EFFORTS TO CHARACTERIZE THE GENOME ORGANIZATION AND GENE EXPRESSION PATTERNS OF THE COTTON ROOT ROT FUNGUS, *PHYMATOTRICHOPSIS OMNIVORA*

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CHAPTER I

REVIEW OF LITERATURE

I. History

Phymatotrichum root rot (PRR), caused by *Phymatotrichopsis omnivora* (Duggar) Hennebert 1973 has caused significant losses to cotton and alfalfa for over a century in the southwestern United States. The causal fungus was first described in 1888 and 1889 by L.H. Pammel, an assistant to W. Trelease at the Shaw School of Botany at Washington University in St. Louis. Mr. Pammel was sent to work on the disease in Texas and reported his findings in two Texas Agricultural Experiment Station Bulletins (Pammel 1888, 1889). Mr. Pammel examined the roots of dead cotton plants and consistently observed strands of mycelium on the roots which led him to believe that this fungus may be responsible for the death of the plants.

Phymatotrichum Root Rot

P omnivora infects over 1,800 species of dicotyledonous plants (Blank 1953). The initial symptoms of cotton root rot include occasional purpling and bronzing of the leaves (Streets and Bloss 1973) followed by a rapid wilting of the plant resulting in its death (Taubenhaus and Ezekiel 1930). A distinguishing characteristic of the presence of PRR in the field is that plants die in a circle. Cotton and alfalfa plants can exhibit foliar

symptoms 10-12 hours after inoculation with *P. omnivora*, compared to trees and shrubs which may require two years before the death of the plant occurs (Taubenhaus and Ezekiel 1930). When hyphae of *P. omnivora* penetrate the root, there is direct injury to cells, resulting in the formation of a brown lesion around the infection site.

II. Disease Triangle

Environment

P. omnivora is restricted to alkaline and calcareous soils (Percy 1983) in the southwestern United States and Mexico (Lyda 1978). Soil pH is a critical requirement for the survival of the fungus. A soil pH range of 7.2-8.0 is ideal for growth and survival of the fungus (Percy 1983). The fungus can grow as hyphae in acidic soils (pH 4.7-6.5), but it does not produce sclerotia, the survival stage (Lyda 1978). Optimal vegetative growth and sclerotia production of *P. omnivora* occur at soil temperatures near 28°C (Lyda 1978).

Hosts

The primary agronomic hosts of *P. omnivora* are cotton (*Gossypium hirsutum* L.) and alfalfa (*Medicago sativa* L.) (Streets and Bloss 1973). Other important hosts include forest, fruit and nut trees, shrubs, herbaceous ornamentals, vegetable crops, grapes and berries (Streets and Bloss 1973). In general, *P. omnivora* is not a problem for most monocotyledonous plants, such as sorghum (*Sorghum bicolor* (L.) Moench). Resistance appears to manifest as a hypersensitive response, during which the fungus comes into contact with the roots and the attempted infection is confined to a localized lesion and there is no more proliferation of mycelial strands (Rush et al.1984). Some scientists have

suggested that monocots are not immune, but because of their fibrous root systems, they can avoid significant damage by the fungus (Lyda 1978; Taubenhaus and Ezekiel 1930).

Pathogen Life-Cycle

The life-cycle of *P omnivora* consists of sclerotia which are the primary overwintering stage, vegetative mycelia, multihyphal strands, and spore mats (Lyda and Kenerley 1993; Streets and Bloss 1973). First described by Neal et al. (1934), sclerotia stay dormant in the soil and germinate, forming vegetative mycelia which then produce multihyphal strands, when soil water potentials increase over -10 bars and soil temperatures rise above 20°C. These multihyphal strands grow outward through the soil until roots of a host plant are encountered (Lyda and Kenerly 1993). If the plant is susceptible to the fungus, then the strands will penetrate the epidermis of the root and colonize host tissues (Riggs 1993). Once the fungus has extensively colonized the taproot it produces more mycelial strands that can continue to infect additional roots or form sclerotia. In some cases, the multihyphal strands can grow towards the soil surface to produce spore mats.

Growth Stages

Conidia (spore mats)

P omnivora will produce spore mats during prolonged periods of heavy rain and warm temperatures late in the summer (Streets and Bloss 1973). The spore mats are composed of large-celled branching hyphae that produce conidiophores, which will then from conidia (Streets and Bloss 1973). Spore mats occur most often in fields with dense

vegetation (Lyda 1978). Significant efforts have been devoted to inducing the germination of conidia *in vivo*; however, these attempts were not successful (Woods et al. 1967; Baniecki and Bloss 1969). Taft et al. (1967) reported successful germination of conidia following sonication. However, Lyda (1973) stated, "This is a rather drastic treatment and I would not expect such to occur in nature".

Mycelial strands

In the early stages of hyphal development, a large central hypha becomes thickened and elongated (Rogers and Watkins 1938). Smaller hyphae begin to form and wrap around the central hypha forming multihyphal strands (Lyda 1978). As the strand matures the mycelium turns from white to buff and then to brown in color (Rogers and Watkins 1938). The external hyphae form distinctive acicular (needle-like) sterile hyphae that sometimes form the characteristic cruciform branching (Streets and Bloss 1973).

Sclerotia

Soilborne sclerotia permit survival of *P. omnivora* during unfavorable conditions such as winter and in the absence of a host (King and Loomis 1929) and have been reported to survive in the soil for up to 12 years (Streets and Bloss 1973). Sclerotia are formed along the mycelial strands in the soil adjacent to infected roots (possibly at depths of 10 to 12 feet) and, like the mycelial strands, are initially white and turn dark brown in color (Lyda 1978).

Taxonomy

When the fungus was first observed on cotton roots by Mr. Pammel, he sent samples to Harvard University where Dr. Farlow identified it as *Ozonium auricomum* (Pammel 1888). The fungus was renamed as a new species *Ozonium omnivorum* Shear (1907) based on the non-sporulating mycelium associated with diseased roots. A conidial stage was discovered on dying alfalfa plants in Arizona (Thornber 1906) and Duggar (1916) named the fungus *Phymatotrichum omnivorum* (Shear) Duggar. A basidial stage was reported in 1969 and the name was changed briefly to *Sistotrema brinkmanni* (Baniecki and Bloss 1969). However, the basidiospores of the *Sistotrema* failed to produce the mycelium of *P. omnivorum* and a sclerotial stage could not be obtained from the basidiospore isolate. As of today, *Phymatotrichopsis omnivora* (Duggar) Hennebert is the name associated with the cotton root rot fungus and it is solely asexual belonging to the phylum *Ascomycota*, class *Pezizomycetes* (Marek et al. 2009).

III. Previous Molecular Investigations into *P. omnivora*

One major characteristic of *P. omnivora* hyphae is the multinucleate cells. There are typically 4 nuclei per cell in small hyphae with up to 20 in large celled hyphae (Neal et al. 1934). Robert Hosford and George Gries (1966) investigated *P. omnivora*'s nuclei for evidence of a parasexual cycle, which was reported in other fungi (Pontecorvo 1956; Käfer 1961). *P. omnivora*'s hyphae were found to contain 3 to 41 nuclei per cell with the number increasing with size of the hyphae (Hosford and Gries 1966). Hyphal tip cells

contained two to eight nuclei per cell, while 6 to 49 nuclei per cell were found in spore mat hyphal cells and one to two nuclei per conidium.

Lipid composition and DNA methylation differ between mycelium and sclerotia.. The lipid and glycogen composition of sclerotia was examined by Gunasekran and Weber (1974) who concluded that lipid composition in sclerotia is 21.7%, while free glycogen in sclerotia comprises 10.1% of the total dry weight and bound (insoluble) glycogen comprises up to 26.6% of the sclerotial dry weight (Ergle 1947). The lipids present in *P. omnivora* sclerotia include mono, di-, and triglycerides and polar lipids are the predominant ones (Gunasekran and Weber 1974). In mycelium the lipid composition was 48.6 mg/g dry weight compared to 52.5 mg/g dry weight for sclerotia (Gunasekran 1974). The DNA in sclerotia was found to be extensively methylated (Jupe et al. 1986). The level of methylation of sclerotial DNA versus vegetative hyphal DNA was determined by digestion with restriction endonucleases inhibited (*Hpa*II) or not inhibited (*Msp*I) by DNA methylation. To investigate carbohydrate metabolism, *P. omnivora* was grown on different carbon sources and tested for the production of the extracellular enzyme amylase (Gunasekran 1980). Amylase production was higher when the fungus was grown on a basal synthetic medium, which was supplied with soluble starch to induce expression. The optimal temperature for mycelial growth was 20°C, but the optimal temperature for amylase production was 30°C.

In 1992, Van Wayne Crouch Jr., a graduate student at Texas A&M University used pulsed-field gel electrophoresis (PFGE) to separate chromosomes of four isolates of *P. omnivora* (Crouch 1992). Two of the isolates were from cotton, one from okra, and one from grape. Crouch identified four chromosomes ranging in size from 1.8 Mb to 6 Mb.

Overall, the chromosome numbers and sizes did not vary among isolates. More than four chromosomes likely were present, since some DNA, possibly chromosomes larger than 6 Mb, appeared to not have migrated out of the wells of the PFGE gels.

Restriction fragment length polymorphism (RFLP) were used to estimate genetic variability among fungi. For *P. omnivora*, genomic DNA from one isolate was cut with five restriction endonucleases and only one polymorphism was detected with homologous probes (Riggs 1993). Riggs also used random amplification of polymorphic DNA (RAPD), another molecular technique, to analyze genetic diversity among a population of *P. omnivora* isolates obtained from the same field. No diversity was detected using seven primers although there were distinct amplification patterns.

IV. Characterizing Fungal Genomes

Pulsed Field Gel Electrophoresis (PFGE)

PFGE is similar to conventional gel electrophoresis, which can separate DNA fragments up to 20 kb in size using a continuous electrical field. However, PFGE separates high molecular weight DNA fragments, from 30 kb to 10 Mb in size, using an alternating electrical current to move the larger DNA into specialized (large pore size) agarose gels (Shwartz and Cantor 1984). The electrical pulses can be configured in alternating diagonal vectors, which allow larger DNA (up to 20 Mb) to migrate into the agarose, resulting in better separations. The predominantly used PFGE technique is contour-clamped homogeneous electric field (CHEF, manufactured by Biorad Laboratories, Richmond, CA), which, depending on the electrical power supply, permits the

autonomous operation of electrodes and automatically shifts the electrical field angles (Sambrook and Russell 2001). The larger size limits of DNA separated by PFGE can be used to separate the chromosomes of some eukaryotes, including fungi. However, such electrophoretic separations require long run times, varying from 24 hours up to 240 hours.

Fungal genomes studied using PFGE Viral and bacterial genomes are routinely analyzed using PFGE (O'Brien et al. 2006). The genome size and chromosomal organization of numerous plant pathogenic fungi also have been characterized using PFGE. PFGE-based visualization of chromosomes is sometimes referred to as molecular karyotyping. The fungus *Tilletia indica* is an economically important pathogen, causing Karnal Bunt of wheat growing around the world, but little was known about its genetic characteristics. Using PFGE, the molecular karyotypes of eight strains of *T. indica* were examined and found to have 11 chromosomes ranging in size from 1 to more than 3.3 megabases (Tooley et al. 1994). However, each isolate exhibited unique karyotypes, indicative of the inherent variability of genomic organization within this species and corresponding to the genetic diversity of the strains examined. *Magnaporthe grisea,* which causes rice blast, possesses unique chromosomes called "mini-chromosomes", ranging in size from 500 to 2,000 kb (Orbach et al. 1996). These mini-chromosomes were highly diverse in size compared to other chromosomes and ranged in sizes from 2,000 kb to more than 10,000 kb among the 38 strains tested. Mini-chromosomes, also known as supernumerary, accessory, dispensable or B-chromosomes, have been characterized in numerous fungi (e.g. *Nectria haematococca, Pyrenophora,*

Metarrhizium, Macrophomina, Fusarium, and *Leptosphaeria*), are not needed for growth and occur in other eukaryotes such as animals and plants (Zolan 1995; Covert 1998).

Another economically important fungus that has little known about its genetics is *Sclerotinia sclerotiorum*, which causes disease in a wide range of plants and possesses multinucleate hyphae (Purdy 1979). Again, like *T. indica,* more genetic information is needed in order to further investigate this fungus for improving disease management. Six strains of *S. sclerotiorum* virulent on sunflower were subjected to molecular karyotyping, which revealed at least 16 chromosomes ranging in size from 1.5 Mb to 4.0 Mb (Fraissiner-Tachet et al. 1995). These high molecular weight chromosomes did not vary among the six field isolates in number or size. In contrast, smaller chromosomes (<1.5 Mb) were highly variable in size between isolates, and while they attempted to separate these mini-chromosomes, they were unsuccessful. In the canola blackleg fungus, *Leptosphaeria maculans*, electrophoretic karyotypes of four unrelated Australian field isolates revealed that all four had a different karyotype pattern. The size ranges of the twelve chromosomal DNA bands were between 0.9 Mb to > 2.2 Mb (Plummer and Howlett 1993).

Other Genome Characterization Methods used in Fungi

Telomere Fingerprinting

PFGE is useful in estimating chromosome numbers and size in various fungi; however, it is can be difficult to achieve sufficient resolution of the bands, which can lead to underestimates of chromosome numbers. An alternative approach is telomere fingerprinting in which genomic DNA is cut with restriction enzymes and the separated fragments are hybridized with a telomere-specific probe. Since each chromosome possesses two telomeres, the number of chromosomes can be counted and estimated (Zolan 1995). A study involving the fungus *Rosellinia necatrix* employed telomere fingerprinting to better understand the genetic and cytological characteristics of its mating type and vegetative incompatibility (Aimi et al. 2002). Thirty field and single ascospore strains of *R. necatrix* were collected and the extracted DNA was digested with *Bal*31 exonuclease and *Mbo*I and then hybridized with a telomere repeat probe (pTel46) from the fungus *Coprinus cinereus*. The number of hybridizing bands from each isolate was counted and the estimated minimum chromosome number was 6-7. Telomere-linked RFLPs were observed among all the strains, however, there was no variation in the telomere patterns of *R. necatrix* strains belonging to the same MCG (mycelial compatibility group). This suggests that isolates must have similar chromosomal organization to anastamose with each other.

Genetic diversity of the gray leaf spot pathogen *Magnaporthe oryzae* was investigated using telomere fingerprinting. The telomere regions of *M. oryzae* were determined to be more variable than internal chromosomal loci (Farman and Leong 1995). Other RFLPs

that might be useful in studying gray leaf spot populations were also examined using 22 isolates of *M. oryzae* from different states (Farman and Kim 2005). A plasmid clone containing an *M. oryzae* telomere sequence (Farman and Leong 1995) was used to probe *Pst*I-digested DNA, which resulted in multiple hybridization signals. Since the haploid number of chromosomes is 7, they observed 14 hybridized telomere signals from all 22 isolates. An extremely high level of polymorphism among the isolates was observed with no more than 3 isolates sharing the same fragment size. The variability was compared to the internal repetitive DNA loci, which were shown to be genetically similar across the isolates.

Other methods

Fuelgen staining has been used to characterize *P. omnivora*'s nuclear morphology in fixed sections of mycelia, spore mats, and sclerotia (Hosford and Gries 1966). The purple stain was used to monitor the appearance and movements of nuclei in stages of *P. omnivora*'s life cycle. The observations of diploid, aneuploid, and polyploidy nuclei in anastomosing hyphae implied that *P. omnivora* could undergo a parasexual cycle during its development.

Oomycetes such as *Phytophthora infestans*, have been studied using flow cytometry methods to better understand the cell cycle, and was found to be useful for identifying phenotypic and genetic changes among populations (Catal et al. 2010). In this study, the researchers used laser flow cytometry, which is capable of measuring 20,000 nuclei stained with propidium iodide. Isolated nuclei from *P. infestans* hyphae were delivered

through a laser flow cytometer. The DNA content was measured by quantifying the intensity of fluorescence and compared to known chicken red blood cell nuclei controls. The outcomes of this analysis revealed high diversity in DNA content of nuclei among the *P. infestans* isolates yielding a single 2C peak which corresponds to the diplophase cycle. Some isolates were found to be heterokaryons because they had multiple distinct populations of nuclei. This technique was used in other fungi and oomycetes as well (Day et al. 2002).

V. Quantitative Polymerase Chain Reaction (qPCR) for Quantifying Fungal Infection and Following Gene Expression of Fungi *In Planta*

Biochemical methods for estimating fungal biomass *in planta*

Ergosterol and chitin concentrations in biological samples has been used to measure total fungal biomass (Gardner et al. 1993; Zeppa et al. 2000). Ergosterol is a component of the fungal cell membranes and is a good indicator of metabolically active fungal growth (Zeppa et al. 2000). However, this method often lacks specificity because epiphytic fungal biomass on a plant surface does not necessarily correspond with meaningful data regarding infection (Bermingham et al. 1995). Antibodies also have been used to determine fungal biomass (Dewey et al. 1997), but obtaining antibodies specific for fungi can be problematic (Ward et al. 1998).

Quantitative Polymerase Chain Reaction (qPCR)

Quantitative PCR (also referred to as real-time PCR), uses a detection method based on a fluorescent dye (SYBR® Green I) that emits a signal once it is bound to double-stranded DNA (dsDNA). One advantage to using this fluorescent dye is that the florescence emitted is proportional to the amount of dsDNA in a reaction (Schena et al. 2004). In general, qPCR products are short amplicons that range from 100-150 base pairs (bp) and quantification of target dsDNAs can be measured directly using a fluorimetric PCR thermocycler.

qPCR and plant pathogenic fungi

Real-time PCR has been used to quantify the fungal biomass of *M. grisea* in rice plants and the amount of fungal biomass was found to increase with time in infected tissues. Also, the proportion of certain fungal RNAs increased over time compared to the total RNAs extracted from infected plant tissue (Qi and Yang 2002). In particular, the 28S rDNA gene was found to be useful for tracking the amount of *M. grisea* DNA present in rice plant tissues and allowed for high-throughput screening useful in a diagnostic setting where multiple samples are often obtained.

Infection of cereals by certain toxigenic *Fusarium* spp. is not tolerated in the food industry, so detecting toxic levels of the pathogen is very important. Trichodiene synthetase (*Tri5*) is an essential enzyme in *Fusarium* spp. for the production trichothecene mycotoxins (Niesen and Vogel 1998). In one study, thirty-two wheat samples infected with 20 species of *Fusarium* were assayed using qPCR to quantify the

presence of the *Tri*5 (Schnerr et al. 2001). Using this assay, down to 0.05 ng of *Tri*5 DNA could be detected in naturally contaminated samples.

Fungal gene expression *in planta* can be quantified using qRT-PCR and can also be used to confirm microarray data. Gene expression during nitrogen starvation *M. grisea* was followed using microarrays (Donofrio et al. 2006). The *in planta* expression of seven genes-of-interest identified from the microarray data were evaluated using qRT-PCR and found to be expressed similarly to that seen during nitrogen starvation.

Expression of nitrogen metabolism genes in the ectomycorrizal fungus *Tuber borchii* during *in vitro* culture and *in planta* symbiosis has been examined using qRT-PCR (Guescini et al. 2003; 2007; 2009). To normalize expression data between samples, the *T. borchii* 18S rDNA was used as an internal reference gene. Using qRT-PCR, the expression levels of nitrate transporter, nitrate reductase, nitrite reductase and nitrogen regulatory element were determined to be 15-, 8-, 10- and 3-fold higher in ectomycorrhizal tissues than in control vegetative mycelia, respectively.

VI. Accomplishments of Thesis Research:

First, several isolates of *P. omnivora* were analyzed using PFGE and telomere fingerprinting in order to attempt to characterize its genome and to determine if chromosomal variation occurs. Second, the cytology of the multinucleate hyphae of *P. omnivora* was examined using the fluorescent DNA stain DAPI to visualize the number of nuclei in each cell of the different isolates. Next, genes-of-interest in the *P. omnivora* genome sequence were identified and categorized according to Gene Ontology. Genes

that were selected are involved in ‘housekeeping’ functions such as chromatin remodeling, fungal cell wall biosynthesis, glycogen storage, virulence and secondary metabolism. Finally, expression of these genes-of-interest in vegetative mycelia, mycelia exposed to host and nonhost root exudates, and 4 and 8 week-old sclerotia was estimated using qRT-PCR.

CHAPTER II

ATTEMPTS TO CHARACTERIZE THE GENOME SIZE AND ORGANIZATION OF *PHYMATOTRICHOPSIS OMNIVORA*

INTRODUCTION

Since the mid 1980s, electrophoretic karyotyping has revealed that most fungi have a vegetative chromosome number between 5 and 20, and the genomes are estimated between 10 to 40 MB (Beadle et al. 2003). The technique Pulsed Field Gel Electrophoresis (PFGE) separates DNA molecules in an agarose gel by alternating electric currents. Because the chromosomes of most fungi are small, this method gives researchers improved resolution when visualizing the DNA. Previous investigations have shown that electrophoretic karyotyping can be used to separate fungal chromosomes based on their size (Orbach et al. 1996; Tooley et al. 1994; Fraissiner-Tachet et al. 1995; Plummer and Howlett 1993). To isolate intact chromosomes from fungal cells without shearing the DNA, intact protoplasts must be isolated from the hyphae, spores, or fruiting bodies , using cell wall degrading enzymes in an isotonic solution containing osmolytes.

Protoplasts are embedded in agarose and treated with a proteinase/detergent solution to lyse the cells and inactivate nucleases, thereby releasing intact chromosomal DNA. This DNA is then separated using specialized electrophoresis designed to separate high molecular weight molecules. Contour clamped homologous electric field (CHEF) electrophoresis is a type of PFGE. CHEF uses electrodes placed in a hexagon pattern, and alternating electrical pulses to separate larger and small chromosomes. It has been demonstrated that fungi can have high variability in chromosome number among different isolates within the same species (Zolan 1995). Based on previously determined phylogeographic differences among isolates, varying numbers of chromosomes among these isolates was expected. To determine if *P. omnivora* shows similar variation in chromosome number among isolates from Texas, Arizona and Oklahoma, protoplasts from each *P. omnivora* isolate were prepared, embedded in agarose and lysed. DNA released from protoplasts was subjected to CHEF to separate and enumerate the chromosomes present. **In the first section of this chapter**, the separation of chromosomes from several isolates of *P. omnivora* by CHEF ultimately failed due technical failures, which will be discussed.

Previous research has shown *P. omnivora* possesses 3 to 41 nuclei per hyphal cell, depending on the size of the hyphae (Neal et al. 1943; Hosford and Gries 1966). There is also evidence that this fungus possesses haploid, diploid, aneuploid, and polyploidy nuclei (Hosford and Gries 1966). It has been hypothesized that *P. omnivora* has developed a parasexual cycle over the course of its evolution, which lead to its heterokaryotic nature (Pontecorvo 1956; Käfer 1961). This means that hyphae from different individuals can fuse and exchange nuclei, which may undergo karyogamy

resulting in genetic recombination. This phenomenon is not uncommon in fungi that do not appear to have a sexual stage identified. **In the second section of this chapter,** the hyphal nuclei of *P. omnivora* were stained using the nucleic acid stain 4',6-diamidino-2-phenylindole (DAPI). Epifluorescence microscopy was used to confirm the mutlinucleate nature of this fungus, which may indicate these isolates are heterokaryotic.

All eukaryotic chromosomes contain telomeres, which are specialized DNA-protein structures at the termini that stabilize chromosomes and are necessary for replication of DNA at the 5' ends (Blackburn 1990). Previous investigations showed that counting the number of telomeres through telomere fingerprinting can be used to estimate the numbers of chromosomes for fungi (Blackburn 1990; Fierro and Martin 1999; Levis et al. 1997; Padmavathi et al. 2003; Zolan 1995). Telomere fingerprinting is possible since a conserved telomere hexanucleotide repeat, TTAGGG, occurs across all filamentous fungi (Blackburn, 1990), and can be used as hybridization probe to detect telomeres in blotted restriction enzyme-digested genomic DNA. **In the third section of this chapter**, two restriction enzymes were separately used to cut genomic DNA from different *P. omnivora* isolates, blotted to nylon membranes and hybridized against a labeled telomere probe $(TTAGGG)_4$. The number of bands hybridizing to the probe in each lane, divided by two, estimates the total number of chromosomes present. Isolate OKAlf8 was used for all initial experiments. Unfortunately, no well resolved telomere bands were identified using the telomere probe. The possible reasons for this failure are discussed.

MATERIALS AND METHODS

Fungal Isolates and Culture: Pure cultures of *P. omnivora* (Table 1) were isolated from diseased cotton or alfalfa plants according to Marek et al. (2009). Isolates were maintained by serial transfer on modified ATCC medium 1078 (M1078), containing per 1,000 ml distilled water: 1 g NH_4NO_3; 0.75 g $MgSO_4$; 0.4 g KH_2PO_4; 0.9 g K_2HPO_4; 0.1 g $CaCl_2$; 40 g glucose; 1 g yeast extract; 1 g peptone; 100 µl Vogel's trace elements (Vogel 1964) and 18 g agar.

TABLE 1. Isolates of *Phymatotrichopsis omnivora* used in this study.

Strain	Host	Origin		
		Location	Date	Collector
OKAlf8	Alfalfa	Belleville, OK	2003	S. Marek
NFAlf4	Alfalfa	Ardmore, OK	2004	S. Marek
ATCC 22316	Cotton	Arizona	1975	H.E. Bloss
ATCC 32448	Peach	Mexico	1973?	H.E. Bloss
Ranch1	Cotton	Austwell, TX	2005	S. Marek
Rick5	Cotton	Austwell, TX	2005	S. Marek
Trumbull4	Cotton	Trumbull, TX	2005	S, Marek
MaudLowe8	Cotton	Austwell, TX	2005	S. Marek
EC59	Cotton	El Campo, TX	2005	S. Marek

Molecular Karyotyping of* P. omnivora *Using CHEF

Protoplast isolation from mycelia: A medium sized cork borer (No.8) was used to cut mycelial agar plugs from 7-10 day old M1078 cultures of *P. omnivora*. Three plugs were used to inoculate each of six deep petri dish (100 × 25 mm) containing 25 ml M1078 broth, carefully wrapped with parafilm and incubated at 28°C incubator for 7-8 days.

Mycelial mats were harvested by vacuum filtration using sterilized filter paper (Fisherbrand 9.0 cm, Waltham, MA) on a Buchner funnel (9.0 cm diameter) with a perforated plate atop a 500 ml filtration flask, all inside a laminar flow hood. The mycelial mats were washed three times with sterile water to remove residual broth. After excess moisture was removed from mycelial mats by vacuum filtration, the mats were used either for protoplast formation or a tissue homogenizer (Fig. 1).

Three mycelial mats were transferred into a 250 ml Erlenmeyer flask containing 60 ml Glucanex enzyme solution (Sigma-Aldrich, St. Louis, MO; 20 mg/ml; 0.2µm-filter-sterilized) in KC buffer (0.64M KCl, 0.2M $CaCl_2$, 26mM MES, pH 8.0). Two flasks containing 3 mats each were used per preparation. The flasks were covered with sterile foam plugs and aluminum foil and placed in a 28°C incubator shaking at 80 rpm overnight. The enzyme/protoplast solution was filtered through sterile Miracloth (EMD Chemicals, San Diego, CA) and the filtrate collected in a 50 ml falcon centrifuge tube. A few drops of this protoplast solution were placed on a microscope slide, cover slipped and protoplast formation evaluated using a microscope. Fourteen ml of the protoplast solution was overlaid with 10 ml STC buffer (1M sorbitol, 50mM Tris-Cl, pH 8.0, 50mM

$CaCl_2$), in 30 ml Corex centrifuge tubes and centrifuged for 5 minutes at 5000 x g. After centrifugation, the supernatant was removed and discarded and a pellet containing the protoplasts had precipitated on the bottom of the tubes. An additional 14 ml protoplast solution and 10 ml STC buffer was added to the previously pelleted protoplasts by resuspending in the same tubes and the process was repeated. The protoplasts from each of the original flasks were collected and pooled in one Corex tube each generally after four centrifugation runs. After the final round of centrifugation, the pellet in one tube was completely resuspended in 1 ml STC buffer and transferred to the other Corex tube and mixed with the other pellet. The concentration of protoplasts was determined using a hemocytometer. The pooled protoplast suspension was centrifuged for 5 minutes at 5000×*g* and the supernatant removed. The final pellet was resuspended in 300 µl GMB buffer (0.9M sorbitol, 125mM EDTA pH 7.5; Carolyn Young, personal communication).

Protoplast plug preparation for CHEF: About 700 µl of 50°C 1% low-melting point agarose (Biorad, Hercules, CA) was mixed with 300 µl protoplast suspension, resulting in 6×10^5 to 6×10^8/ml protoplasts (depending on prep used; Table 5) embedded in 0.7% low melting point agarose and this mixture was pipetted into CHEF plug molds (Biorad). The molds were placed 4°C for 20 minutes. The solidified protoplast-agarose plugs were then transferred to a 50 ml falcon tube containing 10 ml of SE buffer (2% SDS, 0.25M EDTA pH 8.0). The plugs were incubated in a 55°C water bath and then transferred to 10 ml of 10× ET buffer (0.5M EDTA, 10mM Tris pH 8.0) containing 1% sodium lauroyl sarcosine and 20 µl proteinase K (20mg/ml stock solution). The plugs were incubated for 24 hours in a 50°C water bath and then washed three times (1 hour between washes) with 1× ET buffer. The plugs were stored in 10 ml 10X ET buffer at 4°C for up to 6 months.

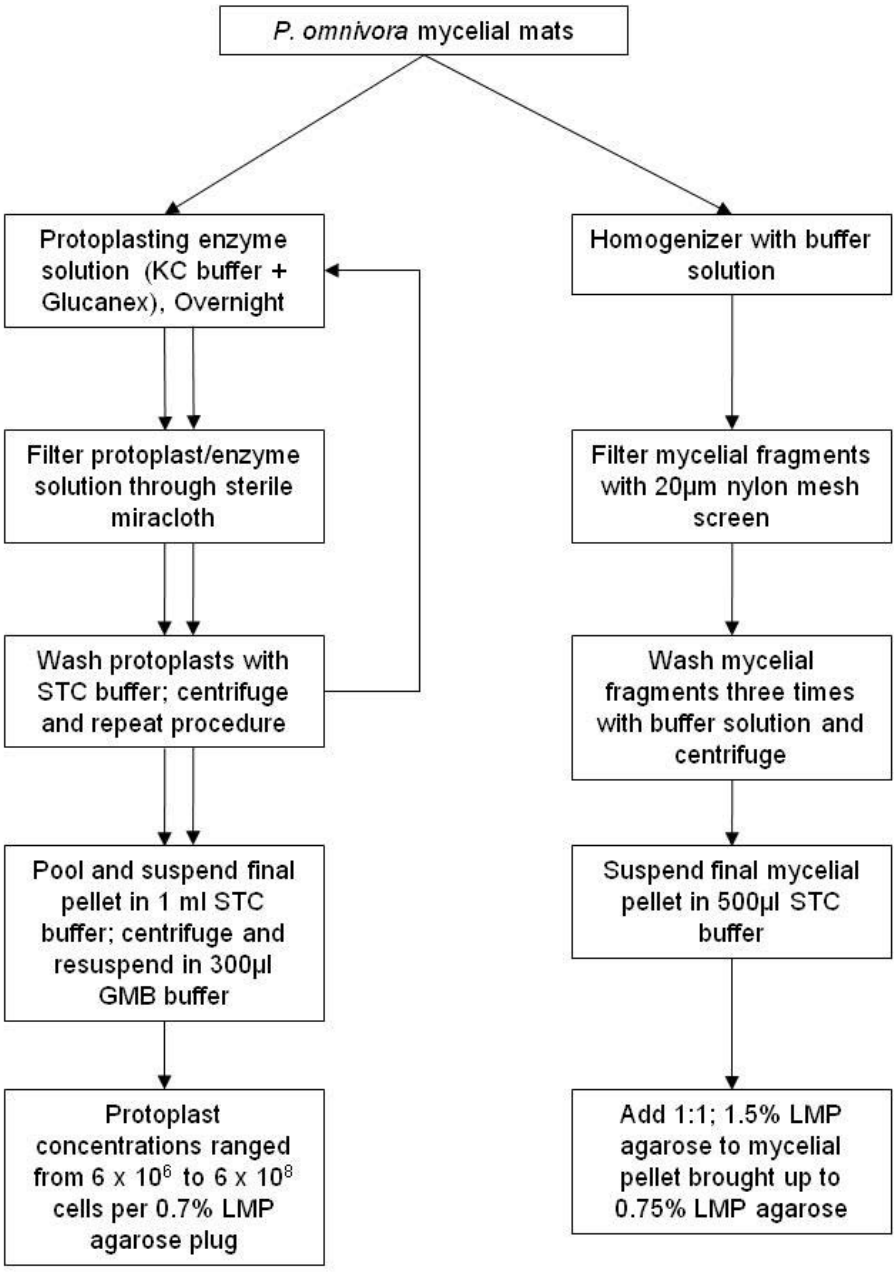


FIG. 1. Flow chart describing protoplast preparation procedures from *Phymatotrichopsis omnivora* mycelia.

Mycelial homogenization for CHEF gels: The following procedure was modified from Crouch (1992). Mycelial mats of *P. omnivora* were ground in 10 ml STE buffer solution (25mM Tris-HCl pH 7.5, 1M sorbitol, 25mM EDTA) using a tissue homogenizer (Kontes, Tissue Grinder 15 ml, Vineland, NJ) and then filtered through a sterile 20 µm nylon mesh screen. The flow through mycelial fragment filtrates were transferred to a 50 ml falcon centrifuge tube and centrifuged for 10 minutes at 5000 x g at room temperature. The supernatant was discarded and the pellet was resuspended in 10 ml STE buffer. This 'washing' step was repeated twice more. The final pellet was resuspended in 500 µl STC buffer. An equal amount of 1.5% low melting point agarose (Biorad) was added to the pellet. The solution was pipette into plug molds (Biorad) and maintained at 4°C to solidify. The plugs were equilibrated in 10 ml of 0.5M EDTA, pH 8.0 for 1 hour, then transferred to a solution containing 10 ml of 1mg/ml proteinase K, 1% sodium lauroyl sarcosine and 0.45M EDTA, pH 8.0 and incubated at 50°C for 20 hours. After proteinase K treatment, plugs were washed several times with 0.5M EDTA, pH 8.0, then stored in 10 ml 0.5M EDTA, pH 8.0 at 4°C for up to 6 months.

PFGE gel preparation: Molten 0.7% agarose (Certified Megabase Agarose, Biorad) in TBE buffer was poured into a CHEF gel casting mold (Biorad) and allowed to solidify. Once the gel solidified and the well comb removed, protoplast-gel plugs were placed into the wells using a scalpel (fine point) and overlaid with molten (50°C) 0.7% agarose-TBE (Certified Megabase Agarose) poured over the wells to seal them. The gel was then removed from the casting stand and placed in the electrophoresis chamber. Two liters of 0.5X TBE buffer (chilled to 4°C) was added into the chamber and PFGE run using the Biorad CHEF mapper system. In each PFGE run, in addition to the *P. omnivora*

protoplast plugs, two lanes were loaded with plugs containing DNA size markers from *Schizosaccharomyce pombe* (3 chromosomes, 3.5 - 5.7 Mb) and *Saccharomyces cerevisiae* (~14 chromosomes, 0.225-2.2 Mb). In addition to being size standards, these acted as positive DNA loading controls and confirmed electrophoresis settings caused the migration and separation of DNA bands of the appropriate size range. Numerous PFGE run parameters were assessed (Table 2).

TABLE 2. CHEF parameters used in experiments with protoplast and mycelial preparations from *Phymatotrichopsis omnivora*.

Experiment	Voltage	Pulse time	Total run time	Angle	Buffer	Agarose %
1[a]	40V	90 min	178 h	120°	0.5X TBE	0.7%
2[b]	60V	3-15 min	168 h	120°	0.5X TBE	0.7%
3[c]	40V	30-60 min	120 h	106°	0.5X TBE	0.7%
4[d]	50V	1200-6000 s	240 h	106°	0.5X TBE	0.7%

[a] adapted from Orbach et al. (1996)

[b] adapted from Tooley et al. (1994)

[c] from this study

[d] adapted from Young (2005)

Nuclear Staining of P. omnivora *Hyphae:* Superfrost microscope slides (Fisherbrand, Waltham, MA) were sterilized in 95% ethanol in a coplin jar. The slides were then dipped in M1078 media, placed onto a 1.8% water agar petri plate and allowed to air-dry slightly in the laminar flow hood. An M1078 agar plug colonized by *P. omnivora* mycelium was placed next to the slide so the fungus would grow towards the slide. The water agar plates with the inoculated slides were incubated at 28°C for 7 days. After the mycelium covered most of the slide, the slide was lifted off the plate and transferred into a coplin jar containing formalin-acetic acid-alcohol (FAA) fixative (10:5:50:35, 37% formalin: glacial acetic acid: 95% ethanol: distilled water) and maintained overnight at 4°C. The slides were then dehydrated through a methanol series (20 min in each, 50%, 70%, and 100% methanol), air-dried 20 minutes in a laminar flow hood to bond hyphae to the microscope slide and then re-hydrated through the reverse order of the previous methanol series ending in water. The slides were then placed in a coplin jar containing 1 μg/ml DAPI for 1 hour and then rinsed in sterile distilled water for 1 hour. The slides were examined using Nikon Eclipse E800 epifluorescent microscope (Melville, NY) using the UV-2E/C DAPI filter and Nomarski differential interference contrast (DIC) to image the nuclei and hyphal cells, respectively. Separate monochrome DAPI and DIC images of each field were captured using a QImaging Retiga 2000R charge-coupled device (CCD) camera (Quantitative Imaging Corp., Surrey, BC, Canada). Monochrome images (grayscale) were artificially colored (DAPI = light blue and DIC = white) and combined as layers using QCapture Pro version 5.1.1.14, the camera's image capture program.

Genomic DNA Extraction from Phymatotrichopsis omnivora *Isolates*: Lyophilized mycelial mats from isolates OKAlf8, NFAlf4, Rick5, EC59, Maudlowe8, ATCC 22316, ATCC 32448, Ranch1, and Trumbull4 were ground in liquid nitrogen using a mortar and pestle and genomic DNA (gDNA) isolated according to the protocol of Möller et al. (1992) modified to increase the volumes of reagents to a maxi-prep scale (50 ml) instead of the mini-prep scale (1 ml) reported in the article. Isolated gDNA was analyzed spectrophotometrically (NanoDrop, Wilmington, DE) and separated by electrophoresis on a 0.5× TBE gel. Aliquots were stored at -80°C until used. The concentrations of DNA in preparations from each isolate before restriction digests are shown in Tables 3 and 4.

TABLE 3. Concentrations and qualities of gDNA isolated from different isolates of *P. omnivora*.

Name	µg/µl	260/280
OKAlf8	0.851	2.01
Maudlowe8	5.411	1.03
Trumbull4	0.100	2.01
EC59	3.409	2.02
Ranch1	0.885	2.01
ATCC 22316	0.677	2.15
ATCC 32448	2.871	2.04
NFAlf4	1.028	1.91
Rick 5	1.332	2.00

TABLE 4. Concentrations of DNA from *P. omnivora* isolates used for each restriction digest prior to dividing in 5 µg aliquots and telomere fingerprinting.

Enzyme	OKAlf8	NFAlf4	Rick5	ATCC 32448
***Bgl*II**	177ng/µl	134.7ng/µl	172.8ng/µl	94.6ng/µl
***EcoR*I**	254ng/µl	162.7ng/µl	51ng/µl	103.8ng/µl

Preparation of Labeled Hybridization Probes: Three DNA probes were generated by PCR from OKAlf8 gDNA. Two gene-specific probes, glycogen synthase 1 (GLYS, 576 bp) and RNA polymerase II, subunit 2 (RPB2, 1113 bp), were amplified with the respective primer pairs, GLYS1-BAC-FOR 5'-GGCAAAAGAGGCATTTACCA-3' and GLYS1-BAC-REV 5'-CGCAAACATTCGTCTCTTCA-3' and PoRPB2-Forward 5'-TCTTGAGTGTAGGTGCCGTTGAGT-3' and PoRPB2-Reverse 5'-CAAATCAGGCACGATTCCTTCGCA-3', which were designed based on *P. omnivora* genome sequences at the University of Oklahoma (http://www.genome.ou.edu/fungi.html) using GoTaq Green Master Mix (Promega, Madison, WI) with the following PCR program: an initial denaturation at 95°C for 5 minutes; followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds , and 72°C for 1 minute, and a final extension at 72°C for 10 minutes. The telomere fingerprinting probe (variable bp lengths) was amplified using the complementary telomeric repeat primers $(CCCTAA)_4$ and $(TTAGGG)_4$, the same polymerase as gene-specific probes and the PCR program from Ijdo et al. (1991). The resulting PCR products were cleaned and labeled using the AlkPhos Direct Labeling and Detection System (GE Healthcare, Piscataway, NJ) according to manufacturer's instructions. Labeled probes were stored in 50% (v/v) glycerol at -20°C. To asses probe detection limits, 5 µl drops of OKAlf8 gDNA in a $\log_{10}$ serial dilution (10^{-1},10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) were spotted onto a positively charged nylon membrane (Millipore, Billerica, MA), UV-cross linked, and hybridized with labeled GLYS, RPB2, and telomere probes according to manufacturer's instructions (GE Healthcare), then membranes were treated with hybridization buffer for 1 hour at

55°C, hybridized with labeled probes (10 ng/µl) for 24 hours at 55°C in hybridization buffer, then washed with hybridization buffer for 10 minutes at 55°C twice.

Southern Blot Hybridization: Five µg gDNA from each isolate of *P. omnivora* were digested with the restriction enzymes *Bgl*II or *EcoR*I overnight at 37°C. The digested DNA was separated by electrophoresis in a 1% agarose gel (TAE buffer), and stained with 0.5 µg/ml ethidium bromide to confirm separation. DNA was then transferred to a positively charged nylon membrane by overnight capillary transfer (Fig 2) according to the protocol by Brown (1993). After overnight transfer, the gel was stained with 0.5 µg/ml ethidium bromide to visualize DNA transfer to the membrane. The membrane was pre-hybridized at 55°C for one hour and labeled probe was added according to manufacturer's instructions (AlkPhos Direct, GE Healthcare). The membrane with labeled probe was allowed to hybridize overnight before post hybridization washes were performed. Two washes were done with hybridization buffer at 55°C for 10 minutes.

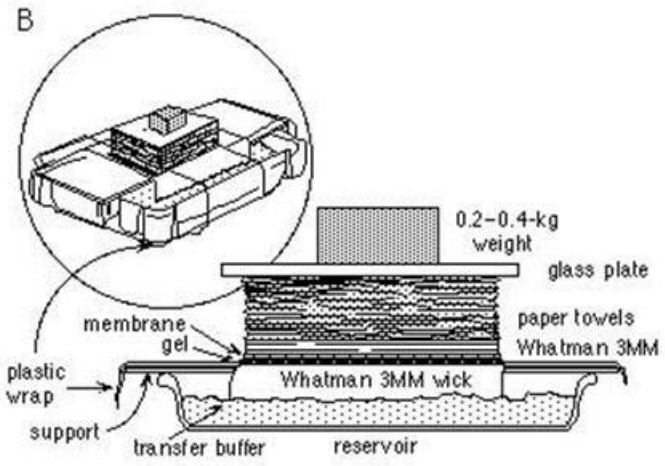


FIG. 2. Capillary transfer of DNA from agarose gel to nylon membrane (from Brown 1993)

Labeled Probe Signal Detection using CDP-Star: Detection of alkaline phosphatase (AP)-labeled probe was performed using the chemiluminescent AP substrate CDP-Star (Tropix, Inc. Bedford, MA) according to the AlkPhos Direct Labeling and Detection System manufacturer's protocol (GE Healthcare). After incubating hybridized blots with CDP-Star blots were exposed to chemiluminescence grade autoradiography film CL-Xposure Film (Thermo Scientific, Waltham, MA) inside a photography cassette for an initial exposure time of 20 minutes. The exposure time was prolonged up to 1 hour depending on signal strength.

RESULTS and DISCUSSION

Molecular Karyotyping Attempts using CHEF of P. omnivora *Protoplasts*

Initial attempts were unsuccessful (data not shown) because the CHEF Mapper power supply needed repairs. The initial CHEF gel analyses after the power supply was repaired were not successful even though the protoplasts numbers seemed promising (1.0 x 10^5-2.1 x10^5 protoplasts/ml and optimal concentration is 6.0 x 10^8/ml). To increase the amount of protoplasts released from the *P. omnivora* mycelial mats, cell was degrading enzymes (Glucanex) concentrations were increased to 20 mg/ml from 10 mg/ml. Table 5 shows the estimated protoplast numbers released after this improvement.

TABLE 5. Protoplast concentrations released from mycelial mats of *P. omnivora* isolates after increasing protoplasting enzyme concentration to 20 mg/ml. Protoplast concentrations were estimated using a hemacytometer.

Strain	Concentration (protoplasts/ml)
OKAlf8	$6.4 X10^5$
NFAlf4	$2.7 X 10^6$
Rick5	$2.6 X 10^6$
Maudlowe8	$1.2 X 10^6$
ATCC 22316	$5.8 X 10^8$

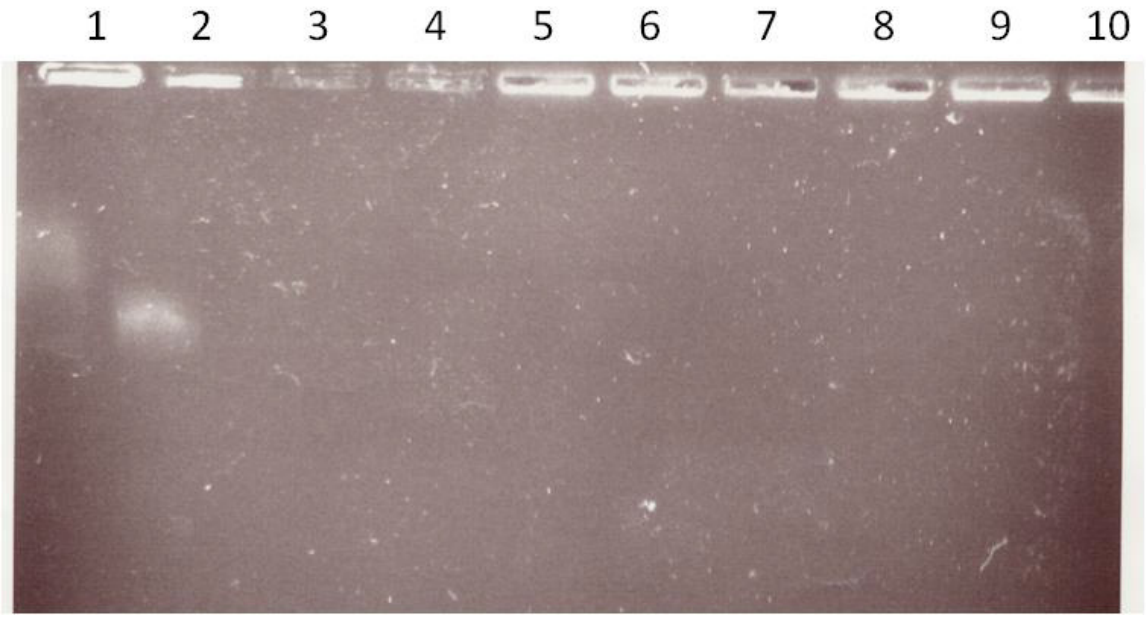


FIG. 3. CHEF analysis carried out after the power supply was repair. Lanes 1 and 2 are chromosome standards from *S. pombe* and *S. cerevisiae*, respectively. Lysed protoplast from *P. omnivora* isolates were loaded as follows: lane 3 OKALF8, lane 4 NFAlf4, lane 5 Rick5, lane 6 EC59, lane 7 Maudlowe8, lane 8 Trumbull4, lane 9 ATCC 33316 and lane 10 ATCC 32448. The gel was run according to Experiment 1 in Table 2 and post-stained with 0.5 µg/ml ethidium bromide.

These improved protoplast preparations were loaded in plugs onto a CHEF gel but electrophoresis resulted in poorly separated chromosome standards and no detectable DNA from protoplasts (Fig 3). Protoplasts (and DNA) were not sufficiently concentrated in the plugs and the CHEF power supply program was incorrect.

In another attempt to separate chromosomes of *P. omnivora*, CHEF plugs were again prepared from the *P. omnivora* protoplast preparations (Table 5) and a CHEF program previously used to separate the chromosomes of *Tilletia indica* (Tooley et al. 1994) was used (Table 2, Experiment 2). Three chromosomes were expected and the 5.7Mb and the 4.6 Mb chromosomes likely remained unresolved as one band, but distinguishable from the 3.5Mb chromosome. This resulted in only two chromosomes from the *S. pombe* standard being resolved (Fig 4, lane 1). Eleven chromosomes were well-resolved from the *S. cerevisiae* standard (Fig 4, lane 2) and ranged from 2.2 Mb to 450 kb. Fifteen chromosomes were expected from *S. cerevisiae* and the smaller chromosomes probably migrated off the gel. The wells at the top of each lane in Fig 4 look like significant DNA failed to move out of the wells. No chromosomes were migrated out of the protoplast plugs from the five *P. omnivora* isolates suggesting that CHEF program did not persist long enough to move large chromosomes (>5.7 Mb) out of the protoplast-plugs or the protoplasts were insufficiently lysed (Fig 4, lanes 3-5) or were not sufficiently concentrated, as indicated by the diffuse staining of the wells (Fig 4, lanes 6 and 7).

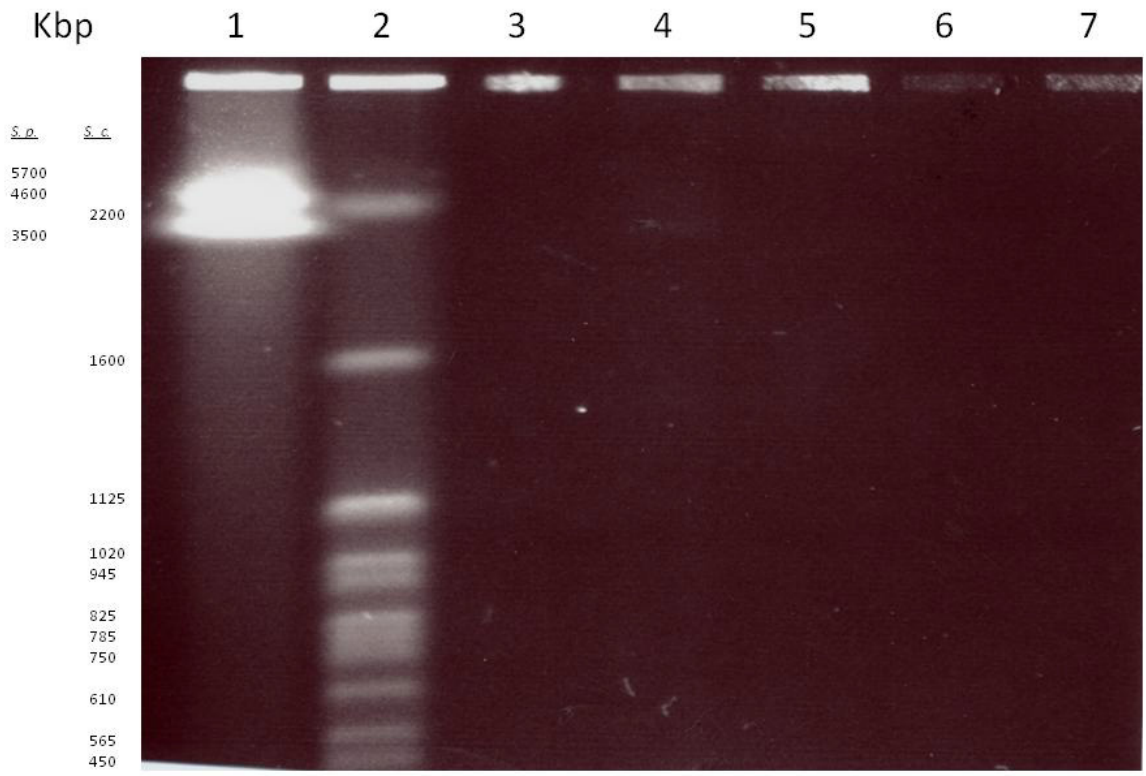


FIG 4. CHEF analysis of lysed protoplasts from *P. omnivora* isolates. Lanes 1 and 2 are the standards from *S. pombe* and *S. cerevisiae*, respectively; lane 3 is OKAlf8, lane 4, NFAlf4; lane 5, Rick5; lane 6, EC59; lane 7, ATCC 22316. The gel was run according the Experiment 2 conditions in Table 2 and post-stained with 0.5 µg/ml ethidium bromide.

Molecular Karyotyping of P. omnivora *Mycelial Homogenates*

After many unsuccessful attempts to separate chromosomes from *P. omnivora* protoplasts, mycelial homogenates were employed for 2 reasons; a) DNA in fragmented hyphal cells should be durable and b) success using mycelial homogenates of *P. omnivora* for PFGE had been reported previously (Crouch 1992). In the first attempt, standard plugs from *S. pombe* and *S. cerevisiae* and six mycelial homogenate plugs from isolates (OKAlf8, NFAlf4, Rick5, EC59, ATCC 22316, and Trumbull4) were loaded and analyzed by CHEF. The DNA failed to migrate from the wells (Fig 5). Another attempt was made using standard plugs from *S. pombe* and *S. cerevisiae* and mycelial homogenate plugs from four isolates of *P. omnivora* (OKAlf 8, NFAlf4, Rick5, and ATCC 22316). This time we chose a different set of conditions with longer pulse times. Unfortunately, all chromosomes from standards and *P. omnivora*, failed to resolve in the gel (Fig 6). Smeared bands in each lane also could indicate DNA degradation occurred. The *S. pombe* and *S. cerevisiae* standards in (Fig 6) show chromosomes migrated out of the wells, but resolved poorly. For *S. pombe*, the expected three chromosomes were resolved, but the *S. cerevisiae* chromosomes remained clustered. For the first time, DNA migrated out of a *P. omnivora* well, isolate OKAlf8 (lane 3, Fig 6). However, no distinct chromosomal bands were apparent, as in the standards. The size and smearing of the band in lane 3 indicates it may correspond with sheared euchromatin released from the homogenized mycelial cells and that some DNA degradation may have occurred. For isolates NFAlf4, Rick 5, and ATCC 22316 (lanes 4-6, Fig 6), no bands were present in

the gel. Significant ethidium bromide-staining of the wells above each lane indicated DNA may failed to migrate out of the wells, possibly due to insufficient cell lysis required to release chromosomal DNA.

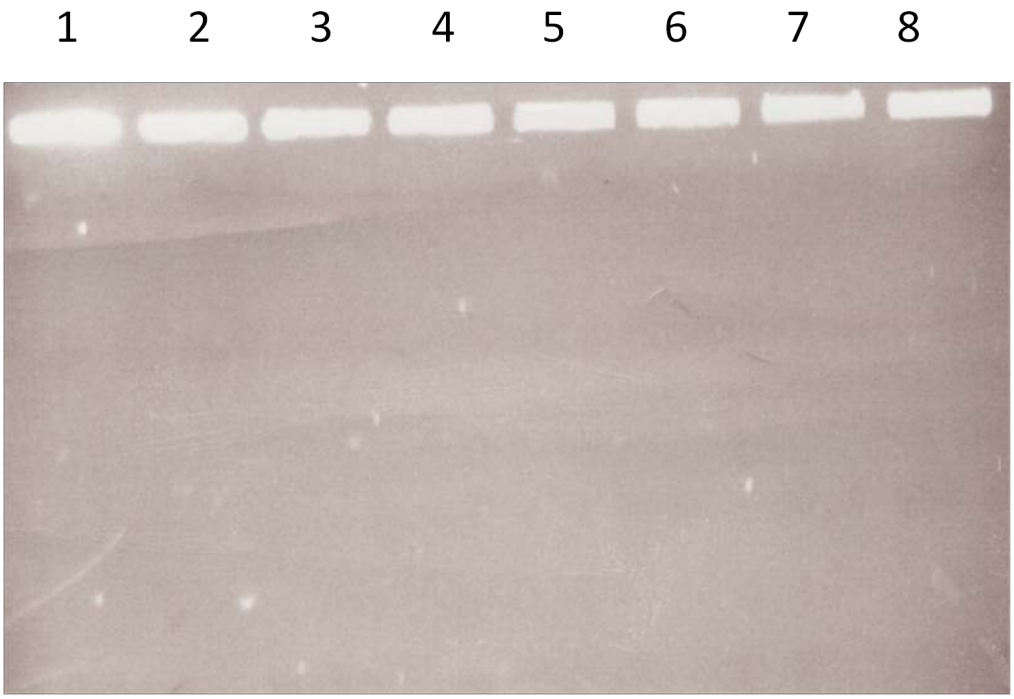


Fig 5. CHEF gel using mycelium plugs. Lane 1, *S. pombe*; lane 2, *S. cerevisiae*, Mycelium plugs from *P. omnivora* isolates are as follows: lane 3 is OKAlf8; lane 4, NFAlf4; lane 5, Rick5, lane 6, EC59; lane 7, ATCC 22316; lane 8, Trumbull4. The gel was run according the Experiment 3 conditions in Table 2 and post-stained with 0.5 µg/ml ethidium bromide.

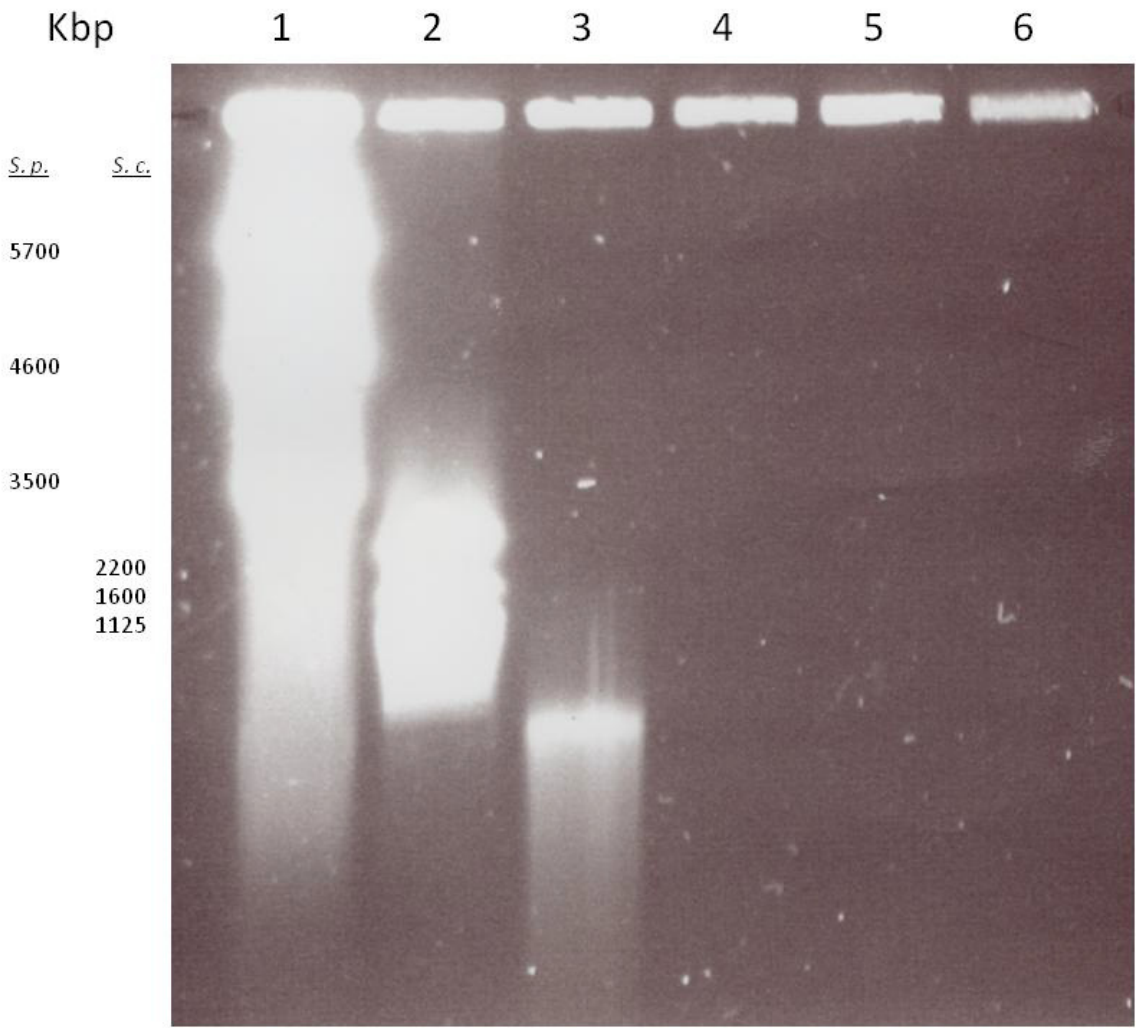


FIG 6. CHEF gel from prepared mycelium plugs. Lane 1, *S. pombe*; lane 2, *S. cerevisiae*; lane 3, OKAlf 8; lane 4, NFAlf4; lane 5, Rick 5; lane 6, EC 59. The gel was run according the Experiment 4 conditions in Table 2 and post-stained with 0.5 μg/ml ethidium bromide.

Staining of Nuclear DNA in P. omnivora *Hyphal Cells*

To determine the number of nuclei present in each *P. omnivora* hyphal cell isolates, EC59, NFAlf4, Ranch1, and Rick5, each isolate was grown on agar coated microscope slides, fixed, stained with DAPI, and examined with an epifluoresence microscope. Visualization of the nuclei as fluorescent blue circles within the hyphal cells indicated DAPI staining was successful (Fig 7 A-D). Thin (young) hyphal cells were expected to possess fewer nuclei than thicker (older) hyphal cells. As expected the number of nuclei per cell observed was relative to the size of the hyphae, except in the case of hyphal cells undergoing branching. In thinner hyphae, 2 to 5 nuclei were observed in each hyphal cell of isolate NFAlf4 and Rick5 (Fig 7B and 7D). In the thicker hyphae of Ranch1 5 to 6 nuclei per cell were observed (Fig 7C). However, in the branching hyphal cells of EC59 (Fig 7A) 24 nuclei per cell were observed. This large number of nuclei in EC59 may be due to the timing of fixation, which captured branching-induced nuclear division before intercalary septation could take place. Also, distended or lobed nuclei were observed in some cells (Fig 7A and 7C), which appeared similar to the aneuploid or diploid nuclei reported in *P. omnivora* (Hosford and Gries 1966). This may be a fixation artifact or due to the asynchronous mitosis of individual nuclei fixed during the M phase of cell cycle (telophase).

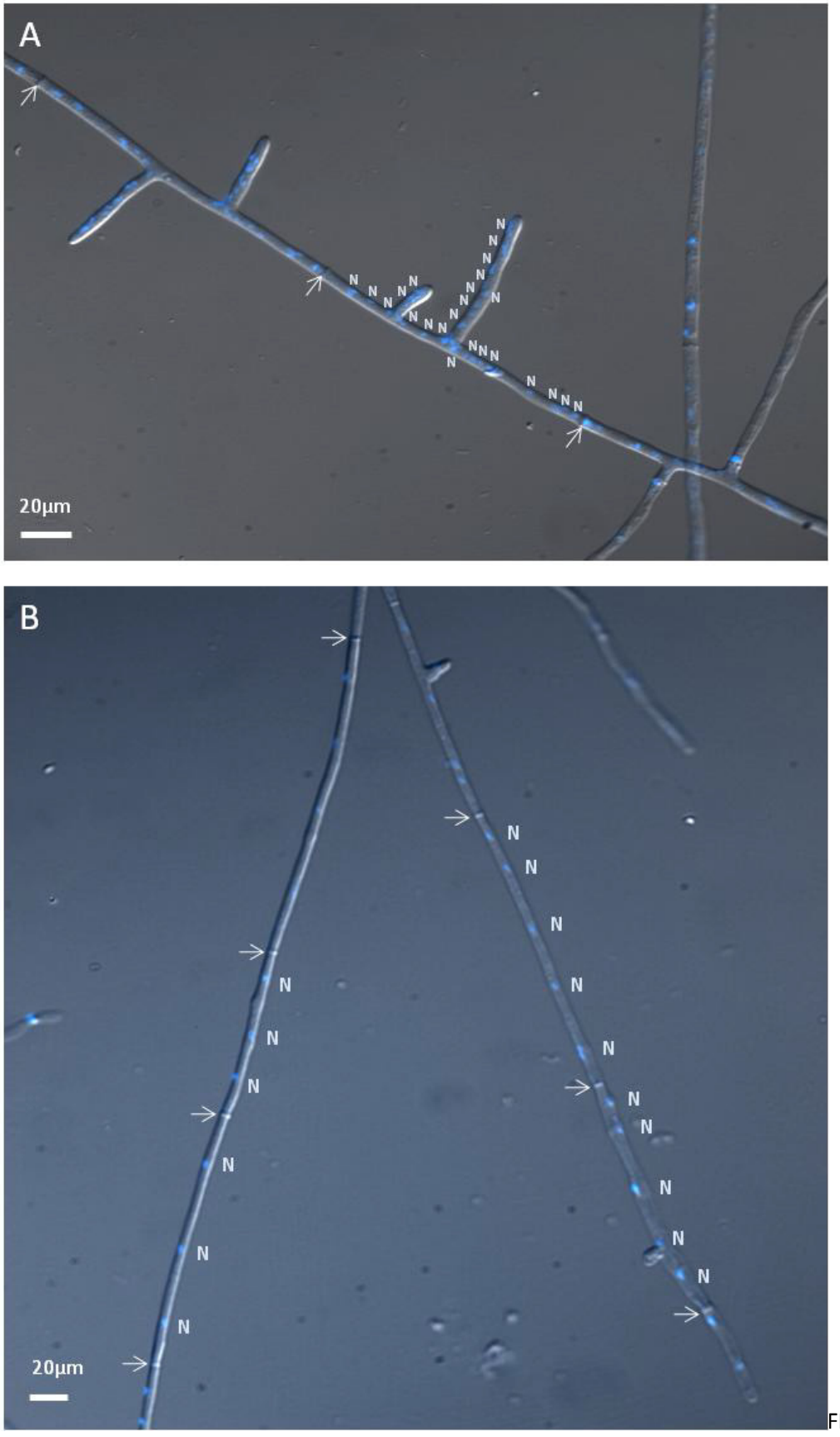
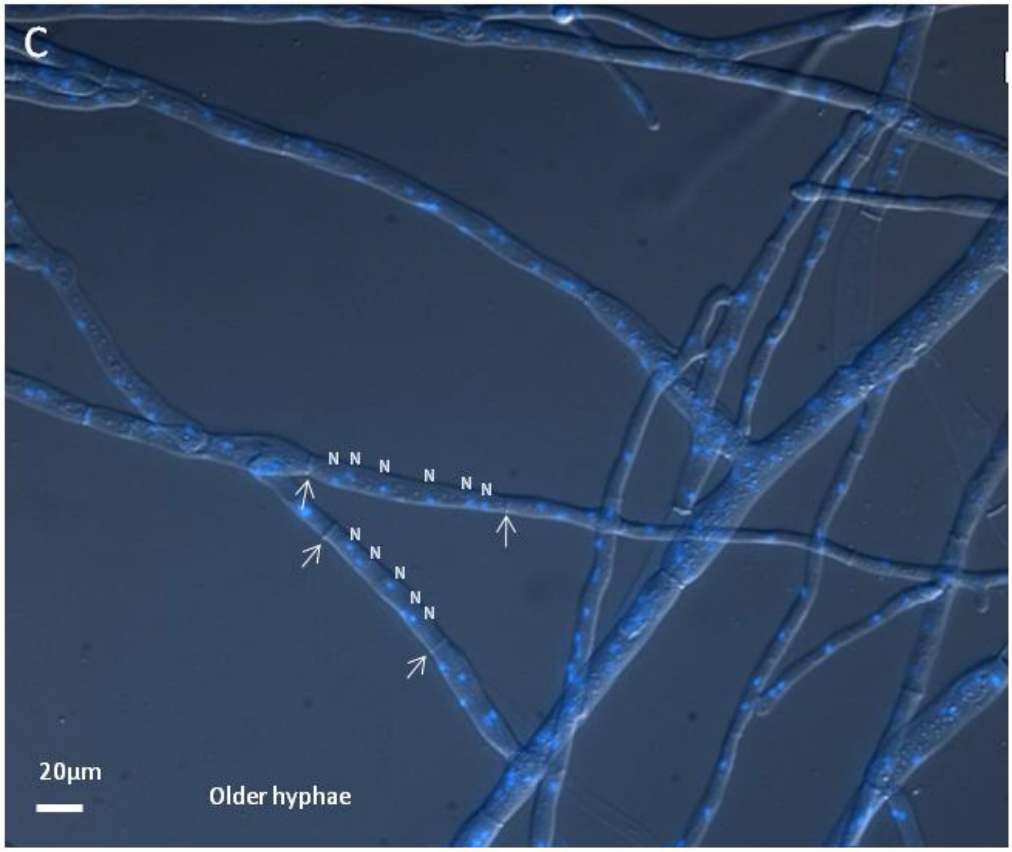


FIG 7



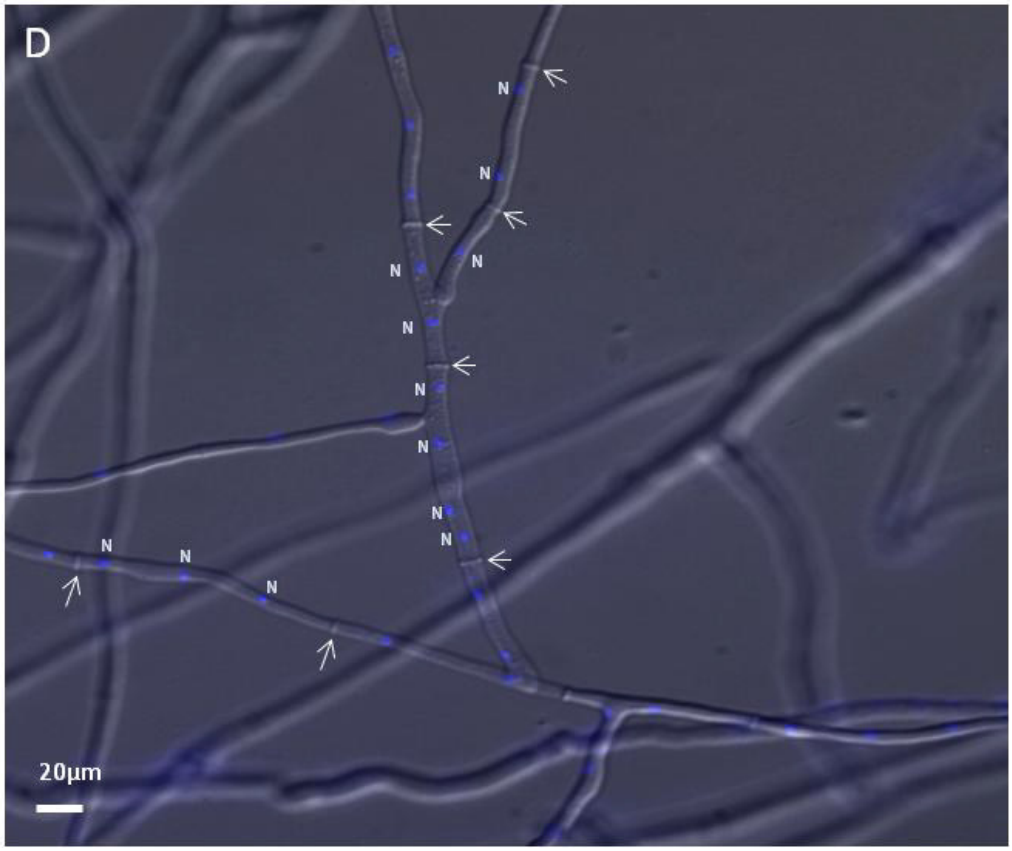


FIG 7

(cont.)

FIG. 7. DAPI-stained nuclei in the hyphal cells of *P. omnivora* isolates, with septa indicated by arrows and nuclei by "N". Hyphal cells of isolates EC59 (A), NFAlf4 (B), Ranch1 (C) and Rick5 (D) all display nuclei and septa. All images were captured at a total magnification of 400×. Scale bars are 20 microns in length.

Southern Hybridization and Telomere Fingerprinting Probes

The detection limits of labeled GLYS, RPB2 and telomere probes were determined by hybridizing the probes to dot blots of serially dilutions OKAlf8 gDNA when using a 20 minute film exposure time (Fig 8). The RPB2 and telomere probes detected $\geq$ 0.01 ng gDNA, while the GLYS probe detected $\geq$ 0.1 ng gDNA (10^{-2} dilution). The RPB2 and telomere probes were more sensitive than the GLYS probe. This was likely due to the proportionally larger amount of AP crosslinked to the RPB2 (1.1 kb) and telomeric (variable lengths, 100 bp – 3 kb) probes compared to the GLYS probe (576 bp)

When the hybridized Southern blots of digested OKAlf8 gDNA were exposed for 1 hour, no specific probe hybridization was observed (data not shown). In another attempt, gDNA from the isolates, Rick5, NFAlf4 and ATCC 32448, in addition to OKAlf 8, was digested, electrophoretically separated, blotted and hybridized to the probes (Table 4), but again no specific bands were observed. A few more repeated attempts also failed. Based on the probes' detection limits determined using gDNA dot blots, no specific bands may have been detected on Southern blots due to insufficient gDNA being transferred to the membrane or due to the relatively diluted state of the gDNA fragments carrying probes' target after digestion, separation and blotting.

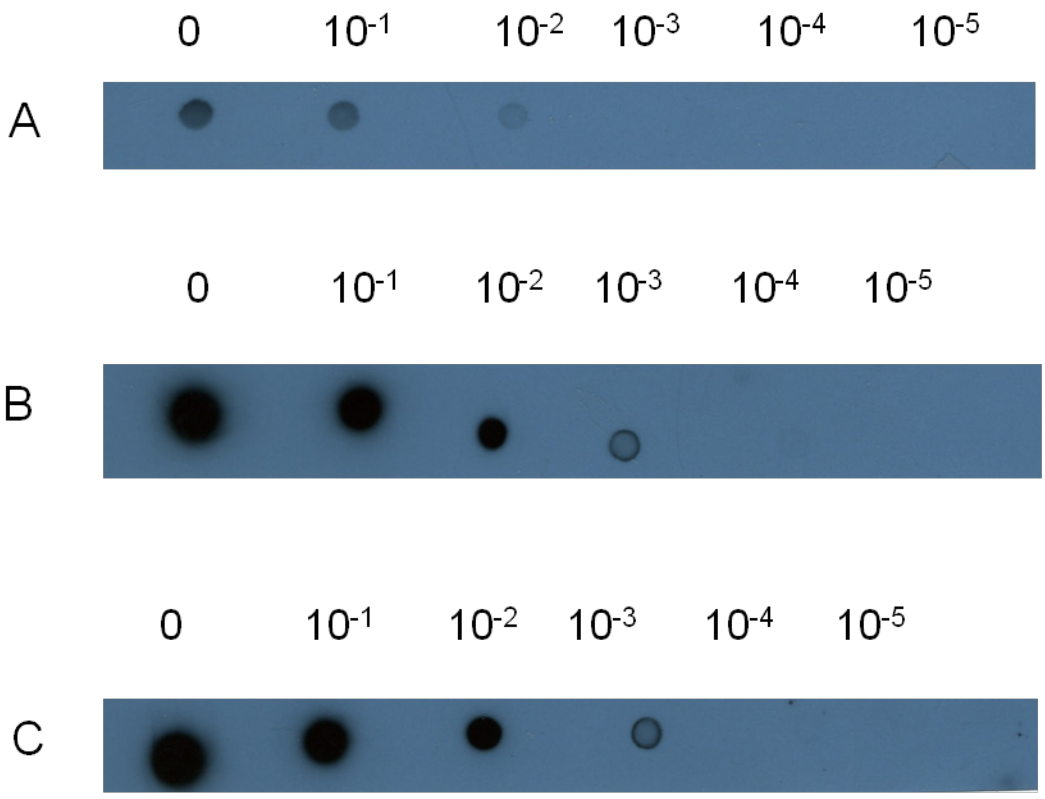


FIG. 8. Dot blots of serial dilutions of OKAlf8 gDNA hybridized with labeled probes to determine detection limits. GLYS (A), RPB2 (B), and telomere (C) probes detection limits shown. Films were exposed for 20 minutes.

FURTHER DISCUSSION AND CONCLUSIONS

Numerous experiments were performed to characterize the genome of *P. omnivora* and its genome organization using CHEF to separate the chromosomes of different isolates from various regions of the southwestern US. One of the challenges was to generate a sufficient number of protoplasts from *P. omnivora* mycelia. At first, 10 mg/ml protoplasting enzyme was used to digest the cell walls of mycelial mats but insufficient numbers of protoplasts were released. Increasing the concentration of enzyme to 20 mg/ml gave more sufficient numbers of protoplasts, though the amounts varied from each isolate (Table 5). According to the CHEF Mapper manual (Biorad), between 10^7 to 10^8 protoplasts/ml should be loaded per well, since 6.0×10^8 protoplasts/ml were necessary from *S. cerevisiae*. Using the increased protoplasting enzyme concentrations, 6×10^6 to 6×10^8 protoplasts/ml were achieved from all the isolates (Table 5). It was reported that 3.3×10^9 protoplasts/ml is sufficient for *Magnaporthe grisea* (Orbach et al. 1996). One reason for the variation in concentrations of protoplasts could be the drastic differences in the weights of the mycelial mats harvested from each isolate (data not shown). Other reasons could be the fragile nature of protoplasts, which are easily damaged by transfers between solutions and mixing with molten agarose, resulting in further reduction in protoplast numbers. Additionally, protoplast numbers may not necessarily equate with nuclear numbers, since some proportions of protoplasts lacked nuclei. Since the chromosomal standards were successfully separated, the CHEF parameters chosen for this study were adequate to separate *P. omnivora* chromosomes based on previously

reported sizes (Hosford and Gries 1966; Crouch 1992; Joshi et al. 2008). During optimization of the methods employed for producing protoplasts, we increased the amount of intact and viable protoplasts released from mycelia. However, unless protoplast concentrations similar to the CHEF standards are achieved, CHEF gels of *P. omnivora* chromosomes will not be possible.

As an alternative approach to analyze the genome of this fungus, we investigated the multinucleate nature of *P. omnivora*. Hyphae from several isolates were grown and fixed on a microscope slides and stained with DAPI. Nuclear counts proportional to hyphal diameter agree with previous findings (Hosford and Gries 1966). It is reasonable to conclude the possibility that the number of nuclei per cell increases as the hyphae get thicker because mitotic replication of nuclei continues as hyphal cells grow and age. This experiment showed that the number of nuclei increases with fungal age and that DAPI stains *P. omnivora* nuclei.

In our final attempt to characterize the genome of *P. omnivora*, we used telomere fingerprinting. Telomere fingerprinting was also unsuccessful even though the telomeric repeat probe quality and detection limit appeared to be sufficient. Also, complete transfer of the DNA to the nylon membrane was confirmed when the gel was post-stained with ethidium bromide and no DNA could be detected. Another possibility is that the restriction digests proceeded too long, resulting in nonspecific DNA degradation. Sufficiently high concentrations of DNA could be isolated from *P. omnivora*. It is not known how many copies of the telomere repeat sequence is present in *P. omnivora*'s chromosomes. If very low numbers of tandem telomeric repeats are present, then this may have been insufficient target sequence for detection.

Few reports are available on the molecular biology of *P. omnivora* and to our knowledge, most of the methods attempted in this chapter have never been used to characterize *P. omnivora*'s genome organization and chromosomes. This chapter has laid some groundwork and described protocols that could be further modified and optimized. This fungus warrants more attention because of the devastating losses it causes to cotton and alfalfa growers.

CHAPTER III

SELECTED GENE EXPRESSION PATTERNS IN *P. OMNIVORA*

INTRODUCTION

Reverse transcription polymerase chain reaction (RT-PCR) is an effective molecular tool to determine specific gene expression levels in response to external stimuli. Complementary DNA (cDNA) is reverse transcribed from total RNA and used as a template for PCR amplification of short DNA sequences using gene specific primers. The PCR product is viewed using agarose gel electrophoresis. This technique does not necessarily amplify PCR products to a discernable level making it a limited tool for accurately measuring changes in gene expression. Real time quantitative RT-PCR (qRT-PCR) was developed to quantitatively measure mRNA levels (reverse transcribed to cDNAs) and requires a specialized fluorometric thermocycler that constantly measures amplicon generation in each reaction. The simplest and most popular chemistry for qRT-PCR is the SYBR® Green (Molecular Probes®), which allows detection of PCR products via the generation of a fluorescent green signal.

SYBR® Green produces a strong fluorescent signal upon binding double-stranded DNA (Schena et al. 2004). As PCR products accumulate, green fluorescence increases and can be measured in 'real time' using a thermocycler that contains optics to constantly measure fluorescence intensity in each reaction. Data acquisition software in a linked computer is then used to analyze data. Since PCR product amplification is detected in real time, this method almost eliminates the need to run gels in order to visualize PCR products. However, as SYBR® Green-based qRT-PCR cannot distinguish multiple, nonspecific or spurious PCR products from a single, specific PCR product, confirmatory electrophoresis gels sometimes must be run. Inclusion of proper negative and positive controls in separate, simultaneous qRT-PCR reactions can reduce misinterpretation of data (Vandesompele et al. 2002).

There are few reports of molecular research investigating *P. omnivora*. Because it is a devastating pathogen of alfalfa and cotton (Streets and Bloss 1973), such molecular approaches to investigate its interactions with its hosts should provide novel insights into its biology and, ultimately, its management. In this study, we examine selected genes' expression in *P. omnivora* using qRT-PCR methods. Characterization of gene responses to environmental and developmental cues will provide the foundation for developing effective management strategies against *P. omnivora*. We chose a set of 26 genes representing the following six functional categories: housekeeping, cell wall biogenesis, survival glycogen biosynthesis, stress response, virulence and secondary metabolism. The goal of this study was to profile significant changes in gene expression of *P. omnivora* during the three life stages, vegetative mycelium, 4 week and 8 week-old

sclerotia, and, in response to either *Sorghum bicolor* (non-host) root exudates or *Medicago truncatula* (host) root exudates. We expected that for each life stage, individual or groups of genes will be expressed differentially and the outcomes of this analysis provide more insights as to how this fungus functions. It was also expected that treatment with host root exudates will induce or repress specific genes' expression that will not be detected following treatment with non-host root exudates. Such genetic bases for a host-specific response will help us define host-pathogen interactions. Thus, it is expected that the identity of and differentiation of genes that can serve as markers for certain life-cycle stages or are related to *P. omnivora* pathogenicity will be achieved. Identification of crucial genes that control development or pathogenicity can be used in the future as targets for biotechnological approaches to controlling disease (e.g. in transgenic crops).

MATERIALS AND METHODS

Fungal Isolates and Cultures:

Sclerotia, vegetative mycelia and mycelia challenged with host and non-host root exudates: *P. omnivora* strain OKAlf8 was used for all experiments in this chapter. Dr. Tim Samuels cultured and harvested sclerotia and root exudate-induced mycelia. Sclerotia were wet sieved from soil cultures of *P. omnivora*, were grown in magenta boxes containing sorghum grains and black clay soil (from the Texas A&M Research and Extension Center, Dallas, TX) for 4 and 8 weeks (Dunlap 1941; Lyda and Kenerley 1993). Sclerotia were harvested by sequentially sieving the soil through a series of mesh screens numbers 10, 18, 30 and then 35. Sclerotia recovered from the surface of the

number 10 screen (4 to 32 g) were transferred to a 50 ml Falcon™ tube containing RNALater™ solution and stored at -80°C.

P. omnivora vegetative mycelium was cultured by inoculating 500-ml Erlenmeyer flasks containing 100 ml M1078 liquid media (recipe in previous chapter) with a 1 cm^2 agar plug of mycelium from a M1078 agar plate. Stationary cultures were incubated for 7-10 days in a 28°C incubator. Mycelial mats were collected by vacuum filtration using a porcelain Büchner filter and Fisherbrand P8 filter paper (9.0 cm diameter, Fisher Scientific, Pittsburgh, PA) until slightly dry, weighed and transferred to 50 ml Falcon™ tubes. Mycelia were immediately flash-frozen in liquid nitrogen and stored at -80°C. Root exudates from non-host *Sorghum bicolor* and host *M. truncatula* were prepared from seedlings as follows: Root exudates from non-host *Sorghum bicolor* and host *M. truncatula* were prepared from seedlings as follows: 10 g of each seeds were surface-sterilized by suspending in 1% $AgNO_3$ and 0.1% Tween-20 solution, mixed in a sonicating water bath for 30 seconds and rinsed 3 times each in sterile ddH_2O for 5 minutes. Seeds were then covered with sterile distilled water in a sterile glass beaker (500 ml), covered with parafilm and incubated at room temperature for 2-4 days (*M. truncatula* for 2 days and *S. bicolor* for 4 days). One hundred ml sterile ddH_2O were added to germinated seedlings, slowly shaken for 2 hours and the seeds removed by filtration through a Fisherbrand P8 filter. The filtrate was then sterilized by filtration through a 0.2 µm filter and was used as "root exudate". Root exudates were prepared and pooled from each plant species and stored 4°C until used for subsequent experiments, usually within 2 days.

To induce *P. omnivora* with root exudates, three 1 cm^2 mycelial plugs from 8-day-old cultures were used to inoculate each of six magenta boxes containing 100 ml of M1078 liquid media and then incubated for 7 days at 28°C. After 7 days, the liquid media was aseptically removed and replaced, in three magenta boxes each, with either 100 ml root exudate from either *S. bicolor* or *M. truncatula* and incubated at 28°C for 7 days. Induced mycelial mats were harvested by vacuum filtration as for vegetative mycelial mats. Mycelia were collected from the filter paper, weighed, stored in RNALater™, flash frozen in liquid nitrogen and stored at -80°C.

Isolation of total RNA from P. omnivora *mycelia and sclerotia*: Dr. Tim Samuels isolated total RNA from root exudate-induced mycelia and sclerotia. Total RNA was isolated from all samples using the PureYield™ Midiprep System (Promega, Madison, WI) according to the manufacturer's instructions with certain modifications. Specifically, 0.2 g mycelia or sclerotia stored at -80°C in RNALater™ were ground to a powder in liquid nitrogen with a mortar and pestle and transferred to a 15 ml Falcon™ tube containing 2 ml RNA lysis buffer and 40 µl beta-mercaptoethanol (BME). The mixture was vortexed vigorously to disrupt clumps of mycelium. If clumps of mycelia still persisted, another 2 ml of RNA lysis buffer and 40 µl BME was added to the tube and clumps disrupted by vortexing. The lysate mixture was incubated at room temperature for 10 minutes and the manufacturer's protocol for recovering RNA from cleared lysates was followed. The final RNA concentrations were determined spectrophotometrically using a NanoDrop 1000 (Thermo Fisher NanoDrop, Wilmington, DE) and the RNA was divided into 5 µg

aliquots that were stored in a -80°C freezer. Total RNA was isolated from three separate treatments.

Preparation of cDNA from total RNA: Tim Samuels prepared cDNA from the total RNA of sclerotia and root-exudate-treated mycelia using the SMART PCR cDNA Synthesis Kit (Clontech, Mountain View, CA) according to the manufacturer's instructions. cDNAs were prepared from total RNA from vegetative mycelia using the SuperScript III™ First-Strand Synthesis System kit (Invitrogen, Carlsbad, CA) using the manufacturer's instructions. Briefly, oligo dT primers were annealed to template in 10 µl reactions containing, 5 µg template RNA, 1 µl 50µM oligo dT primer; (n = 20), 1 µl 10mM dNTP mix, and DEPC-treated ddH_20 to 10 µl were incubated at 65°C for 5 min and then cooled on ice for 1 minute. For cDNA synthesis, 10 µl of the cDNA synthesis mix (10× RT buffer, 25mM $MgCl_2$, 0.1 M DTT, RNaseOUT, and SuperScript III RT) was added to each annealing reaction and cDNA extension reactions carried out in a thermal cycler with the program, 50°C for 50 minutes and then 85°C for 5 minutes. One µl of 40 U/µl RNase H was added to each tube and incubated at 37°C for 20 minutes. All cDNA was stored frozen at −20°C or −80°C.

PCR: cDNAs from each *P. omnivora* treatment were used as templates for PCR. PCR primers amplifying 65-146 bp of the selected 26 predicted protein-coding genes and the ribosomal RNA internal transcribed spacer 1 (rRNA-ITS1) (Tables 8 and 9) were designed using PrimerQuest℠ web software (www.idtdna.com/Scitools/Applications/Primerquest/). All primers amplified the

predicted amplicons from *P. omnivora* genomic DNA and optimal melting temperatures determined by gradient PCR prior to qRT-PCR analyses (data not shown). Twenty-five µl qPCR reactions contained 12.5 µl 2× SYBR Green™ Master Mix (either from SA Biosciences, Frederick, MD or Fermentas, Glen Burnie, MD), 100 nM each primers, 10 ng cDNA and 10.5 µl ddH_2O. All reactions were performed in 96-well PCR plates sealed with real time PCR sealing film (MidSci, St. Louis, MO) and carried out using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The PCR program was as follows: 95ºC for 10 min, followed by 90 cycles of 94ºC for 30 sec, 60ºC for 30 sec, and 72ºC for 33 sec. The relative fluorescence intensity of each well was individually measured after the 72ºC extension step. The instrument's SYBR green (FAM) filter was used to measure PCR product amplification and the ROX filter was used to measure the passive reference dye to normalize reaction volumes (variation due to pipetting error).

All PCR reactions were carried out in triplicate with 'no DNA' negative control and genomic DNA positive control reactions included for each primer pair. Threshold cycle (Ct) values were obtained using the Absolute Quantitation program in the software associated with the ABI7500 system (Applied Biosystems, Foster City, CA). Several modified versions of the comparative Ct method detailed below were employed.

Analyses: For all qRT-PCR reactions using cDNA derived from vegetative mycelia, Ct values for respective induced genes (*ig*) and internal standard (*ITS1*) were each normalized using the equation $90 - Ct$, since a Ct of 90 would imply no expression, and these values were averaged ($\bar{x} = 90 - \text{Ct}$). To calculate the dCt value, the average

values describing the levels of induced gene (*ig*) expression relative to the average values describing the levels of *ITS1* expression, we employed the equation dCt = $\bar{x}_{ig} / \bar{x}_{ITS1}$. The standard errors among were calculated using Statistics Analysis Software (SAS Institute, Cary, NC). The dCt values for each gene were plotted using Microsoft Excel 2007.

For all qRT-PCR reactions using cDNA derived from sclerotia, Ct values for each *ig* was subtracted from each Ct value for *ITS1* to obtain *dCt* values for 4 week-old (*4wks*) and 8 week-old (*8wks*) sclerotia. To generate the comparative relative quantification of dCt_{4wks} and dCt_{8wks} values for each *ig*, the ddCt values were calculated using the equation ddCt = $dCt_{8wks} - dCt_{4wks}$ in all possible combinations for each *ig*. Finally, to determine relative comparative transcript level of each *ig* in 4 week-old versus 8 week-old sclerotia, each ddCt value was used to generate a range of transcript levels for each gene using the recommended formula 2^{-ddCt} (Livak, and Schmittgen 2001). This method typically used to compare expression of an endogenous control gene to a target gene over time sample (ABI manual). In this case the 2^{-ddCt} value compares changes in relative expression of each *ig* in developing sclerotia after 4 and 8 weeks. The range of final 2^{-ddCt} values for each *ig* were presented as box plots (http://www.bloggpro.com/box-plot-for-excel-2007/) to holistically represent the variation of the data collected (Bamunusinghe et al. 2009)

All qRT-PCR reactions using cDNAs derived from root exudate-treated mycelia were analyzed identically to gene expression data from the sclerotia. The dCt_{sb} and dCt_{mt} values were obtained for samples treated with *S. bicolor* (sb) and *M. truncatula* (mt) root exudates, respectively, and ddCt values for each *ig* calculated using the equation ddCt = $dCt_{mt} - dCt_{sb}$. The range of final $2^{-dd\,CT}$ values for each *ig* was presented as box plots as for sclerotia.

TABLE 6. Characterization of the selected candidate genes' ontology

Gene (abbreviations)	Contig no.[a]	Functional Classification[b]	Significance[c]	Gene ontology terms[d]		
				COGEME	AmiGO	
					Term	Accession number
Translation elongation factor-1α (TEF1)	100253	**Housekeeping: translation**	Good promoter?	Translation (initiation, elongation and termination)	Translational elongation	GO:0006414
Orotidine 5'-phosphate decarboxylase (PYRG)	86363	**Housekeeping: RNA**	Selectable marker	Pyrimidine base and ribonucleotide metabolism	Orotidine 5'-phosphate decarboxylase activity	GO:0004590
Histone H3/5 (HIS3)	107903	**Housekeeping: DNA**	Nuclear	organization of chromosome structure	Chromatin assembly/disassembly	GO:0006333
Chitin synthase-3 (CHS3)	52183	**Cell wall: biosynthesis**	Membrane	Biogenesis of cell wall (cell envelope)	Cell wall chitin biosynthetic process	GO:0006038
Endochitinase-1 (CHT1)	182472	**Cell wall: plasticity**	Membrane	Biogenesis of cell wall (cell envelope)	Chitinase activity	GO:0004568
Hydrophobin-1 (HYP1)	147188	**Cell wall: aerial hyphae**	Structural cell wall protein	Cytoplasmic degradation	Hyphal growth	GO:0030448
SOFT (SO)	98521	**Cell wall: fusion and repair**	Heterokaryosis	Intracellular communication	Biological process	GO:0008150
Glycogen synthase-1 (GS1)	99297	**Survival: glycogen biosynthesis**	Sclerotial energy storage or turgor maintenance	Oligosaccharide / polysaccharide biosynthesis	Glycogen (starch) synthase activity	GO:0004373
Glycogen synthase-2 (GS2)	170361	**Survival: glycogen biosynthesis**	Sclerotial energy storage or turgor maintenance	Oligosaccharide / polysaccharide biosynthesis	Glycogen (starch) synthase activity	GO:0004373

Gene (abbreviations)	Contig no.[a]	Functional Classification[b]	Significance[c]	Gene ontology terms[d]		
				COGEME	AmiGO	
					Term	Accession number
Glycogen synthase kinase (GSK)	37785	**Survival: inhibits GS**	Sclerotial energy storage or turgor maintenance	Regulation of C-compound and carbohydrate utilization	Negative regulation of biosynthetic process	GO:0045719
MAP Kinase (HOG1)	92509	**Stress: osmotic**	Adaptation to salt	Osmosensing, key kinases	Response to osmotic stress	GO:0006970
Copper oxidase (CuOX)	170488	**Stress: oxidative**	Laccase	Biosynthesis of melanins	Laccase activity	GO:0008471
Superoxide dismutase (SODA)	142263	**Stress: oxidative**	Fe-Mn SOD, detox	Defense against oxidative stress	Superoxide dismutase activity	GO:0004784
Catalase (CATA)	112126	**Stress: oxidative**	Detox H_2O_2	Defense against oxidative stress	Response to oxidative stress	GO:0006979
Glutathione peroxidase (GSHP)	195073	**Stress: oxidative**	Detox peroxides	Defense against oxidative stress	Response to oxidative stress	GO:0006979
Cutinase 1 (CUT1)	71968	**Virulence: penetration**	Host cuticle degradation	Degradation of exogenous ester compounds	Pathogenesis	GO:0009405
NADPH oxidase 1 (NOXA)	53840	**Virulence: ROS production**	Symbiosis-pathogenesis switch	Disease, virulence and defense	Superoxide anion generation	GO:0042554
noxA regulator (NOXR)	102292	**Virulence: ROS production**	Symbiosis-pathogenesis switch	Disease, virulence and defense	Superoxide anion generation	GO:0042554
Mitogen-activated protein kinase (MAPK2, Pmk1, Fus3)	17630	**Virulence: signalling**	Signal transduction	Signal transduction, key kinases	Pathogenesis	GO:0009405

Gene (abbreviations)	Contig no.[a]	Functional Classification[b]	Significance[c]	Gene ontology terms[d]		
				COGEME	AmiGO	
					Term	Accession number
Pectin lyase (PL)	36239	**Virulence: host cell wall**	Colonization	Degradation of exogenous polysaccharides	cell wall modification during multidimensional cell growth	GO:0042547
β-glycosidase (BGL3)	72017	**Virulence: host cell wall**	Colonization	Degradation of exogenous polysaccharides	carbohydrate metabolic process	GO:0005975
Ceratoplatanin-3 (CPT3)	85987	**Virulence: phytotoxin**	Snod1 protein	Virulence, disease factors	Pathogenesis	GO:0009405
Multidrug Efflux (MFS1)	75442	**Secondary metabolism**	Xenobiotic pump	Drug transporters	Transporter activity	GO:0005215
Multidrug Efflux 2 (MFS2)	125665	**Secondary metabolism**	Trichothecene efflux	Drug transporters	Transporter activity	GO:0005215
Zinc finger trasnscription factor (SIRZ, CnjB)	5192	**Secondary metabolism**	Transcription factor	Transcriptional control	Regulation of transcription, DNA-dependent	GO:0006355
SAM-dependent methyltransferase (LAEA)	125066	**Secondary metabolism**	Global regulator of secondary metabolism	Fungal development	phosphoethanolamine N-methyltransferase activity	GO:0000234

[a] Contig numbers correspond to sequences in the 454LargeContigs database at ftp://ftp.genome.ou.edu/pub/crr

[b] From this study, based on multiple sources; serves as convenient categories for the clustering of genes based on main function of interest used for selection

[c] To the study of *Phymatotrichopsis omnivora*.

[d] From blastx searches of the GO websites Phytopathogenic Fungi and Oomycete EST Database (cogeme.ex.ac.uk) and AmiGO! The Gene Ontology (amigo.geneontology.org).

TABLE 7. Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) primers amplifying selected candidate genes.

Gene ID (contig)[a]	Gene Product	Primer Name[b]	Primer Sequence	Amplicon Size (bp)
ITS1 (63109)	ITS-rRNA	PoITS22F PoITS22R	TCAGTGTACCTCTCCACGTTGCTT AAGAGTTTGGAGAATTCCTCCCGTGG	131
TEF1 (100253)	Translation elongation factor 1-alpha	TEF1-RTP-FOR TEF1-RTP-REV	GTCGAAATGCACCACGAGCAACTT ACGTTTCCACGACGGATTTCCTTG	102
HIS3 (107903)	Histone H3/5	HIS3-RTP-FOR HIS3-RTP-REV	TTCAAGTCCGATCTCCGCTTCCAA ATGGCACACAAGTTGGTGTCTTCG	105
PYRG (86363)	Orotidine 5'-phosphate decarboxylase	PYRG-RTP-FOR PYRG-RTP-REV	GGAGCGATGTCATCATTGTG GATACCCCAAACTCGAGCAA	142
CHS3 (52183)	Chitin synthase 3	CHS3-RTP-FOR CHS3-RTP-REV	GTCGTGCCAATTGCAGAAAGCGTA TCTACTTCGTCGCCTCGATCATGT	129
CHT1 (182472)	Endochitinase 1	CHT1-RTP-FOR CHT1-RTP-REV	CCAGCAATGTACGCTTTAACGGCA TTGACTTCTGGAACCTCATGGCCT	146
HYP1 (147188)	Hydrophobin	HYP1-RTP-FOR HYP1-RTP-REV	AGGTTGATCGGTGTGCACTGGATA TACGAAGACTTGCGGTAACGCTCA	133
SOFT (147188)	Protein involved with hyphal fusion and septal pores	SOFT-RTP-FOR SOFT-RTP-REV	GTGGAATGTTGGCATAGCTCTGGT TATGGATGCGGCTTCGGGACAATA	144
GS1 (99297)	Glycogen synthase 1	GLYS1-RTP-FOR GLYS1-RTP-REV	ATTGGAAAGAGGCTGTTTGACCGC CCGCTTCAACGCAAACATTCGTCT	127
GS2 (170361)	Glycogen synthase 2	GLYS2-RTP-FOR GLYS2-RTP-REV	ACGAGGAGGCAGAGAATCAACCAA AGCCAGTTGCCTAGCTTTGACGTA	106
GSK (37785)	Glycogen synthase kinase	GLYSK-RTP-FOR GLYSK-RTP-REV	TGTTGATCACTGGCTGCCTCAAAC TTAAACCGTATCTGGACCACCCAC	65

Gene ID (contig)[a]	Gene Product	Primer Name[b]	Primer Sequence	Amplicon Size (bp)
HOG1 (92509)	MAP Kinase	HOG1-RTP-FOR HOG1-RTP-REV	AAGGATCAACTCACAGGGCAGTCT GTCGCAAATGCTTCAACAACTTCAG	116
CuOX (170488)	Copper oxidase (laccase?)	CUOX-RTP-FOR CUOX-RTP-REV	AGTCCTTCTGCACCGGCATTGATA AAATGGCCTTCAACTGCTCGCTTG	131
SODA (142263)	Superoxide dismutase A	SODA-RTP-FOR SODA-RTP-REV	TTCAAGGAAGCGGATGGAGTTGGT TGGGTGATAATCAGCAAGTGCCCA	68
CATA (112126)	Catalase A	CATA-RTP-FOR CATA-RTP-REV	ATCATTTAGAGGGTGCGAGGAGCA ATCGGCTTCAAGTGTCCAAATGCC	137
GSHP (195073)	Glutathione peroxidase	GSHP-RTP-FOR GSHP-RTP-REV	ATGTCTGGACCCTCAATTCCCGTT AAATTCCTCGTTTCCCGCGAAGG	107
CUT1 (71968)	Cutinase 1	CUT1-RTP-FOR CUT1-RTP-REV	TCATTCTTTCGGGCTACAGATGGC TTGGGCTCCTTGGCTAGTTTGGTA	105
NOXA (53840)	NADPH oxidase 1	NOXA-RTP-FOR NOXA-RTP-REV	TCCATTCGCAGGAAGAGAGTGGAA TCCCTGTAAACCCTCTCGCAGAAA	106
NOXR (102292)	noxA regulator	NOXR-RTP-FOR NOXR-RTP-REV	TGCTTGGAGAGTTTACCGAAGCCA GCACTTCGCACGAGAACAACTTGA	124
MAPK2 (17630)	MAP kinase	MAPK2-RTP-FOR MAPK2-RTP-REV	ATCTGCTGCGAGCACCGAAGATAA GCAAGAATGCACCCAACACTCCAA	142
PL (36239)	Pectin lyase	PL-RTP-FOR PL-RTP-REV	TCCAGCAACACCTGCCCTAATGAT ACGTCAATGTAACCTGTTCCCGCT	126
BGL3 (72017)	β-glycosidase	BGL3-RTP-FOR BGL3-RTP-REV	TGTTGACGCCCAAGACATCAGGTA TGGTAGCAAGTCCGTTAACACCGT	124
CPT3 (85987)	Ceratoplatinin 3	CPT3-RTP-FOR CPT3-RTP-REV	TGGTAGCAAGTCCGTTAACACCGT GCCGTCAATGCTATTGCGGTTTCT	108
MFS1 (75442)	Multidrug efflux 1	MFS1-RTP-FOR MFS1-RTP-REV	CGCTGCTATTACTTGCTTGCTGCT TCATAGCCTACCCACCACTGCAAA	145
MFS2 (125665)	Multidrug efflux 2	MFS2-RTP-FOR MFS2-RTP-REV	AACCACGAGGATAGGGCTGTGATT TTCTCCGGGAACTCGCCAATTTCT	138

Gene ID (contig)[a]		Gene Product	Primer Name[b]	Primer Sequence	Amplicon Size (bp)
SIRZ (5192)		transcription factor	SIRZ-RTP-FOR SIRZ-RTP-REV	TCAACAATCGTTCGCCGCA AACGAACGTATTTGCCGGG	74
LAEA (125066)		transcription factor	LAEA-RTP-FOR LAEA-RTP-REV	CTGGTGCCACTGTGCTAAATTGCT GGTGCAAGACGTTGGCGATTACTT	135

[a] Contig numbers correspond to sequences in the 454LargeContigs database at ftp://ftp.genome.ou.edu/pub/crr; color coding corresponds to the functional classification of each gene (black = housekeeping, orange = cell wall maintenance, brown = survival energy storage, blue = stress response, red = virulence, and violet = secondary metabolism).

[b] Primers with names ending an 'F' or 'FOR' are forward primers and those with names ending with 'R' or 'REV' are reverse primers

Gene Prediction from P. omnivora Genome Sequences and Classification of Selected Genes into Gene Ontology (GO) Groups: A collection of candidate "genes-of-interest" were selected from recent literature and based on potential usefulness for functional genomics studies. Two hundred forty-four genes, or gene families, were identified in the *P. omnivora* genome database using tblastx searches (http://www.genome.ou.edu/blast/crr_blastall.html) with the DNA sequences of known fungal genes-of-interest and retrieved contigs with high similarity to the known gene were then used to reciprocally search GenBank (http://www.ncbi.nlm.nih.gov/) to confirm its identification. A subset of 26 genes was selected based on their likelihood for differential expression during various life stages and in response to various stimuli. All twenty-six genes were classified into one of the following categories: a) housekeeping, b) cell wall, c) survival glycogen, d) stress response, e) virulence, or f) secondary metabolism based on the selection criteria used for each gene. To determine the functional gene ontologies (GO) of each candidate gene, the sequence of each was used to perform blastx searches of both the Phytopathogenic Fungi and Oomycete EST (expressed sequence tag) Database (cogeme.ex.ac.uk) and AmiGO! The Gene Ontology (amigo.geneontology.org). GO terms from COGEME and AmiGO associated with each gene are presented in Table 6.

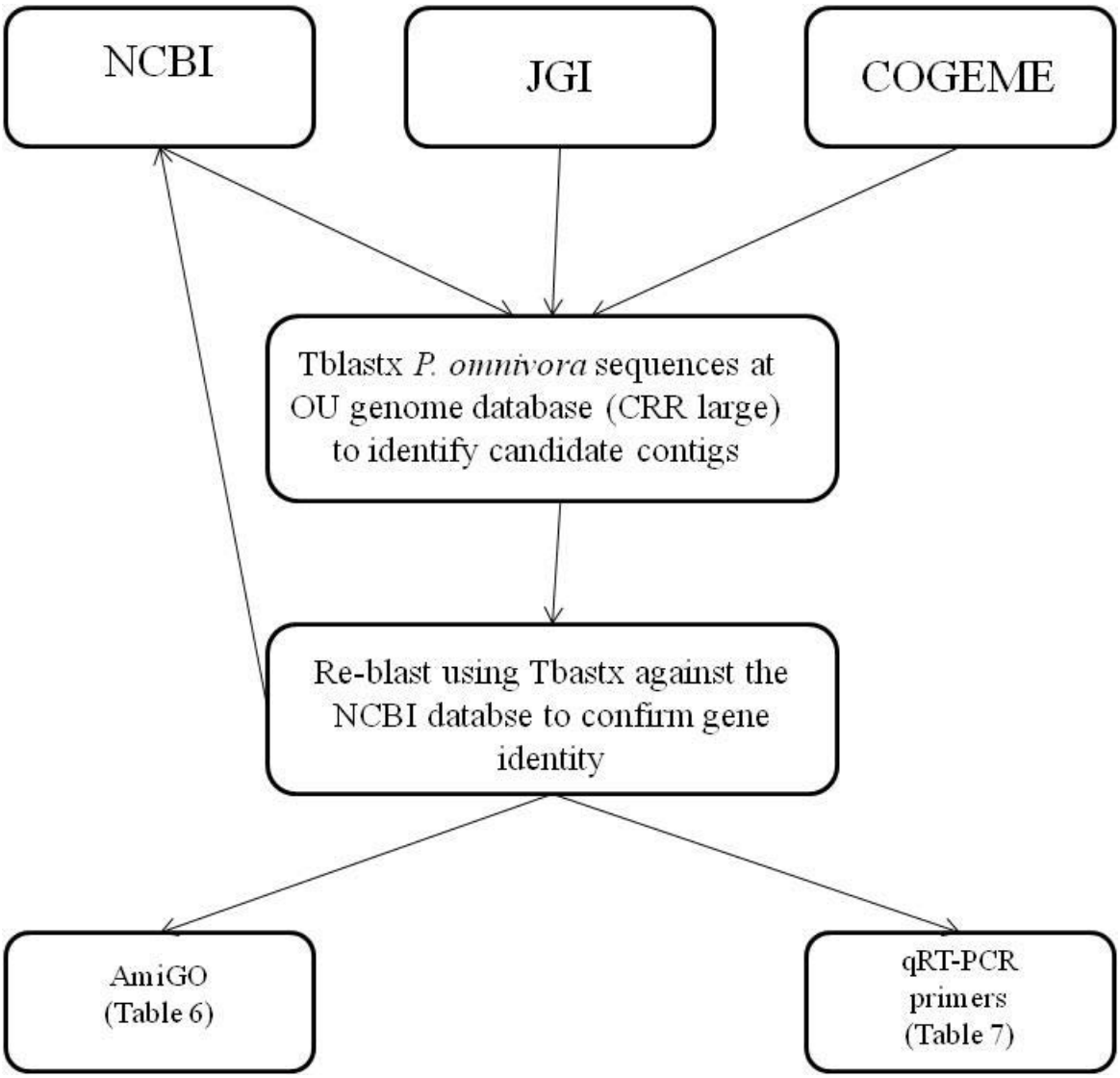


Fig 9. Flow chart describing the process of candidate gene selection and functional gene ontologies. JGI, http://www.jgi.doe.gov/; NCBI, http://www.ncbi.nlm.nih.gov/; COGEME, http://cogeme.ex.ac.uk/.

RESULTS

Validation of ITS1 as the Housekeeping Gene to Use as Internal Control for qRT-PCR

The Ct values of the three protein-coding housekeeping genes, PYRG, TEF1, and HIS3 and the rRNA ITS1 were used to compare the expression of these genes in *P. omnivora* across all treatments (e.g. vegetative or root exudates induced mycelia or sclerotia) to determine which gene would provide the best internal reference for normalization of gene expression data (Fig. 10). Expression of rRNA ITS1 was the highest (average Ct ≈ 20) and most stably expressed gene across all treatments, distantly followed by TEF1 (average Ct ≈ 42). Both PYRG and HIS3 expression levels were too variable or too low to be employed as internal controls. Because of its consistently high expression, ITS1 was selected as the internal control for all subsequent experiments.

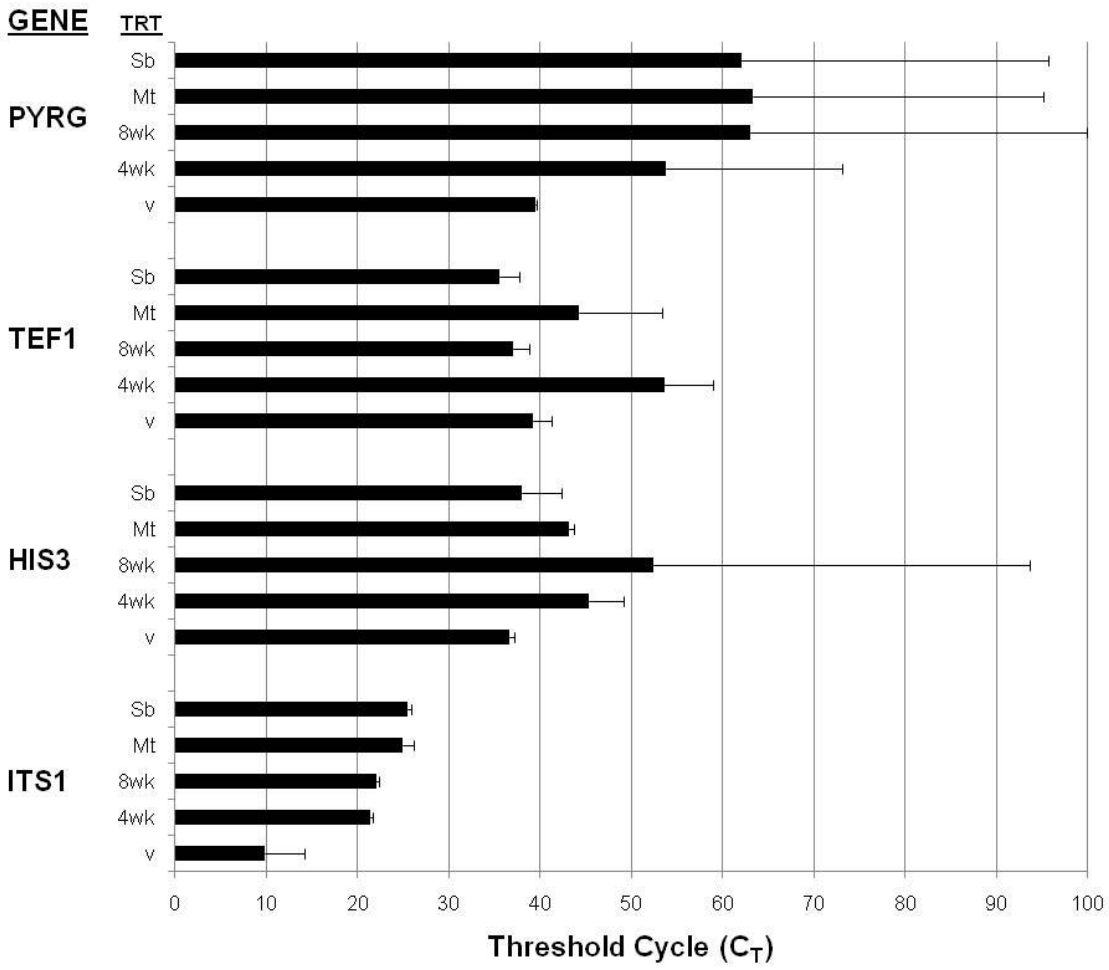


FIG 10. Threshold cycles of housekeeping genes expressed in *Phymatotrichopsis omnivora* mycelia treated (TRT) with *Sorghum bicolor* (Sb) or *Medicago truncatula* (Mt) root exudates, 8 week-old (8wk) or 4 week-old sclerotia, or vegetative mycelia (V). The Ct value is inversely proportional to gene expression level. Error bars represent the standard deviation across reps.

Gene Expression in Vegetative Mycelia

To observe differences in gene expression levels within the vegetative mycelia of *P. omnivora,* qRT-PCR was carried out on the 26 genes-of-interest and normalized as a ratio to ITS1 expression (Fig. 11). Most genes' expression levels fell between the values range from 0.6 to 0.8 with housekeeping and survival glycogen gene expression not significantly different from one another. Among the cell wall biosynthesis genes, chitin synthase 3 (CHS3) was expressed the highest while endochitinase (CHT1) was the least expressed. NADPH oxidase A (NOXA) was expressed the lowest of all the oxidative stress genes, while the others had relatively the same expression levels. The virulence genes, cutinase (CUT1) and pectin lyase (PL), had the lowest expression levels among not only the virulence genes, but also out of all the genes. The secondary metabolism genes had varying levels of expression with multidrug efflux pump 2 (MFS2) being expressed the least and multi-drug efflux pump 1 (MFS1) the highest.

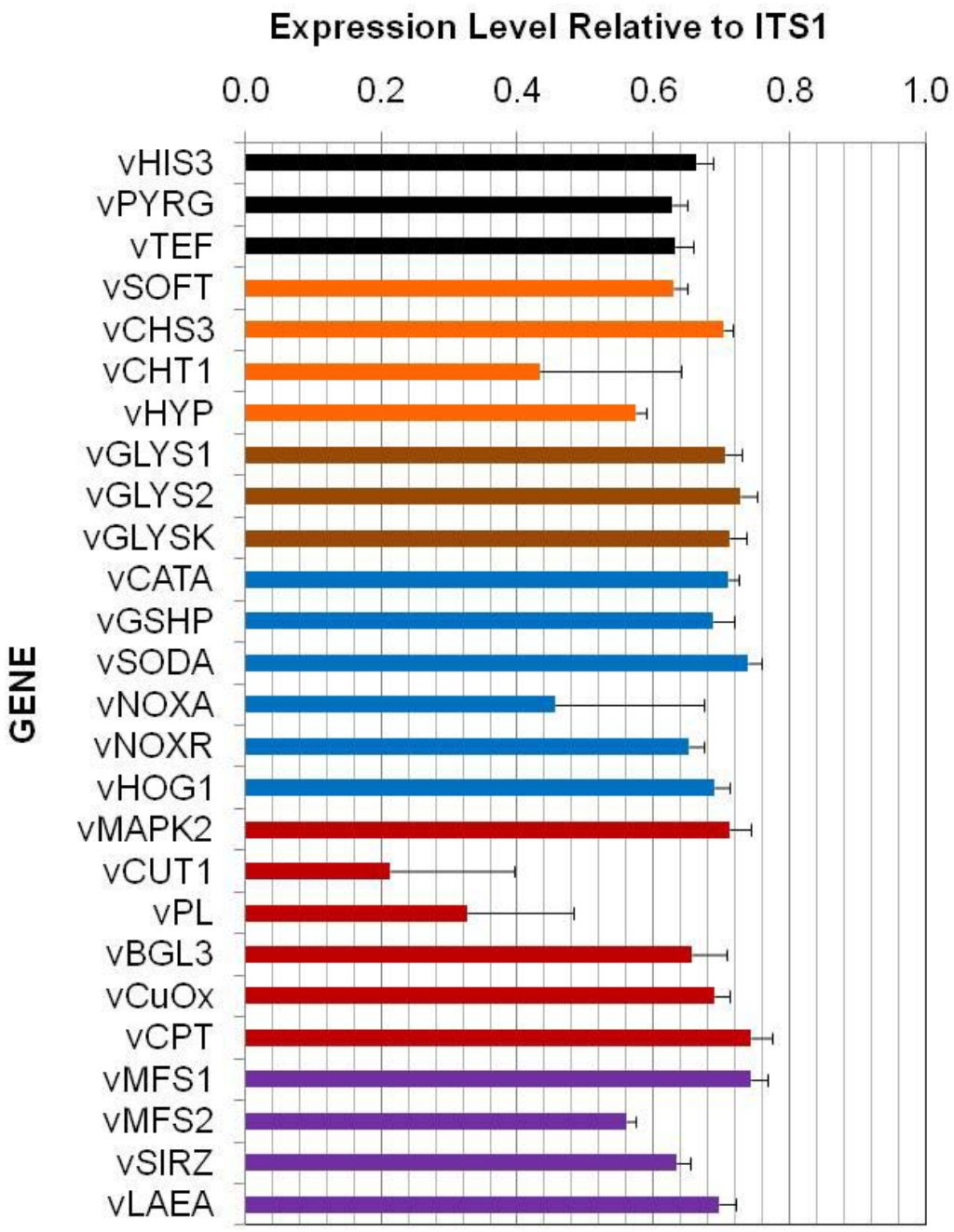


FIG 11. Relative expression of 26 genes-of-interest normalized with the internal reference ITS1. Gene expression bars are categorized according to functional GO term, color-coded as in Table 8. Relative gene expression is expressed as ratio to ITS1 expression (i.e. $[90 - Ct_{ig}] / [90 - Ct_{ITS1}]$). Error bars represent standard errors of ratios.

Comparison of Gene Expression in Mycelia Treated with Host or Nonhost Root Exudates

In this study, differential gene expression in *P. omnivora* mycelia challenged with root exudates from a host, *M. truncatula* or a nonhost, *S. bicolor*, was followed using qRT-PCR (Fig. 11). It was expected that genes categorized as involved in virulence, oxidative stress, and secondary metabolism would show greater expression levels with host root exudates than nonhost root exudates. Across all genes, relative gene expression ranges (max-to-min whiskers) and medians varied across 40 and 23 orders of magnitude, respectively, in response to *M. truncatula* and *S. bicolor* root exudates.

Surprisingly, the median expression values of 20 out the 26 genes were skewed towards mycelia treated with the nonhost root exudates (Fig. 12). Most markedly, the gene for glycogen synthase 2 (GS2) was much more highly expressed in nonhost root exudates-treated mycelia, with the full range of expression values (box and whiskers) skewed. To a lesser extent, histone H3 (HIS3), and pectin lyase (PL) were also more highly expressed in the nonhost treatment. Genes with the majority of both quartiles skewed towards the nonhost treatment included the following: cell wall-related chitin synthase 3 (CHS3) and *soft* (SO), the survival-related glycogen synthase 1 (GS1) and glycogen synthase kinase (GSK), the stress-related catalase A (CATA), glutathione peroxidase (GSHP), NADPH oxidase A (NOXA), superoxide dismutase (SODA) and osmotic MAP kinase (HOG1), virulence-related MAP kinase 2 (MAPK2), cutinase (CUT1) and ceratoplatinin (CPT) and the secondary metabolism genes, multidrug efflux pump 1 (MFS1) and 2 (MFS2) and Zn-finger transcription factor (SIRZ). Only translation elongation factor-1α (TEF1), β-

glycosidase 3 (BGL3), and laccase (CuOX) appeared to be slightly skewed towards expression in host root exudates treated mycelia. The remaining genes expression values ranged across the center line (x-axis) and were interpreted as showing little expression differences between *M. truncatula* and *S. bicolor* root exudates.

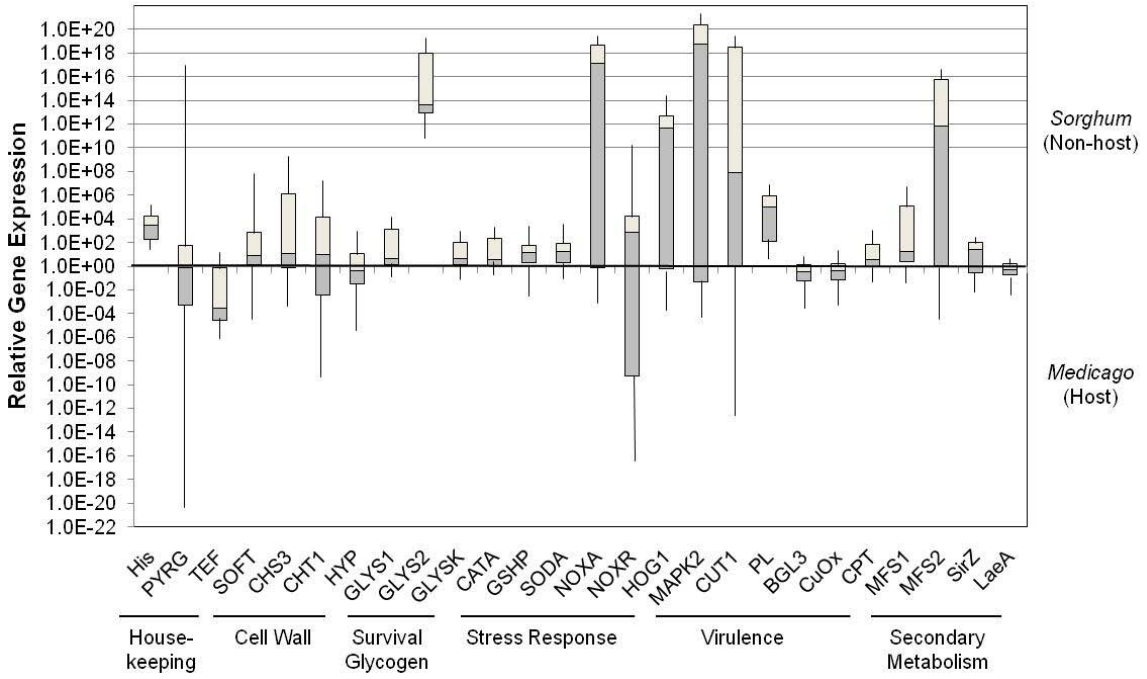


FIG 12. Box plot of gene expression levels in *Phymatotrichopsis omnivora* mycelia challenged with root exudates from either nonhost *Sorghum bicolor* or host *Medicago truncatula*. The distribution of comparative gene expression values is represented in the box plot, with light and dark gray boxes represent the upper and lower quartiles of the range around the median value, respectively, and the whiskers extending to the maximum and minimum values. Outliers were rare and are not presented. Genes are grouped according to 'GO' functional terms in Table 6.

Comparison of Gene Expression in 4 Week-Old or 8 Week-Old Sclerotia

In this study, gene expression levels in sclerotia formed by *P. omnivora* grown on sorghum grains and black clay after 4 and 8 weeks were compared (Fig. 13). It was expected that gene expression would be higher in 4 week-old than 8 week-old sclerotia, since after 8 weeks sclerotia should be mostly dormant. Also since glycogen is the predominant energy store in the sclerotia of *P. omnivora*, it was expected that the survival glycogen genes would be expressed higher in one of the sclerotia ages. Across all genes, relative gene expression ranges (max-to-min whiskers) and medians varied by 40 and 20 orders of magnitude, respectively, in the different aged sclerotia.

Unexpectedly, the relative expression medians of 15 out of the 26 genes were higher in 8 week-old sclerotia and 9 genes' expression medians were higher in 4 week-old sclerotia (Fig. 13). In 8 week old sclerotia, the expression ranges of TEF1 and SIRZ were entirely higher than in 4 week-old sclerotia. Also, both quartiles of the expression ranges of CHT1, SODA, NOXA, MAPK2, BGL3, CuOX, CPT1, and LAEA were higher in 8 week-old sclerotia. The expression of all remaining genes was skewed higher in 8 week-old sclerotia. In 4 week-old sclerotia, only the expression values of the MFS2 and CUT1 genes were entirely higher. The remaining 7 genes with median gene expression higher in 4 week-old sclerotia included survival glycogen synthases GS1 and GS2 and GS kinase, stress response genes NOXR and HOG1, and virulence-related CUT1 and PL were expressed higher in 4 week sclerotia.

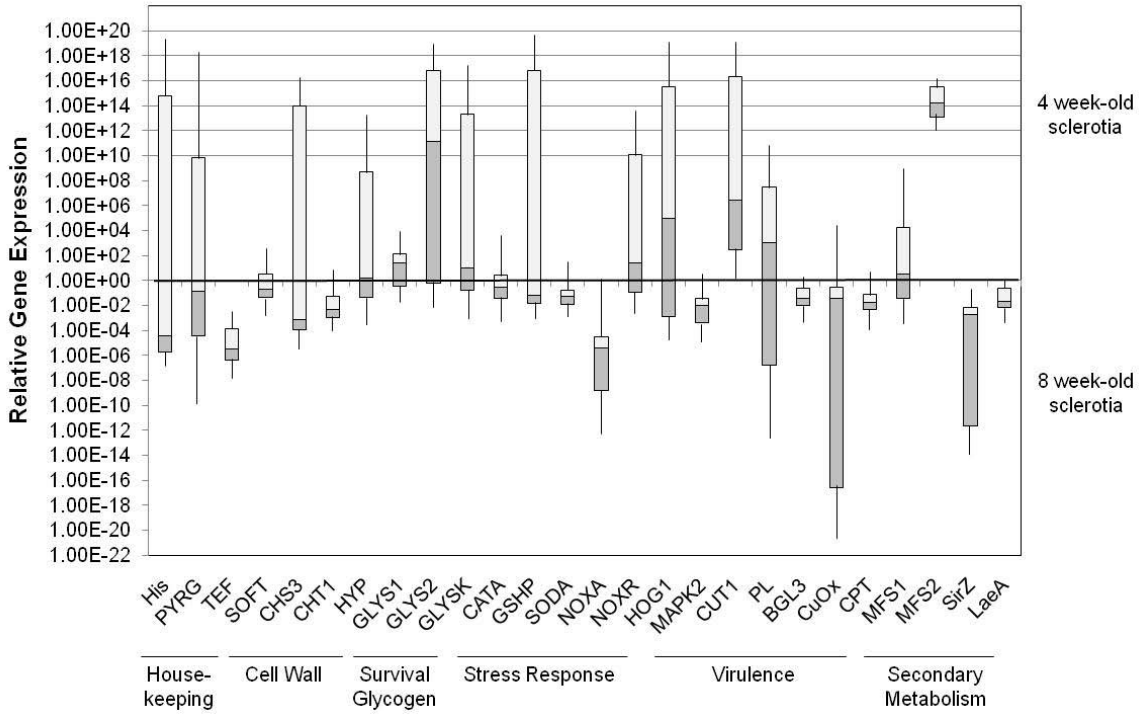


FIG 13. Box plot of gene expression levels in 4 week-old and 8 week-old sclerotia of *Phymatotrichopsis omnivora*. The distribution of comparative gene expression values is represented in the box plot, with light and dark gray boxes represent the upper and lower quartiles of the range around the median value, respectively, and the whiskers extending to the maximum and minimum values. Outliers were rare and are not presented. Genes are grouped according to 'GO' function terms in Table 6.

DISCUSSION

ITS1 as the Internal Reference Gene: The use of ITS1 as the internal reference gene for normalization of gene expression data was made possible due to several polyA and polyT stretches in the rDNA of *P. omnivora*, which facilitated reverse transcription of rRNA with the initial oligoT primers (data not shown). Similarly, rRNA was validated as the most stable housekeeping for normalization of qRT-PCR expression in rice (Jain et al. 2006). Also, since the ITS-1 sequence is highly specific to *P. omnivora* (data not shown), the primers used in this study could potentially be used to help quantify *P. omnivora* biomass during infection and normalized gene expression during mixed infections of host roots with other fungi.

Methods of cDNA Preparation Limited Cross Treatment Comparisons: Originally, gene expression in vegetative mycelia was to be compared to that in sclerotia and root exudate-treated mycelia. However, while total RNA was isolated using identical kits across treatments, cDNAs were prepared from sclerotia and root exudate-treated mycelia using a kit (Clontech's SMART PCR cDNA^{TM} Synthesis) designed to generate full-length cDNAs for downstream library construction. The cDNAs were prepared from vegetative mycelia using a kit (Invitrogen's SuperScript^{TM} III First-Strand Synthesis System for RT-PCR) designed to increase cDNA yields for RT-PCR. The difference in overall cDNA levels between these two preparations is evident in the ITS1 expression levels in Fig. 10,

with Ct_{ITS1} values from sclerotia and root exudates-treated mycelia 2-fold greater than that from vegetative mycelia.

Unfortunately, this gene expression bias due to cDNA synthesis kit differences prevented sufficient normalization of expression data using ITS1 levels to permit gene expression comparisons across treatments. Also, because of large differences in SMART cDNAs prepared from sclerotia or root-exudate treated mycelia, relative gene expression comparisons were only made between 4 week and 8 week-old sclerotia or between mycelia treated with root exudates from either *S. bicolor* or *M. truncatula*.

Selection and Differential Expression of Genes of Interest in Phymatotrichopsis omnivora: The expression of twenty-six genes falling into various functional classes ranging from housekeeping to virulence was analyzed in the vegetative mycelium, sclerotia and mycelia treated with host and nonhost root exudates. More specifically, differential gene expression in sclerotia after 4 and 8 weeks of maturation or in mycelia challenged with root exudates from *M. truncatula* (host) and *S. bicolor* (non host) were examined.

Relative expression of housekeeping genes: The selected housekeeping genes histone H3 (HIS3), orotidine-5'-monophosphate decarboxylase (PYRG), and translation elongation factor 1α (TEF1a) in this study are involved in nucleic acid maintenance and have been studied extensively in other fungal genomes. Chromatin modifications involving histones like HIS3 control many major functions in fungal cells, such as cell differentiation, metabolic function, and cell development, all of which influence a fungus' ability to cause disease in plants and adapt to environmental changes (Brosch et

al. 2008). The TEF1 gene is essential for translation in fungal cells and was also examined because it possesses a good promoter useful for gene expression systems, as in the fungus *Aspergillus oryzae* (Kitamoto et al. 1998). PYRG is essential for pyrimidine biosynthesis and, in the model system *A. nidulans*, has been proven to be a very good positive selection marker in transformation systems when auxotrophic mutants are available as demonstrated by Kanamasa et al. (2003). In vegetative mycelia of *P. omnivora*, all three genes were expressed similarly (Fig 8) and indicates that during the vegetative growth in rich media, such as M1078, *P. omnivora* does appear to be starved and nucleic acid metabolism appears homeostatic to maintain DNA packaging (HIS3), RNA biosynthesis (PYRG) and translation (TEF1) for hyphal growth. In mycelia challenged with nonhost *S. bicolor* root exudates, HIS3 is expressed at higher levels than PYRG and TEF1 (Fig 11). In sclerotia, HIS3 and PYRG are express similarly in both 4 and 8 week-old sclerotia, albeit highly variably and with slightly more expression in 8 week old sclerotia, while TEF1 is expressed more in the 8 week-old sclerotia (Fig. 12). Slightly higher expression of HIS3 in the 8 week-old sclerotia may be required for compaction of chromatin in preparation for dormancy. Similarly, DNA methylation is higher in dormant sclerotia *P. omnivora* than in metabolically active cells (Jupe et al. 1986). Less clear, is why TEF1 expression, indicative of active metabolism, is higher in 8 week-old sclerotia.

Relative expression of cell wall related genes: The hyphal cell wall protects a fungus from the environment and plant defenses during fungal-plant interactions. Cell wall related genes chosen for this study all perform important functions in hyphal development. Most of the scientific knowledge about chitin synthases' comes from

studying yeast. Class I is a repair enzyme during budding, where as class II is involved in division of septae and class IV generates chitin at bud scars and in the lateral wall (Choquer et al. 2004). Chitin synthase III (CHS3) is a major biosynthetic enzyme of chitin, the predominant polysaccharide in fungal cell walls and is crucial to the structural integrity (Souza et al. 2009). CHS3 was the most consistently expressed of all the cell wall-related genes across all studies (Figs 10-12). CHS3's high expression in vegetative mycelium (Fig 10) suggests that *P. omnivora* is actively growing and must synthesize cell wall materials to maintain this growth. In root exudate challenged mycelia, CHS3 is more highly expressed in response to *S. bicolor* root exudates than to *M. truncatula* root exude (Fig 11). One reason for this result could be that the fungus is finding more nutrition in the sorghum exudates than the *M. truncatula* root exudates.

One of the more interesting cell wall-related genes is the SOFT (SO) gene. The SO gene was first described in the model system *Neurospora crassa* as responsible for not only hyphal fusion, but also septal plugging to prevent cell nutrients from leaking out of damaged hyphae (Fleibner and Glass 2007). Also, the SO homolog (*Aso1*) in *Alternaria brassicicola* is required for hyphal fusion and pathogenicity on cabbage (Craven et al. 2008). The SOFT gene is expressed in vegetative mycelia in relatively high levels (Fig 10). However, expression was similar in both root exudate treatments and both 4 and 8 week-old sclerotia (Figs 11 and 12). The other two cell wall-related genes were endochitinase 1 (CHT1) and hydrophobin (HYP), which degrade chitin, contributing to wall plasticity and coat aerial hyphae with hydrophobic proteins, respectively. In vegetative mycelia, CHT1 and HYP were expressed at lower levels than CHS3 and SOFT (Fig 10). In host-nonhost root exudate treated mycelia, expression levels of CHT1 and

HYP did not differ (Fig 11). CHT1 seemed to be expressed slightly more in 8 week-old sclerotia and HYP expression was expressed higher in the 4 week sclerotia (Fig 12).

The importance of the cell wall-related genes to fungi is the subject of much current research and CHS enzymes are being targeted for drug development to inhibit fungal growth in plants and humans (Souza et al. 2009). Hydrophobins are small proteins that self assemble on the surface of hyphae, fruiting structures and aerial structures and prevent the lost of moisture from coated structures (Kershaw and Talbot 1997). Also, hydrophobin *MHP1* plays essential roles in the pathogenicity-related development and surface hydrophobicity of the rice blast fungus *Magnaporthe grisea* (Kim et al. 2005). Interestingly, HYP was expressed in *P. omnivora* under all treatments even though aerial structures were not formed. Sclerotia may use HYP to prevent desiccation in the soil during droughty periods.

Relative expression of survival glycogen genes: One of the most epidemiologically important life stages of *P. omnivora* is the resting stage facilitated by the formation of specialized structures called sclerotia. These thick-walled, multicellular structures form in the soil when environmental conditions are unfavorable and there is little to no nutrients on which the fungus can survive. In these sclerotia, the fungus deposits high levels of glycogen and other lipids for energy storage (Ergle 1947). In this study we chose two glycogen synthase genes (GS1 and GS2) and a glycogen synthase kinase (GSK) gene. In vegetative mycelia, all three genes are expressed at similar levels (Fig 8), while growth on nutrient rich media could be made possible as a way to store the excess available carbon nutrition. In root exudate challenged mycelia, GS2 is much more highly expressed in response to exudates from nonhost sorghum than those from host *M.*

truncatula (Fig 11), and GS1 and GSK were only slightly more expressed in the sorghum root exudates. The reason for this result could be that *P. omnivora* obtains more nutrients from the sorghum root exudates and GS2 is specifically induced by this excess nutrition. Or, some other signal in sorghum root exudates induces nonhost-dependent glycogen biosynthesis specifically through GS2. In sclerotia, GS1 is only expressed a slightly higher after 4 weeks than after 8 weeks. GS2 and GSK were expressed even higher in 4 week-old sclerotia than in 8 week-old sclerotia (Fig 12). From these findings it seems glycogen storage occurs mostly in the 4 week-old sclerotia and after sufficient glycogen has accumulated, glycogen biosynthesis pathways are repressed in 8 week-old sclerotia. Previous studies showed that free glycogen in 30-day old sclerotia makes up 10.1% of the dry weight of sclerotia and bound glycogen makes up to 26.6% of sclerotial dry weight (Ergle 1947).

Relative expression stress response genes: Reactive oxygen species (ROS) defense plays an important role during fungal development as fungi are constantly exposed to physical and chemical stresses in the environment including pH and osmotic fluctuations, ionizing radiation, UV, and ROS production during plant infection (Gessler et al. 2007). In vegetative mycelia, most of the oxidative stress response genes are expressed at similar levels, except for NADPH oxidase A (NOXA), which was expressed at lower levels (Fig 9). In sclerotia, only the NOXA gene is expressed more highly in the 8 week-old sclerotia (Fig 12), while GSHP, NOXR and HOG1 are expressed slightly more in 4 week-old sclerotia. Catalase A (CATA) and superoxide dismutase (SODA) were expressed at similar levels at each time point. Although the functions of these genes in *P. omnivora* are only presumed, previous studies in other fungi permit inferences to be

drawn as to what role these genes may play in *P. omnivora*. For example, the HOG1 gene is important for the osmoregulation of fungal cells, which is crucial for the homeostatic balance of water and solute levels across membranes and cell walls (Hohmann et al. 2007). The HOG1 MAP kinase is activated at the plasma membrane by numerous stresses (e.g. osmotic, oxidative and heavy metal) and once activated by hyperosmotic shock, migrates into the nucleus where it can trigger gene expression for water and glycerol uptake. HOG1 has also been shown to be required for the pathogenicity of both plant and animal pathogenic fungi (Weiwei et al. 2008; Alonso et al. 2006). The NADPH oxidase (NOXA) and its regulator (NOXR) may play a role when *P. omnivora* is penetrating a plant's root, while at the same time overcoming the defenses produced by the plant. NoxA and NoxR have been studied in the fungus *Epichlöe festucae* with its grass host *Lolium perenne* (Takemoto et al. 2006). Interestingly, when NoxA is deleted, *E. festucae*, normally a mutualistic endophyte of the grass, becomes a pathogen (Tanaka et al. 2006). NoxR interacts with the GTP-binding protein RacA and is a key regulator for NoxA, which controls the symbiotic relationship. To our knowledge *P. omnivora* is a plant pathogen and has no symbiotic relationship with any plant, but the NoxA and NoxR genes may have multiple roles in different fungal-plant interactions.

Among mycelia challenged with root exudates, NOXA and HOG1 are expressed higher in response to sorghum root exudates, while NOXR is expression occurs over a wide range in both sorghum and *M. truncatula* root exudates (Fig. 11). The other stress-relatecd genes, CATA, GSHP and SODA are expressed only slightly more in sorghum root exudates but (Fig 11). Catalases catalyze the break down hydrogen peroxides into

water and molecular oxygen in most species of fungi (Gessler et al. 2007). Catalase A has been described in *S. cerivisiase* and is localized in peroxisomes (Charay and Natvig 1989; Schlibes et al. 2006). In the model system *N. crassa,* catalases are located in the cytosol and perform a variety of functions from defense against extreme temperature-induced peroxides and denaturants and aid spore germination (Michan et al. 2002). Superoxide dismutases (SODs) are among the first genes to be triggered in fungi upon infecting a plant and are believed to defend against plant-produced ROS (Gessler 2007). SODs dismutate superoxide, a highly reactive ROS, into oxygen and hydrogen peroxide (which must be catabolized by catalases), thereby preventing mutagenic DNA damage. It is hypothesized that when filamentous phytopathogenic fungi, *Rhizoctonia solani, Sclerotinia sclerotiorum, Sclerotium rolfsii* and *Sclerotinia minor,* form sclerotia; in response to oxidative stress (Pastoukis and Georgiou 2007). In yeast, glutathione peroxidases, *GPX1, GPX2* and *GPX3* play roles during carbon starvation, oxidative stress and as a redox-transmitter that activates the oxidative stress defense response (Tanaka et al. 2006).

Relative expression of virulence genes: Out of all the virulence genes looked at in this study, the most notable differences in gene expression in vegetative mycelia, are the low expression of pectin lyase (PL) and the cutinase 1 (CUT1) genes (Fig 10). Pectin lyases (PLs) are sometimes referred to as pectin transeliminases are one of many cell wall degrading enzymes (CWDE) secreted by plant pathogenic fungi to digest host cell walls (Juge 2006). Fungi use cutinases, cutin-specific lipid esterases, as a means to penetrate and degrade the external cuticle layer of plant cell walls (Verrips et al. 2000). Other CWDEs likely used by *P. omnivora* to break down plant cell walls include a number of

different enzymes such as cellulases and polygalactuonases (Muñoz and Bailey 1998). The relatively low expression of PL and CUT1 in vegetative mycelia may be due to catabolite repression of these genes during growth in rich media (Wubben et al. 2000).

In root exudate challenged mycelia, the MAPK2, PL and CUT1 are expressed the highest in response to nonhost sorghum root exudates (Fig 11). Numerous studies have identified *Pmk1/Fus3*/*Kss1* MAP kinase (MAPK2) pathway in many plant and human pathogenic fungi as a major signaling pathway regulating pathogenicity, (Zhao et al. 2007; Cho et al. 2006). The other three virulence genes examined in this study, beta glycosidase (BGL3), copper phenoloxidase (CuOx, laccase) and ceratoplatanin (CPT, sometimes referred to as SnodProt1), are all expressed at slightly higher levels in response to nonhost sorghum (Fig 11). Snodprot1 was identified as a small secreted protein phytotoxin in the wheat pathogen *Stagonospora nodorum* (Skinner et al. 2001) and orthologs in *M. grisea* (MSP1), *Botrytis* (*bcspl1*) and *Ceratocystis* (CFP) have been shown to be important for virulence (Jeong et al. 2007; Chague et al. 2006; Pazzagli et al. 1999). CuOx could be a type of laccase (a ligninolytic enzyme), which in the vascular wilt pathogen, *Fusarium oxysporum*, may be used to penetrate the lignified xylem elements of the host plants. One study identified six laccase genes in *F. oxysporum*, of which three of them *lcc*1, *lcc*3, and *lcc*9, are expressed in roots and stems of infected plants (Cordoba-Cañero and Roncero 2007). *P. omnivora* does not specifically infect the xylem of its hosts, but vascular discoloration and rapid wilting of leaves indicate the vascular system of the plant is affected.

In the sclerotia, the virulence genes MAPK2, BGL3, CuOx, and CPT seem to be expressed the highest in 8 week-old sclerotia, while CUT1 seems to be expressed higher

in 4 week-old sclerotia (Fig 12). Since these genes are all considered virulence factors it is interesting to find these genes to be expressed during sclerotial formation since sclerotia are survival structures and do not directly interact with plants. Perhaps the virulence-related gene products in sclerotia are mobilized to hyphal germ tubes when sclerotia break dormancy and germinate in response to root exudates.

Relative expression of secondary metabolism genes: Multi-drug transporters in phytopathogenic fungi actively pump small molecular weight toxins from their cytoplasms, thereby protecting against plant phytoalexins and secreting potentially autotoxic phytotoxins (Sun et al. 2006; Daub and Chung 2007) http://www.apsnet.org/online/feature/cercosporin/). Thirty-one putative multidrug ABC-transporters were identified in the genome sequence of *P. omnivora* (data not shown). Two of these multidrug efflux transporter genes, MFS1 (a xenobiotic transporter) and MFS2 (trichothecence efflux transporter), were examined in the study. *Fusarium* spp. can produce trichothecenes, which can harm animals and plants (Desjardins et al. 1993). A well known trichothecene is T-2 toxin, which is a regulated contaminant of moldy grains that can lead to the rejection of harvested grains. It is not known if *P. omnivora* produces trichothecenes like *Fusarium* spp. But, since *Fusarium* is a common constituent of the soils inhabited by *P. omnivora*, perhaps MFS2 protects *P. omnivora* from trichothecenes produced by competing *Fusarium* spp. In the rice blast fungus *M. grisea*, a multi drug transporter (ABC3) plays a vital role in pathogenesis because *M. grisea* mutants lacking this gene were completely nonpathogenic, but still capable of forming appressoria (Sun et al. 2006). Thus these genes should be of interest for future investigations studying their roles in the pathogenicity of *P. omnivora* on its host(s). In

vegetative mycelia, MFS1 is at a higher level than MFS2 (Fig 10). On the other hand, in sclerotia, MFS2 is much more highly expressed in 4 week-old sclerotia than 8 week-old sclerotia (Fig 12). In root exudate challenged mycelia, MFS1 and MFS2 were expressed to higher levels in response to sorghum root exudates (Fig 11). This could be attributed to *P. omnivora* deriving more nutrition form sorghum root exudates, or may have been due the presence of small molecules secreted by sorghum roots that induced the expression of MFS1 and MFS2.

Two other secondary metabolism-related genes examined are regulatory genes, a zinc finger transcription factor (SIRZ) and a methyltransferase (LAEA). These are expressed more in 8 week-old sclerotia than in 4 week-old sclerotia (Fig 12). However, SIRZ and LAEA show very little change in expression in response to root exudates (Fig 11). Biosynthesis of sirodesmin PL phytotoxin is controlled by a gene cluster consisting of 18 genes in the phytopathogenic fungus *Leptosphaeria maculans* (Gardiner et al. 2004). Transcriptional control of this biosynthetic cluster is regulated in *L. maculans* by the $Zn(II)_2Cys_6$ (zinc finger) transcriptional regulator, SirZ (Fox et al. 2008). The biosynthesis of a related secondary metabolite, gliotoxin, is regulated in *A. fumigatus* by the orthologous zinc finger transcription factor GliZ and PlGliZ (putative regulator of gliotoxin). The LAEA methyltransferase has been studied in several *Aspergillus* spp. and has been shown to be a global regulator in secondary metabolite biosynthesis (Bok and Keller 2004).

CONCLUDING REMARKS

There is limited literature describing a qRT-PCR experiment, like the one described in this chapter, which looks at the expression level of twenty six genes across life stages. The selected genes represented a wide range of functions and in spite of the large experimental error, trends in differential gene expression were identified that warrant further investigation. Even though this data is preliminary, lessons learned during these studies (e.g. reduce pipetting error, use only one appropriate kit to prepare RNA and cDNA) could be followed to reduce experimental error. If successful, then this experiment could be repeated with a closely related fungus (e.g. Pezizomycetes, *Rhizina* or *Tuber*) to investigate how gene expression trends have diverged or converged during evolution. If a successful genetic transformation system is successfully developed for *P. omnivora*, then the function of the genes studied could be investigated through gene deletion and overexpression studies. Most of the fungal systems described in the literature cited were highly tractable genetically and had numerous genomic tools available. In the absence of such molecular genetics tools, more descriptive biochemical experiments can be designed to study the activities of the enzymes encoded by the genes described here and how these change in response to nutritional and developmental changes. More molecular investigations into *P. omnivora*'s interactions with its hosts are needed. *P. omnivora* is a difficult fungus to manipulate genetically perhaps, because it is multinucleate heterokaryon, which may make transformation difficult. We think that this pathogen still warrants investigation because cotton root rot is endemic to many Oklahoma alfalfa and cotton fields and negatively impacts growers to the point that entire

fields must be abandoned or replanted with grasses. This qRT-PCR experiment is a good start to future investigations into the gene functions and pathogenicity of *P. omnivora*.

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VITA

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Candidate for the Degree of

Master of Science

Thesis: EFFORTS TO CHARACTERIZE THE GENOME ORGANIZATION AND GENE EXPRESSION PATTERNS OF THE COTTON ROOT ROT FUNGUS, *PHYMATOTRICHOPSIS OMNIVORA*

Major Field: Plant Pathology

Biographical:

Education:

Completed the requirements for the Master of Science in Plant Pathology at Oklahoma State University, Stillwater, Oklahoma in July, 2010.

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Experience: I have been a graduate research assistant for 4 years where I have primarily worked on fungi. Experiments have ranged from culturing fungi, to DNA/RNA extraction, transformation, and real-time PCR. I was a teaching assistant in 2007 for an introductory plant pathology course where I prepared materials for labs, graded exams and responded to students' questions outside of class.

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Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: EFFORTS TO CHARACTERIZE THE GENOME ORGANIZATION AND GENE EXPRESSION PATTERNS OF THE COTTON ROOT ROT FUNGUS, *PHYMATOTRICHOPSIS OMNIVORA*

Pages in Study: 97 Candidate for the Degree of Master of Science

Major Field: Plant Pathology

Scope and Method of Study: Several isolates of the fungus *Phymatotrichopsis omnivora* from cotton and alfalfa were used in attempts to study the fungus' genome structure and expression profiles of twenty-six genes during different growth stages. Pulsed field gel electrophoresis (PFGE), telomere fingerprinting, nucleic acid staining, and real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) were employed to investigate the study's objectives.

Findings and Conclusions: Efforts to characterize the genome of *P. omnivora* using PFGE and telomere fingerprinting were unsuccessful. However, chromosomal standards were separated by PFGE in two experiments. We confirmed the multinucleate nature of our *P. omnivora* isolates using fluorescent nucleic acid staining and microscopy, which showed younger hyphae have fewer nuclei than older hyphae and newly branching hyphal cells contained up to 24 nuclei. The gene ontology (GO) categorization and life stage expression patterns of twenty-six different genes were followed using qRT-PCR. However, because cDNAs were prepared using two different kits, sample bias prevented comparisons of gene expression across treatments. Of four housekeeping genes examined, expression of ITS-1 rRNA was most consistent across treatments and was used as an internal control to normalize expression of all other genes. Gene expression levels differed among the life stages of *P. omnivora* investigated. In vegetative mycelia, two virulence-related genes appeared repressed, while individual genes in all categories were highly expressed. In mycelia challenged with host (*Medicago truncatula*) or nonhost (*Sorghum bicolor*) root exudates, some genes involved in secondary metabolism, virulence and glycogen synthesis were expressed higher with nonhost root exudates. In sclerotia, genes involved in glycogen biosynthesis and secondary metabolism were expressed higher in 4 week-old sclerotia than 8 week-old sclerotia. Unfortunately, due to the high level of error, all gene expression data can only be considered preliminary. However, these findings point to interesting new directions for future gene expression and genomics studies comparing *P. omnivora* with other fungal pathogens and related fungi in the Pezizomycetes.

ADVISER'S APPROVAL: Dr. Stephen Marek