration of 0.25%, 0.5%, 0.75% and 1.0%; 2) LB agar plates containing beta-glucan (Sigma-Aldrich, MO) at a final concentration of 0.25%, 0.5%, 0.75%, and 1.0%and 3) LB agar plates containing carboxymethylcellulose (CMC, Sigma-Aldrich, MO) at a final concentration of 0.25%, 0.5%, 0.75%, and 1.0%. The plates were then incubated overnight at 37°C. Four replicates were performed.

The following day plates were stained with Congo red (Sigma, MO.) at a final concentration of 0.2% for 30 minutes and subsequently flooded with 10% NaCl (Fisher Scientific, MA.) for another 30 minutes to destain the plates. Clear zone diameters were then measured [Figure 4].

Dinitrosalicylic Acid Assay

10mL of liquid culture at an OD of 2.0 prepared as described above were transferred to a flask containing 20mL of fresh LB media with 0.5% laminarin and 1mM IPTG each. The flask was then incubated at 37°C and 200RPM for 60 minutes. At 15 minute intervals, 1mL of the culture was then taken and centrifuged at 9000RPM for 1 minute. The supernatant was then transferred to a tube containing 1mL of dinitrosalicylic acid (DNS) reagent and heated to 95°C for 10 minutes. The OD was then measured at 562nm and recorded. Three replicates were performed.

A similar experiment was performed using beta-glucan at a concentration of 0.5% with three replicates. A standard glucose curve was also performed prior to experiments.

Phylogenetic Analysis

A full-length amino acid sequence of PinsEXO1 was used as a query sequence to BLAST against various databases including the Broad Institute, NCBI, and Fungidb. A total of 31 protein sequences from oomycete species were then recorded (appendix A). NCBI Conserved Domain and Pfam databases were utilized to identify conserved protein domains. Phylogenetic analysis of *PinsEXO1* was performed to determine the evolutionary relationships between *PinsEXO1* and the other homologous oomycete proteins using the Molecular Evolutionary Genetic Analysis (MEGA) software. Prior to uploading the sequences to MEGA, sequences were aligned using the ClustalW2 software (EMBL-EBI 2015). The Maximum Likelihood method was used to construct the tree with 1000 bootstraps to estimate the accuracy of the tree. Additionally, trees were also built using only the cellulase domain [Figure 10] and only the BG1C domain [Figure 11] for comparison.

Gene Expression Analysis

RNAseq Data Analysis

In order to determine whether or not the glucanase genes collected are expressed, publically available RNAseq data was collected for genes from the following organisms: *Phytophthora ramorum, Phytophthora sojae, Pythium ultimum,* and *Saprolegnia declina.* For *P. ramorum,* RNAseq data was found to be already available on Fungidb (Kasuga, et al).

Data for *Phytophthora sojae,Pythium ultimum* and *Saprolegnia parasitica*was collected via the NCBI SRA database. The resulting data files were then uploaded to Galaxy, generating several Tophat and Cufflinks analysis files. The Tophat data for accepted hits along with the Cufflinks data for assembled transcripts was then uploaded to the Fungidb Genome Browser for visualization. *P. ultimum* RNAseq data was also found to be available on the Michigan State Genome Browser for the *P. ultimum* genome, under a number of different treatment conditions. Specifically, RNAseq data was analyzed for *PYU1_G011796* (identified as glucanase).

Results

Functional Analysis

Agar Plate Assays

Laminarin , beta-glucan and CMC were used to test for substrate specificity of *PinsEXO1*.Significant clear zones formed by the reaction between the *PinsEXO1* enzyme and substrate were developed on laminarin and beta-glucan agar plate assays that had been inoculated with JM109 containing the gene for *PinsEXO1*[Figures 4&5, Table 1]. Substrate concentration did not seem to have a significant effect on clear zone diameters. On assays that had been performed with JM109 containing the empty pUC19 vector, clear zones were either minimal or, more commonly, not present. On assays that had been performed with cellulase enzyme as a positive control, large clear zones were observed. Clear zones were not observed when CMC was used as substrate in all agar plates (data not shown).

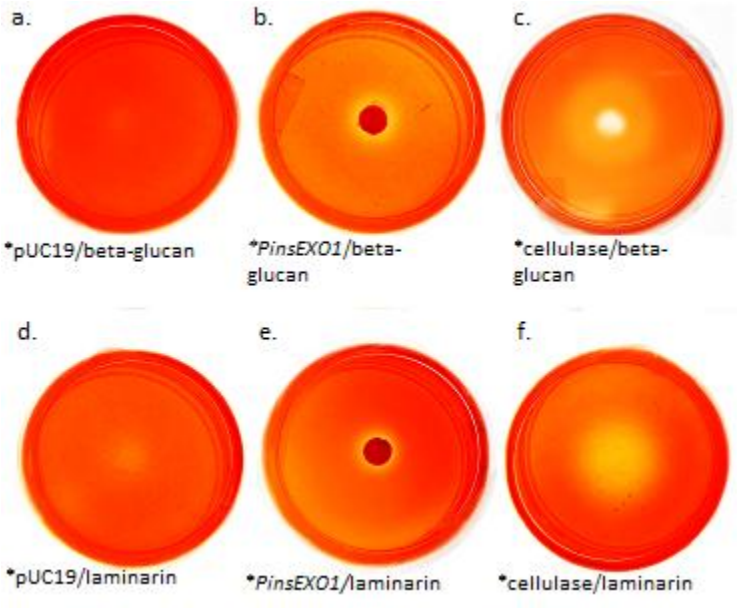


Figure 4: Glucanase agar plate assays stained with Congo red following incubation. The top row displays beta-glucan agar plates inoculated with pUC19 (a), pRSET+PinsEXO1 (b), and cellulase (c). The bottom row displays laminarin agar plates inoculated with pUC19 (d), pRSET+PinsEXO1 (e), and cellulase (f).

Table 1: Measurement of clear zone diameters in millimeters.

	Clear zone diameter		
%Laminarin	pRSET PinsEXO1	pUC19 (-)	Cellulase (+)
0.25%	8mm	0mm	15mm
0.50%	13mm	0mm	20mm
0.75%	10mm	5mm	16mm
1.00%	11mm	4mm	15mm

	Clear zone diameter		
%beta-Glucan	pRSET PinsEXO1	pUC19 (-)	Cellulase (+)
0.25%	12mm	0mm	14mm
0.50%	10mm	0mm	16mm
0.75%	10mm	0mm	17mm
1.00%	12mm	0mm	16mm

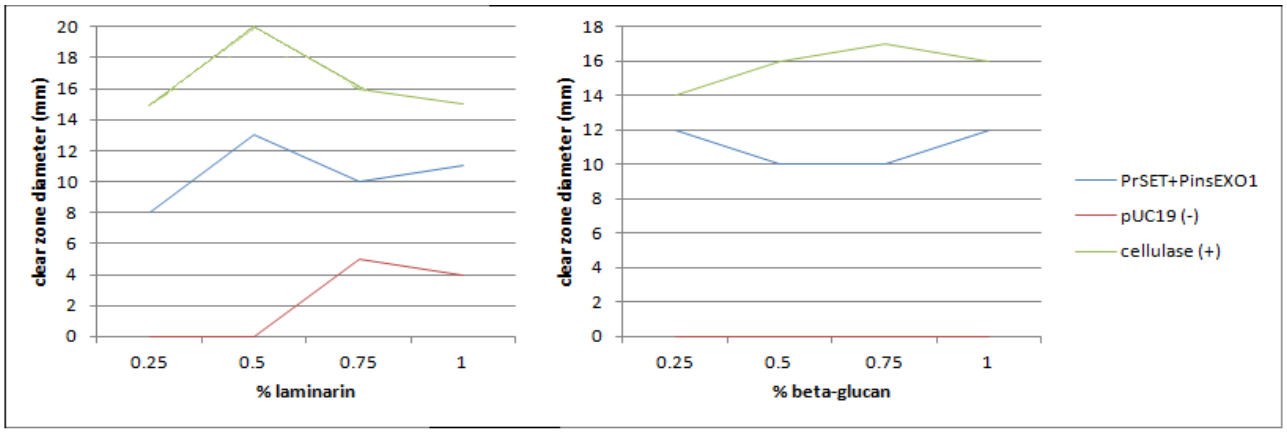
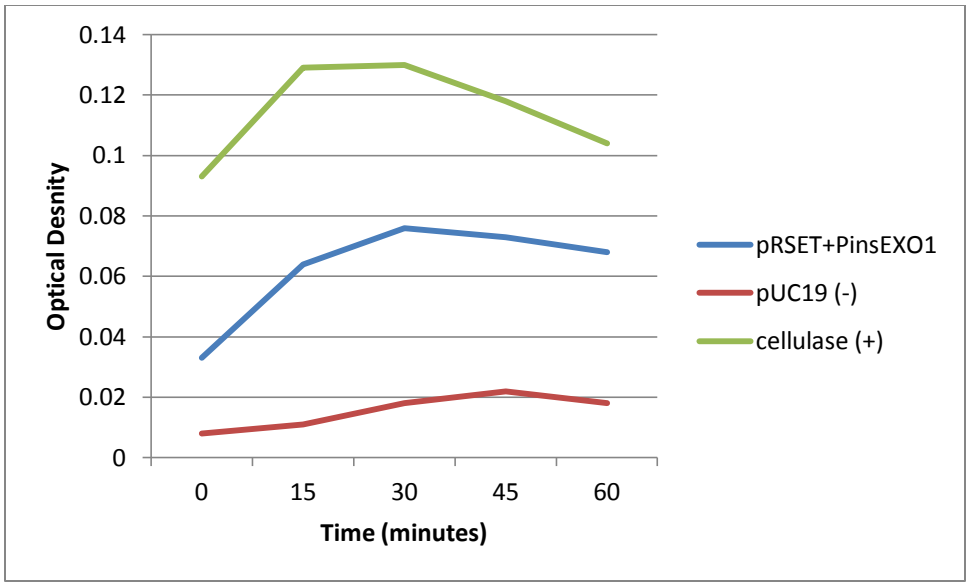


Figure 5: Line graph of agar plate assay clear zone diameters. *PinsEXO1* and the positive control consistently showed large clear zones compared to the negative control, whose clear zones were minimal or absent entirely.

Dinitrosalicylic Acid Assays

When glucose binds to dinitrosalicylic acid reagent, it results in red color that absorbs light strongly at 540-590nm. In cultures containing the *PinsEXO1* gene, the optical density increased at each interval before leveling off after 30 minutes. In media containing cellulase, the optical densities measured were significantly higher, but showed the same pattern of increasing at each interval for 30 minutes[Figure 6a]. Cultures containing the empty pUC19 vector showed consistently low optical densities. Similar patterns were observed using both laminarin [Figure 6a] and beta-glucan [Figure 6b] as the substrateswhile CMC did not yield any red color or absorbance at 540-590nm (data not shown).

a



b

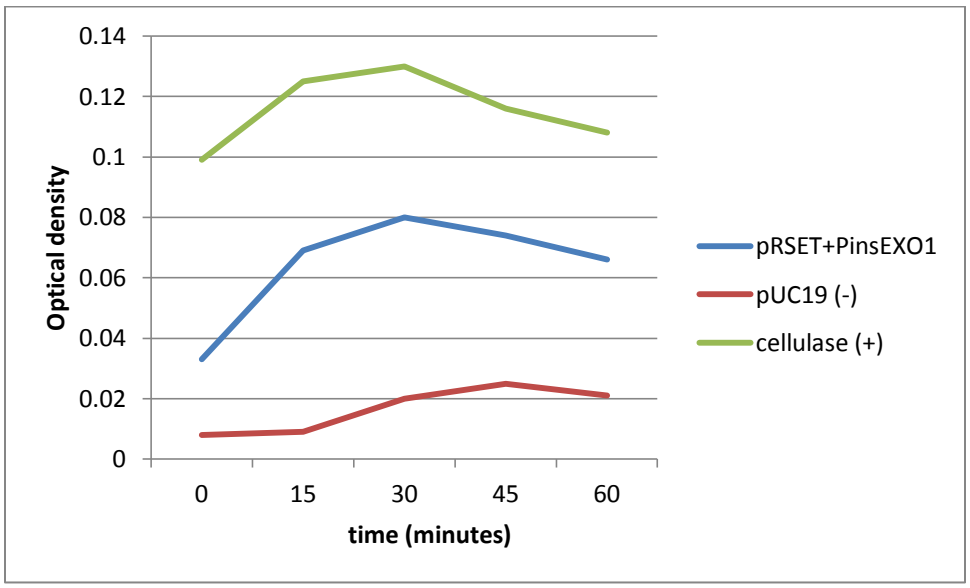


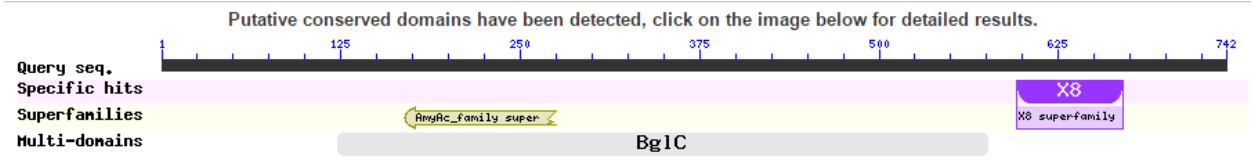
Figure 6: a) Optical densities of the DNS assays using laminarin as substrate. b) Optical densities of the DNS assays using beta-glucan as substrate. In both cases, JM109 cultures containing *PinsEXO1* show much higherglucan-hydrolytic activity compared to JM109 cultures containing the empty pUC19 vector, whose consistently low optical densities suggest little to no glucan-hydrolytic activity.

Phylogenetic Analysis

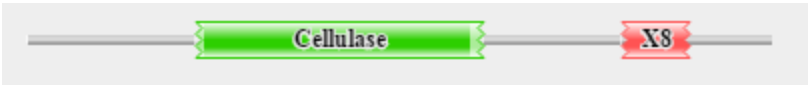
Protein Structure

Almost all of the collected glucanase genes were found to contain three protein domains: X8, the carbohydrate binding domain; BG1C, the endoglucanase domain; and the cellulase domain. The only exception was *PI-T30-2* from *P. infestans,* which did not contain the X8 domain. Among the glucanases of oomycetes collected, the order of the domains was also highly conserved. In all genes, the cellulase domain came first in the sequence. The X8 domain was the final domain in the sequence and the endoglucanase domain was in between the two, occasionally overlapping of domains was observed [Figures 7&8].

a



b



c

MLRSRTWMALAVAVMTLSGSVSAQQAAAIPDPSPTATAPPATQPPAPAPAPAPVSTPAAEPEAKAEPKPKQKKE
VVRDAKWYWEHEEDPRFAAATGSFKAFTPDDATQTCSHDTHATPFNKQVRGANLGGWLVLEPWITPSLFYQFL
GTQERFGDSAPNKTAMDSYTFCTALGKEEANRQLRVHWANWVTEDDIAEMAAAGVNSLRVPVGDWMFTPYEP
YIGCTDGAIEELDRVADLAHKYGMDILIDIHGLIGSQNGFDNSGKTSAVKWTSIASTQPVGTTTFEHWPIRQAEW
AGTFDPAKHAYTSINYGNLNQSLTAVEEIVKRYASHPAVLGLQPVNEPWELTPIKVLKTYYWKSYKRVKALAPH
WKFVLHDSFRFGREFWLDFMRGCPDIAIDTHIYQAWMNPGTKEDFYSNACQQKYTITDIENAVMPVIVGEWSLG
TDNCAMWLNGFNDNLPGFPKVICQLRHCPVESTYLGKGFPGTPLDTTKPIQGPYGTGTSGPSFGLCPVNSNLTFG
QKTPEDELKFMKNLMSKKLNAWLLGHGFYFWNFKTELDTRWDFLALVRAGVMPKNISDYDDADGIFDACERE
DKGDFVCRAKRGVKPFELENGLAYACNAEGVDCSNVKQKYLTLLEQCDYAFNEYWHQYREKGATCDFGGAA
HLLSVPSNTTATGVKPSQINTKSAGIDSADASGASGWWPPTTAALIAYIVGGVVVLAAVGGAIFQWRQRRLRRQ
QYNPIGRP

Figure 7: Cellulase, BG1C and X8 domains found in *PinsEXO1*. a.) Protein domains analyzed viaNCBI. b.) Protein domains analyzed viaPfam. c.) Amino acid sequence of *PinsEXO1* showing the following domains: Cellulase (green text); BG1C (gray highlight); and X8 (red text).

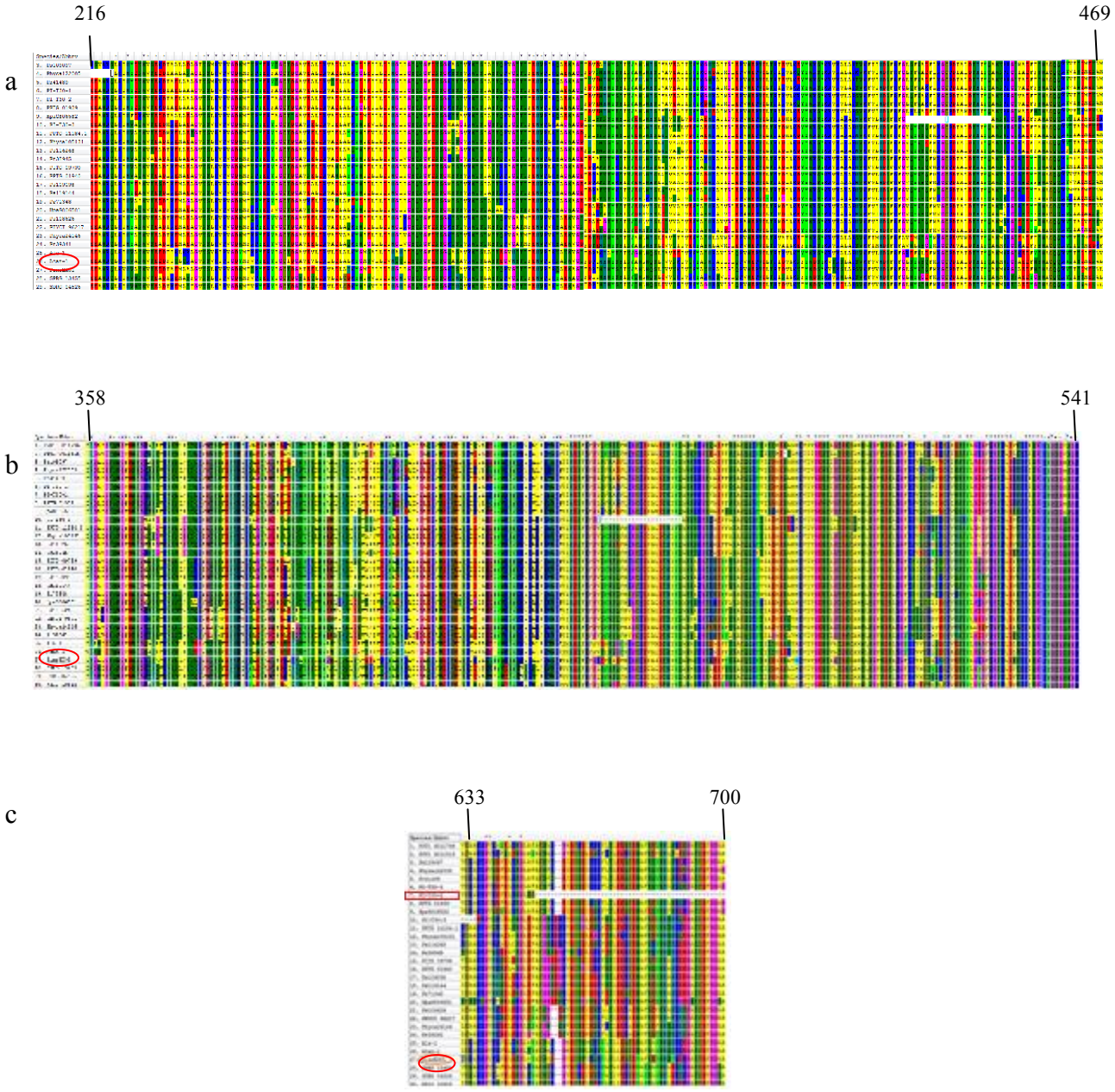
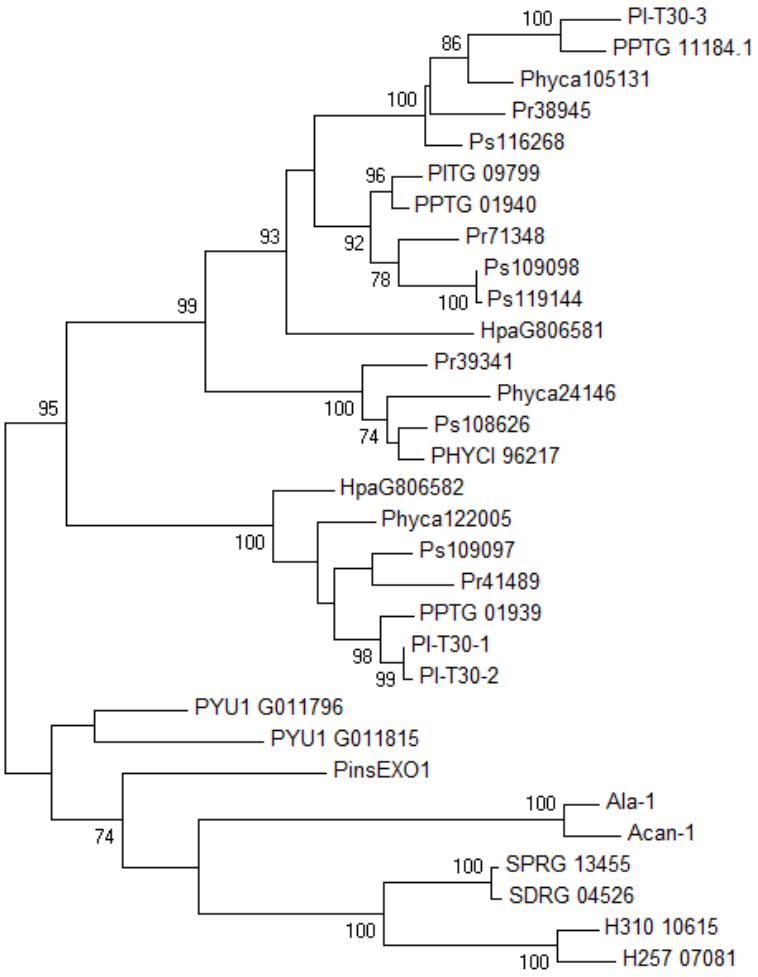


Figure 8: Alignment of a) the cellulase domain, b) BG1C domain and c) the X8 domain among the collected oomycete glucanases. *PinsEXO1* is circled in red. All proteins contained X8 except for *PI-T30-2* from *P. infestans* (indicated in red square).

Phylogenetic Tree

Three different approaches were used to identify the relationship of glucanase among the oomycetes; full-length sequences [Figure 9], the cellulase domain alone [Figure 10], and the BG1C domain alone [Figure 11]. The full-length sequences of 31 glucanase proteins, including *PinsEXO1*, were used to construct a phylogenetic tree using the Maximum Likelihood method. Proteins from animal pathogens *Saprolegnia declina, Saprolegnia parasitica, Aphanomyces invadans,* and *Aphanomyces astaci* showed a higher degree of homology to *PinsEXO1* than other proteins from plant pathogens such as *Phytophthora spp.* and *Hyaloperonospora arabidopsidis*. However, proteins from plant pathogens *Pythium ultimum, Albugo candida,* and *Albugo laibachii* also showed a higher degree of homology with *PinsEXO1*.

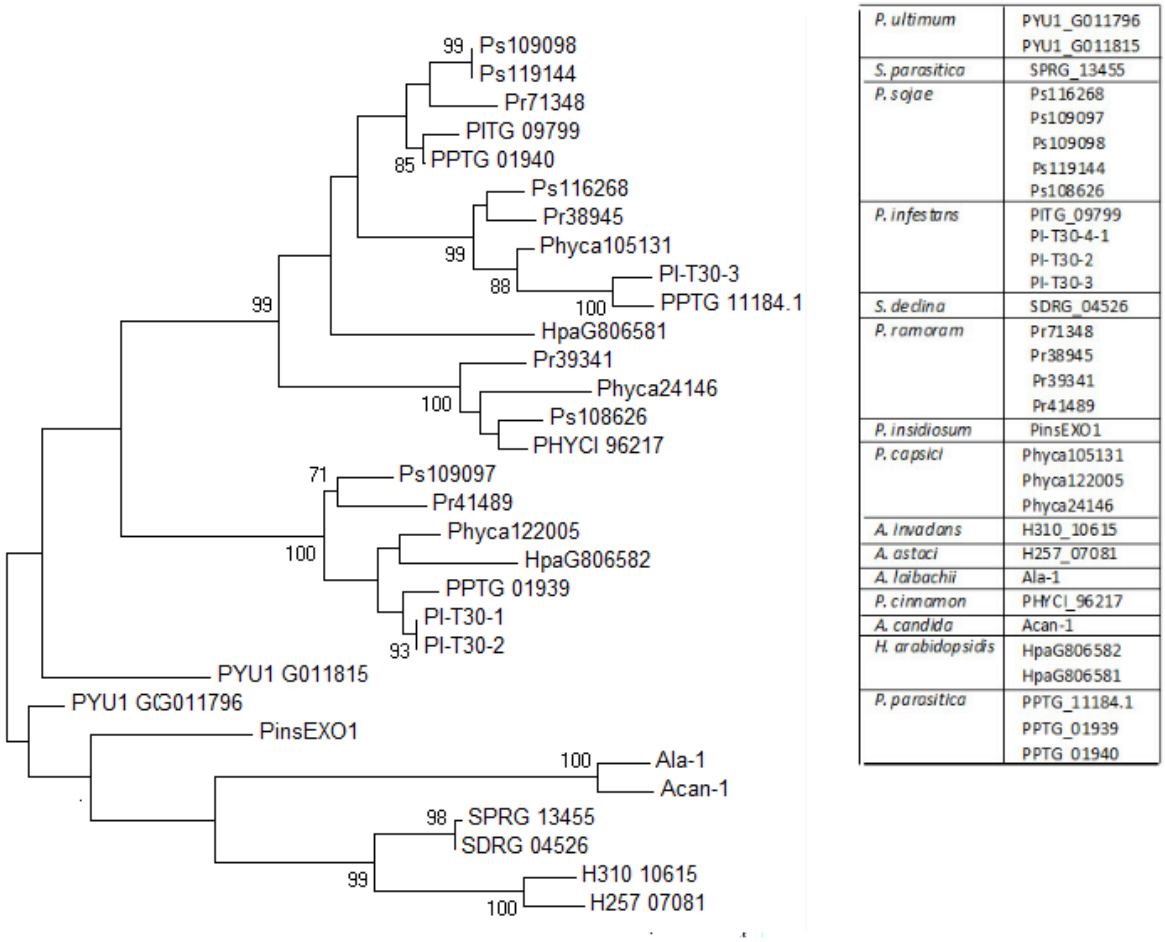


P. ultimum	PYU1_G011796 PYU1_G011815
S. parasitica	SPRG_13455
P. sojae	Ps116268 Ps109097 Ps109098 Ps119144 Ps108626
P. infestans	PITG_09799 PI-T30-4-1 PI-T30-2 PI-T30-3
S. declina	SDRG_04526
P. ramorum	Pr71348 Pr38945 Pr39341 Pr41489
P. insidiosum	PinsEXO1
P. capsici	Phyca105131 Phyca122005 Phyca24146
A. invadans	H310_10615
A. astaci	H257_07081
A. laibachii	Ala-1
P. cinnamon	PHYCI_96217
A. candida	Acan-1
H. arabidopsidis	HpaG806582 HpaG806581
P. parasitica	PPTG_11184.1 PPTG_01939 PPTG_01940

Figure 9: Phylogenetic tree built using full-length glucanase sequences. The phylogenetic tree was constructed using 31 full-length protein sequences and the Maximum Likelihood method. *PinsEXO1* shows a high degree of homology with other proteins from animal pathogens, as well as proteins from another *Pythium spp.*, along with *Albugo spp.*

Cellulase domains were found in all 31 proteins using the Pfam database. The cellulase domain sequences were then used to construct a Maximum Likelihood tree. Similar to the full-length sequence tree, the cellulase domain from *PinsEXO1* showed a high degree of homology with the cellulase domains from animal pathogen proteins, cellulase domains from *Pythium*

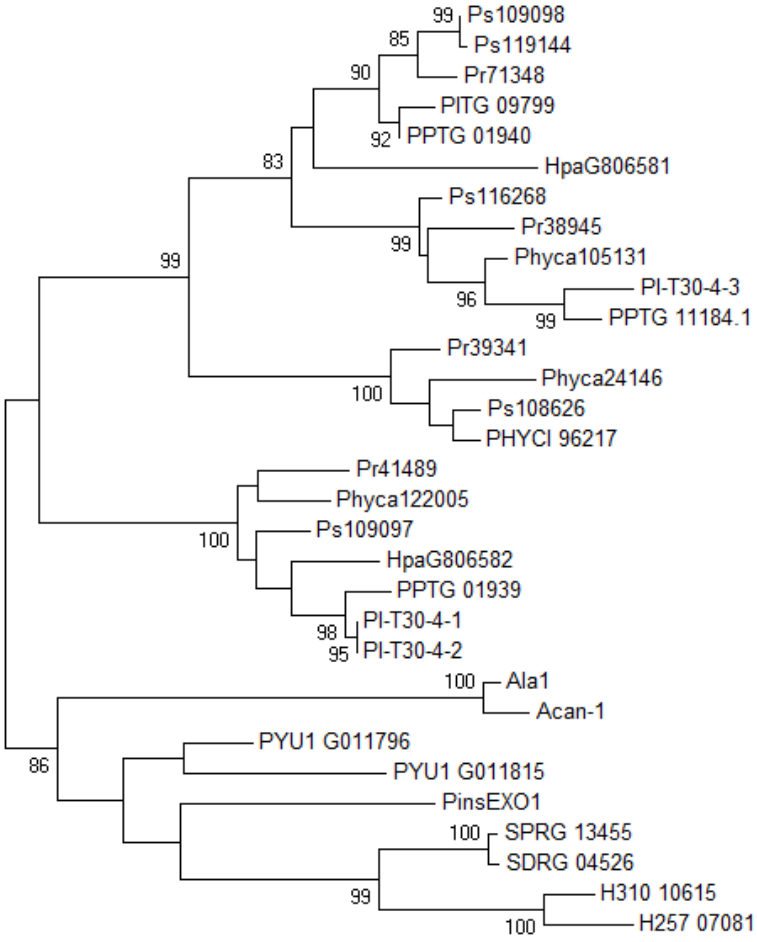
ultimum proteins, and cellulase domains from *Albugo spp.* proteins. Cellulase domains from *Phytophthora spp.* and *H. arabidopsidis* showed a more distant homology [Figure 10].



P. ultimum	PYU1_G011796 PYU1_G011815
S. parasitica	SPRG_13455
P. sojae	Ps116268 Ps109097 Ps109098 Ps119144 Ps108626
P. infestans	PITG_09799 PI-T30-4-1 PI-T30-2 PI-T30-3
S. declina	SDRG_04526
P. ramorum	Pr71348 Pr38945 Pr39341 Pr41489
P. insidiosum	PinsEXO1
P. capsici	Phyca105131 Phyca122005 Phyca24146
A. invadans	H310_10615
A. astaci	H257_07081
A. laibachii	Ala-1
P. cinnamon	PHYCI_96217
A. candida	Acan-1
H. arabidopsidis	HpaG806582 HpaG806581
P. parasitica	PPTG_11184.1 PPTG_01939 PPTG_01940

Figure 10: Phylogenetic tree built using cellulase domain sequences. The phylogenetic tree was constructed using only the cellulase domain sequences of the 31 proteins. Though similar to the full-length sequence tree, one notable difference is *PYU_G011815* sharing a higher degree of homology with the proteins from *Phytophthora spp.* This may be due to the fact that both *Phytophthora spp.* and *Pythium ultimum* are plant pathogens and may use these proteins for a similar function, such as breakdown of the plant cell wall.

In addition to the cellulase domain, the BG1C domain was also found in all 31 protein sequences. The BG1C domain is an endoglucanase domain. The presence of BG1C, along with cellulase and X8, strongly suggest that all 31 proteins are glucanases. BG1C domains were identified using the NCBI database. The sequences of the BG1C domains were then used to construct a Maximum Likelihood tree. The BG1C domain from *PinsEXO1* was shown to be most closely related to BG1C domains from other animal pathogens [Figure 11].



P. ultimum	PYU1_G011796 PYU1_G011815
S. parasitica	SPRG_13455
P. sojae	Ps116268 Ps109097 Ps109098 Ps119144 Ps108626
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S. declina	SDRG_04526
P. ramorum	Pr71348 Pr38945 Pr39341 Pr41489
P. insidiosum	PinsEXO1
P. capsici	Phyca105131 Phyca122005 Phyca24146
A. invadans	H310_10615
A. astaci	H257_07081
A. laibachii	Ala-1
P. cinnamon	PHYCI_96217
A. candida	Acan-1
H. arabidopsidis	HpaG806582 HpaG806581
P. parasitica	PPTG_11184.1 PPTG_01939 PPTG_01940

Figure 11: Phylogenetic tree built using BG1C domain sequences. The tree was constructed using only the sequences of the BG1C (endoglucanase) domain. The high degree of homology

shared between the BG1C domain from *PinsEXO1* and the BG1C domains from other animal pathogens suggests that glucanases may play a similar role in oomycete animal pathogens.

Expression Analysis

RNAseq Data Analysis

Pr38945 and Pr41489 were identified as glucanase genes *P. ramorum* via BLASTp using *PinsEXO1* as the query. The RNAseq data for *Pr38945* [Figure 12] and *Pr41489* [Figure 13] was found to be available on the Fungidb database. They are found to be expressed in chlamydospore, zoospore, mycelia growth in tomato media, and mycelial growth in V8 media. The expression of both *Pr38945* and *Pr41489* was relatively low in the zoospore stage. *Pr41489* showed higher levels of expression compared to *Pr38945*.

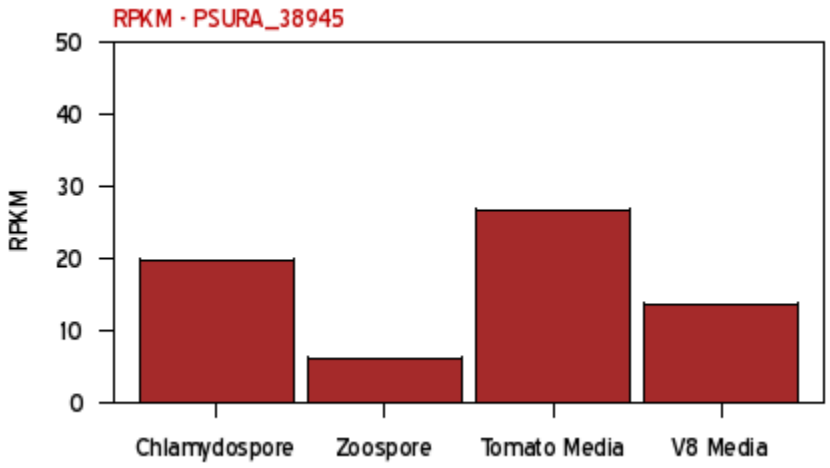

Figure 12: RNAseq analysis of *Pr38945* derived from Fungidb (Kasuga, et al,). Expression is relatively low in the zoospore stage and slightly higher in the chlamydospore, mycelia growth in tomato media, and mycelia growth in V8 media.

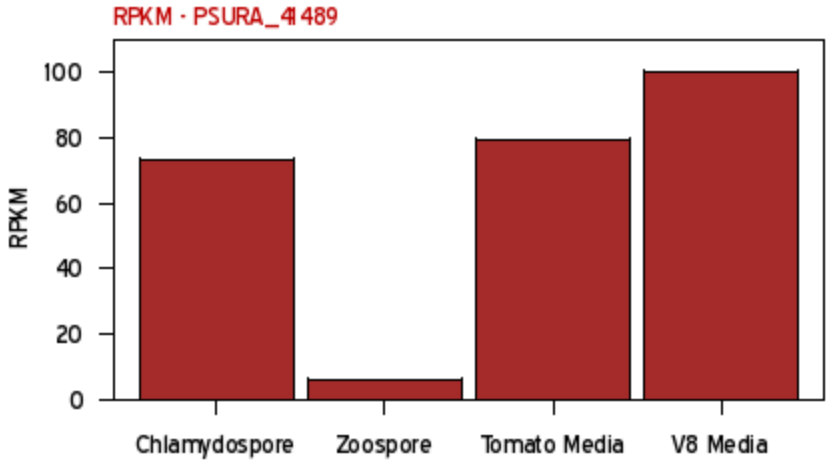

Figure 13: RNAseq analysis of *Pr41489* derived from Fungidb (Kasuga, et al). Expression is low in the zoospore stage, and very high in the chlamydospore, mycelia growth in tomato media and mycelia growth inV8 media stages.

The RNAseq data for *Pr38945* and *Pr41489* could also be visualized using the genome browser from Fungidb. Below, Figures 14 and 15 display the data for RNA expression of *Pr38945* and *Pr41489* respectively.

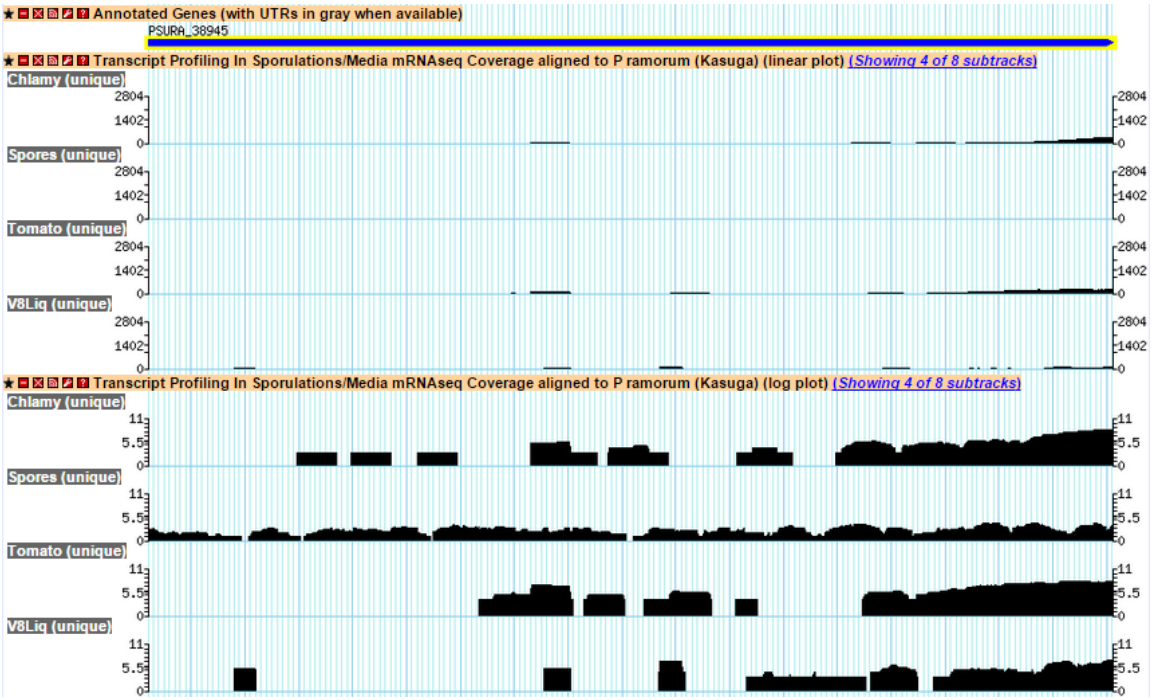

Figure 14: RNAseq analysis of *Pr38945* from *P. ramorum* as represented by linear and log histogram plots viewed on the Fungidb genome browser.

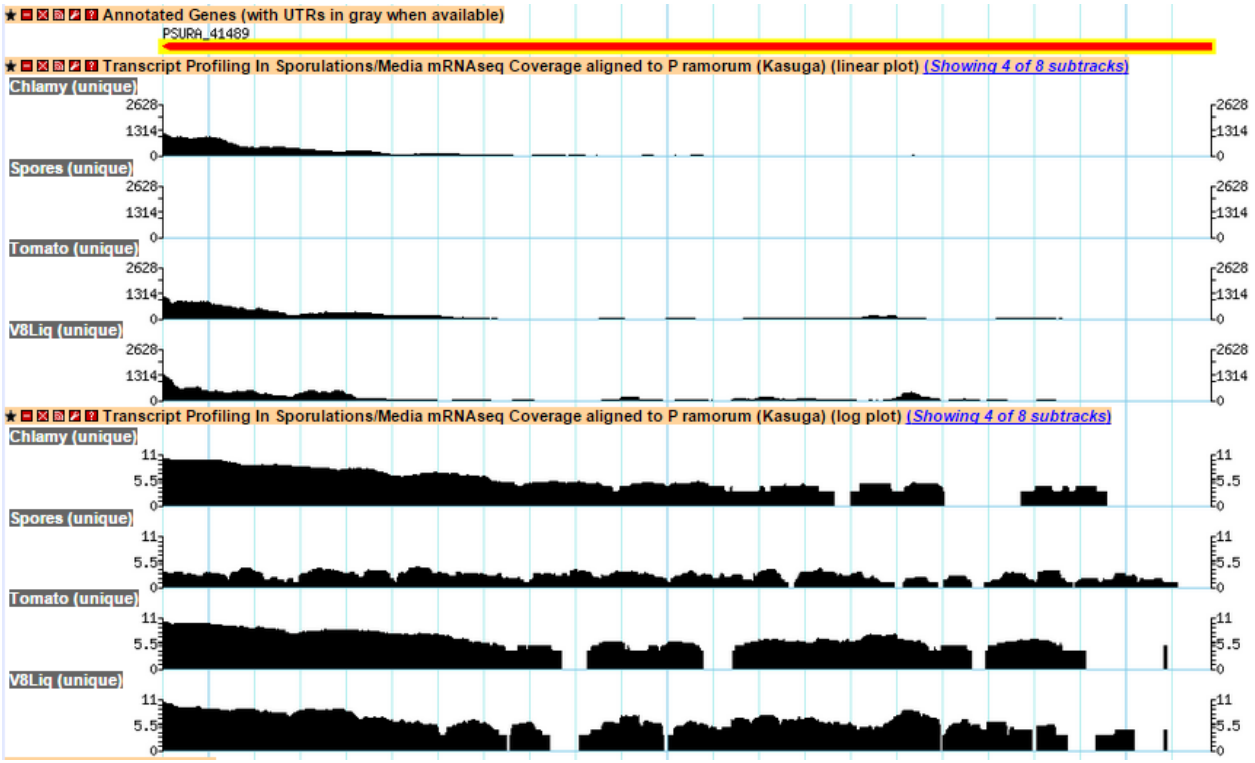

Figure 15: RNAseq analysis of *Pr41489* from *P. ramorum* as represented by linear and log histogram plots viewed on the Fungidb genome browser.

Expression data of *P. ultimum* from the following treatments were available; hypoxia, Arabidopsis infection, 0°C, 35°C, room temperature, mefenoxam control, and mefenoxam treatment. *PYU1_G011796,* identified as a glucanase, is shown to be expressed in all treatments tested [Figure 16].

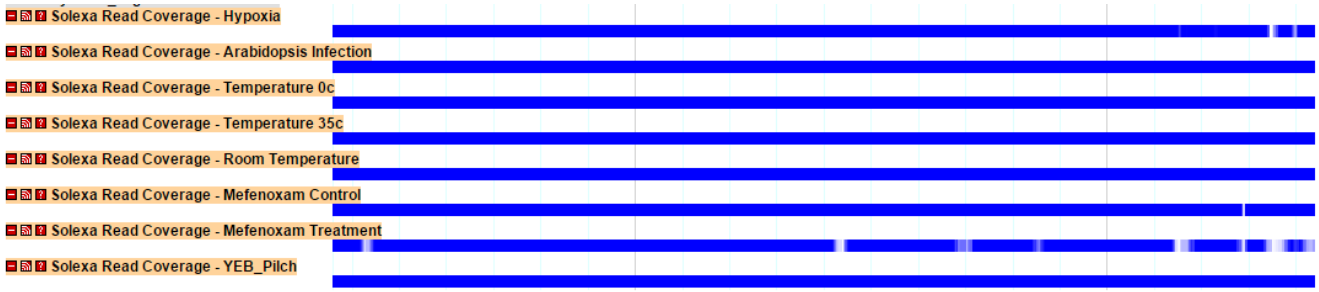

Figure 16: Wiggle plots representing expression data of *PYU1_G011796* from *P. ultimum* viewed on the Michigan State Pythium database genome browser. *PYU1_G011796* is shown to be expressed in all treatments tested (MSU Pythium database).

Expression data for *P. ultimum* could also be found on the NCBI SRA database under the accession number SRX020087 [Figure 17a], SRX022712 [Figure 17b], and SRX022708 [Figure 17c]. These data files were uploaded to Galaxy and visualized on the Fungidb genome browser. Below is the visualization of expression of *PYU1_G011796* represented by a histogram plot [Figure 17]. Expression of *PYU1_G011796* is shown to be relatively high in the generic sample (isolation source unknown) [Figure 17a] and control samples [Figure 17c], compared to *Arabidopsis* seed contact samples [Figure 17b].

Figure 17: Expression data for *PYU1_G011796* as viewed on the Fungidb genome browser. The data suggests that *PYU1_G011796* is expressed in *P. ultimum.* a) SRX020087; b) SRX022712; c) SRX022708.

Expression data for *S. parasitica* was found on the NCBI SRA database under the accession number RSRX155938 [Figure 18a], SRX155933 [Figure 18b], and SRX155932 [Figure 18c]. Using the same procedure as *P. ultimum* SRA, a visualization of the expression of *SPRG_13455* represented by a histogram plot is shown in Figure 18. Expression was observed in the generic sample (isolation source unknown) [Figure 18a], during mycelial growth [Figure 18b] and in cysts/zoospores [Figure 18c]. Expression levels during mycelial growth were higher compared to cysts/zoospores.

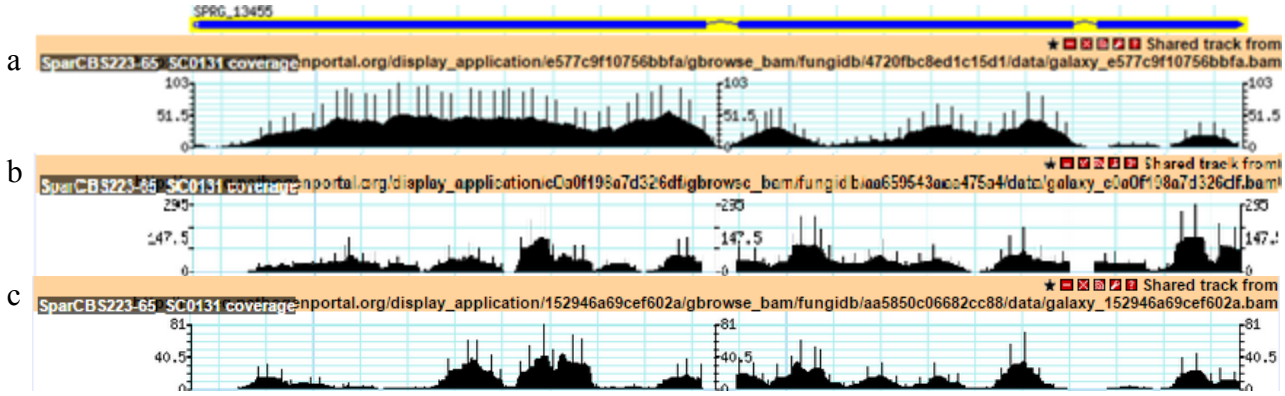

Figure 18: RNAseq data of *SPRG_13455* from *S. parasitica* viewed on the Fungidb genome browser. The data suggests that *SPRG_13455* is expressed in *S. paraisitca,* and that it contains two introns. a) SRX155939; b) SRX155933; c) SRX155932.

Expression data for *P. sojae* was found on the NCBI SRA database under the accession number SRX020742 [Figure 19a], SRX020740 [Figure 19b], and SRX020741 [Figure 19c] (isolation sources unknown). The data file was uploaded to Galaxy and visualized on the Fungidb genome browser. A visualization of the expression of *Ps116268* represented by a histogram plot is shown in Figure 19. Expression of *Ps116268* is detectable, but at a low level.

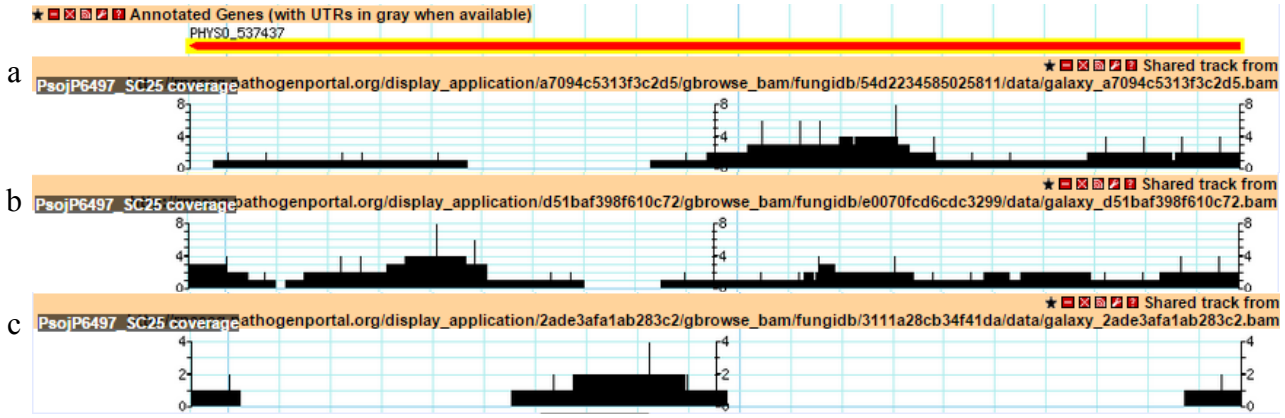

Fig 19: RNAseq data of *Ps116268* from *P. sojae* viewed on the Fungidb genome browser. a) SRX020742. b) SRX020740. c) SRX020741. The data suggests that *Ps116268* may be expressed.

Discussion

It has been hypothesized and supported that plant pathogenic oomycetes use glucanase and cellulase enzymes in order to weaken or break down the cell wall of their hosts in order to facilitate infection (Brouwer, et al, 2014)(Zerillo, et al, 2013). An animal pathogen such as *Pythium insidiosum*, however, would not require glucanase for this function. One possibility as to why *P. insidiosum* expresses glucanase during infection is that it may be involved in remodeling its own cell wall during hypheal growth stages (Krajaejun, et al, 2010). This is supported by previous research that has shown that, in addition to glucanases, oomycete genomes are also rich in beta-glucan-synthases, another class of genes likely involved in cell wall metabolism (Zerillo, et al, 2013). If so, this would make glucanases such as *PinsEXO1* a potential drug target, as inhibiting its function could inhibit the growth of the oomycete.

Oomycete cell walls consist of cellulose and beta-glucans (Gaastra, et al, 2010)(Zerillo, et al, 2013). Among these beta-glucans, those with 1,3 linkages are the most abundant, as compared to those with 1,6 and 1,4 linkages. (Melida, et al, 2012). Here, it has been demonstrated through enzyme activity assays that *PinsEXO1* is capable of hydrolyzing both laminarin and beta-glucan. As these are both beta-1,3-glucans, this may support the hypothesis that *PinsEXO1* is involved in remodeling of the oomycete cell wall. Additional 1,3-glucan substrates, such as the oomycete cell wall component mycolaminarin, can be tested using either the more common agar plate assay, or the more sensitive DNS assay.

PinsEXO1 did not show hydrolytic activity with carboxymethylcellulose (CMC) in this study. In another study, however, *PinsEXO1* was found to be capable of hydrolyzing Avicel, a crystalline form of cellulose (Keeratijarut, et al, publication pending). *PinsEXO1* has been classified as an exoglucanase, which have been shown to be more hydrolytically active with cellulose in its crystalline form as opposed to endoglucanases, which are more hydrolytically active with cellulose in its soluble form (such as CMC) (Dashtban, et al, 2010). This may explain why *PinsEXO1* was not capable of hydrolyzing CMC in this study, yet has been reported to be capable of hydrolyzing Avicel.

Previous research has shown that *Phytophthora spp.* has a significantly higher number of carbohydrate-active enzymes (CAZymes) in its genome compared to *Pythium spp.,* to the extent that plant pathogenic *Pythium* species cannot rely on the complex carbohydrates in the plant cell wall as a carbon source as *Phytophthora* can (Zerillo, et al, 2013). The full genome sequence of *Pythium insidiosum* is needed to provide further insight as to how its CAZyome differs from that of plant pathogenic oomycetes. Transcriptome analysis of *P. insidiosum* has shown that several glycosyl hydrolase and carbohydrate binding proteins are up-regulated when exposed to 40 °C, as would be the case during infection of a mammal (Krajaejun, et al, 2014).

Glucanases similar to *PinsEXO1* are found in a variety of oomycetes, both plant and animal pathogens. Here, three phylogenetic trees have been constructed to compare the homology of *PinsEXO1* to proteins from both plant and animal pathogens. When comparing the full-length sequences, it is unsurprising that *PinsEXO1* shares a close evolutionary relationship to *PYU_G011796* and *PYU_G011815*from *Pythium ultimum* and *Ala-1* and *Acan-1* from *Albugo spp.*, as they are close evolutionary relatives. *PinsEXO1* also shares a high degree of homology with glucanases from the animal pathogens, such as: *SPRG-13455* from *S. parasitica; SDRG-*

04536 from *S. declina; H310-10615* from *A. inadans;* and *H257-07081* from *A. astaci.* This may suggest that these pathogens use glucanase for a similar function, possibly distinct from plant pathogens. This can be further supported by the fact that plant pathogens tend to have significantly more glycosyl hydrolase genes than animal pathogens, suggesting that glucanases play a more significant role in plant infection (Jiang, et al, 2013).

A comparison of the cellulase and BG1C protein domains from the various glucanases further supports that animal pathogens may be using glucanase for a similar function. The BG1C domain is classified as an endoglucanase domain (Marchler-Bauer et al, 2013) and was found in all 31 proteins, indicating that they are all glucanases. Phylogenetic analysis shows that the BG1C domain of *PinsEXO1* shares a higher degree of homology with BG1C domains from animal pathogens than plant pathogens, including those from *Pythium ultimum.* Phylogenetic analysis of the cellulase domains provides similar results, with the major exception being *PinsEXO1*'s homology with *Ala-1* and *Acan-1* from *Albugo spp.* Interestingly, the cellulase domain *PYU_G011*, a glucanase from *Pythium ultimum*, shows more homology with *Phytophthora spp.* This is consistant with *P. ultimum*'s niche role as a plant pathogen, despite being more closely related to *P. insidiosum*. It may suggest that *PYU_G011815* plays a role in plant infection as opposed to cell-wall metabolism.

The RNAseq data analysis suggests that the genes analyzed (*Pr38945* and *Pr41489* from *P. ramorum; PYU1_G011796* from *P. ultimum; SPRG_13455* from *S. parasitica;* and *Ps116268* from *P. sojae*) are expressed in their respective organisms. *Pr38945* and *Pr41489* were shown to be more highly expressed in their growth life stages than in their zoospore life stages, which may suggest that they are involved in infection, as mycelial growth occurs when *P. ramorum* has infected a plant host. It may also suggest that *Pr38945* and *Pr41489* are involved in cell wall

remodeling during mycelial growth. *SPRG_13455* also showed higher levels of expression during in mycelium compared to zoospores. This may support the hypothesis that glucanase genes are involved in cell wall remodeling during mycelia growth in animal pathogens as well.

To conclude, two functional analyses were developed and performed to test the substrate specificity of recombinant *PinsEXO1* from live *E. coli* cultures. *PinsEXO1* is capable of hydrolyzing beta-glucan and laminarin, but not carboxymethylcellulose (CMC). A phylogenetic analysis was performed with 31 proteins from plant and animal pathogenic oomycetes. Analysis of the protein domains confirmed that the 31 proteins were glucanases. The resulting phylogenetic trees displayed a close homology between *PinsEXO1* and glucanses from animal pathogenic oomycetes. Finally, RNAseq data was collected to confirm the expression of glucanase in different oomycete species, and to compare expression levels during different treatments and life cycle stages. Glucanse from *P. sojae* showed very low levels of expression compared to the other oomycetes studied. Glucanse from *P. ultimum* was expressed in a variety of treatment groups including different temperatures and hypoxia. Glucanases from *P. ramorum* and *S. parasitica* were shown to be more highly expressed during mycelial growth as compared to zoospores.

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Organism	Gene	Sequence	Introns	Domain	Length	Pathogen
P. ultimum	PYU1_G011796	MLGGVRVK	0	X8, cellulase, BgIc	737aa	plant
P. ultimum	PYU1_G011815	MLRSAVLS	0	X8, cellulase, BgIc	694aa	plant
S. parasitica	SPRG_13455	MKWTVLA	2	X8, cellulase, BgIc	695aa	animal
P. sojae	Ps116268	MKLSSLLQ	0	X8, cellulase, BgIc	697aa	plant
P. infestans	PITG_09799	MKVLLQVA	0	X8, cellulase, BgIc	692aa	plant
S. declina	SDRG_04526	MKWTVLA	2	X8, cellulase, BgIc	695aa	animal
P. sojae	Ps109097	MLVASRLK	1	X8, cellulase, BgIc	744aa	plant
P. ramorum	Pr71348	MDMYTFC	0	X8, cellulase, BgIc	567aa	plant
P. ramorum	Pr38945	MTLSSAAP	0	X8, cellulase, BgIc	597aa	plant
P. sojae	Ps109098	MDMYTFC	0	X8, cellulase, BgIc	567aa	plant
P. sojae	Ps119144	MDMYTFC	0	X8, cellulase, BgIc	567aa	plant
P. sojae	Ps108626	MDTHSFCT	0	X8, cellulase, BgIc	567aa	plant
P. infestans	PI-T30-4-1	MLVGSSLK	2	X8, cellulase, BgIc	694aa	plant
P. ramorum	Pr39341	MVLRNSLL	0	X8, cellulase, BgIc	578aa	plant
P. ramorum	Pr41489	MLVGSRLK	0	X8, cellulase, BgIc	762aa	plant
P. infestans	PI-T30-4-2	MLVGSSLK	0	X8, cellulase, BgIc	619aa	plant
P. infestans	PI-T30-4-3	MDTYTFCT	2	X8, cellulase, BgIc	523aa	plant
P. insidiosum	PinsEXO1	MLRSRTW	0	X8, cellulase, BG1C	742aa	animal
P. capsici	Phyca105131	MRLLHVAT	0	X8, cellulase, BgIc	676aa	plant
P. capsici	Phyca122005	MVGSRLKT	3	X8, cellulase, BgIc	699aa	plant
P. capsici	Phyca24146	MVKSLFPV	0	X8, cellulase, BgIc	678aa	plant
A. invadans	H310_10615	MKGRGVM	1	X8, cellulase, B,gIc	672aa	animal
A. astaci	H257_07081	MKGPSAM	?	X8, cellulase, BgIc	678aa	animal
A. laibachii	Ala-1	MRRVFLAS	?	X8, cellulase, BgIc	678aa	plant
P. cinnamoni	PHYCI_96217	MVLRNALI	?	X8, cellulase, BgIc	683aa	plant
A. candida	Acan-1	MHTFCFSR	?	X8, cellulase, BG1C	695aa	plant
H. arabidopsidis	HpaG806582	MGALLAAA	?	X8, cellulase, BG1C	715aa	plant
H. arabidopsidis	HpaG806581	MKLSLQPLT	?	X8, cellulase, BG1C	703aa	plant
P. parasitica	PPTG_11184.1	MQLKPLL	0	X8, cellulase, BG1C	681aa	plant
P. parasitica	PPTG_01939	MLVGSRLK	0	X8, cellulase, BG1C	751aa	plant
P. parasitica	PPTG_01940	MKVLLQVA	0	X8, cellulase, BG1C	692aa	plant