DIFFERENTIAL GENE EXPRESSION IN WHEAT ROOTS IN RESPONSE TO INFECTION BY THE 'TAKE-ALL' FUNGUS

(*Gaeumannomyces graminis* var. *tritici*)

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FORMAT OF THESIS

This thesis presented in the Molecular of Plant-Microbe Interactions style and format allowing for independent chapters (Chapter II and Chapter III) to be suitable for submission to scientific journals. Two papers have been prepared from research data collected at Oklahoma State University to partially fulfill the requirements for the degree of Doctor of Philosophy. Each paper is complete in itself containing an abstract, introduction, results, discussion, materials and methods, acknowledgments and literature cited sections.

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NOMENCLATURE

Ggt	*Gaeumannomyces graminis* var. *tritici*
cDNA	complementary DNA
ds	double-strand
mRNA	messenger RNA
PCR	Polymerase Chain Reaction
R/T	room temperature
ss	single-strand
SSH	suppression subtractive hybridization

Chapter I

Introduction and Literature Review

Historical Perspective

The root disease "take-all" is regarded as the most damaging disease affecting wheat (*Triticum aestivum* L.) worldwide (Yarham et al., 1989, McCay-Buis, 1993). Take-all effects crops worldwide, as evident in 1983 when the 1st International Workshop on take-all of cereals was convened consisting of 63 participates from 9 countries (Hornby, 1998). The global distribution includes; Argentina, Australia, Austria, Belgium, Canada, Chile, Czechoslovakia, Denmark, East Africa, England, France, Germany, India, Italy, Japan, Kenya, Morocco, Netherlands, New Zealand, Norway, Poland, Scotland, Spain, Sweden, Switzerland, Uruguay, United States, and Wales (Sprague, 1950).

The name take-all was first used to describe the effect on crops in Australia in 1870 (Butler, 1961). However, the first record of take-all effecting wheat was not recorded until 1912 in the United Kingdom (Massee, 1912). The pathogen responsible for take-all was previously, although erroneously, named *Ophiobolus graminis* (Sacc.). It was renamed in 1972 to *Gaeumannomyces graminis* (Sacc.) Arx & Olivier var. *tritici* Walker (Walker, 1972). The further taxonomic nomenclature as described in Mathre (1992) includes the following: division, Amastigomycota; subdivision, Ascomycotina; class, Ascomycetes; subclass, Hymenoascomycetidae I; order, Diaporthales; family, Diaporthaceae.

Take-All Disease

Take-all disease of wheat is described as infecting root, crown, and foot tissues (Huber and McCay-Buis, 1993). Visual symptoms of wheat take-all include: stunted plants, yellowing leaves, decreased leaf area, reduction in secondary tiller formation and white-heads caused by premature ripening (Hornby and Fitt, 1981) (Figure 1).

Figure 1. Visual symptoms of take-all disease the Plant Pathology farm in Stillwater, OK, May 2000

Light to dark brown or black lesions and necrotic tips are often seen in roots. Blacken roots are a good indication of severe take-all infection (Huber and McCay-Buis, 1993) (Figure 2).

Figure 2. Below ground root symptoms of take-all disease in samples taken from the Plant Pathology farm at Stillwater, OK, May 2000

The foot area is black in appearance by mycelium of the fungus and the majority of the roots are heavily melanized (Huber and McCay-Buis, 1993) (Figure 3).

Figure 3. Below Ground Foot and Crown Symptoms Of Take-All Disease In Experimental Field Stillwater, OK, May 2000

Wheat can be infected at all stages of development in environmentally favorable conditions (Huber and McCay-Buis, 1993). The most severe symptoms of take-all occur in young plants infected just after planting (Ohio State University Fact Sheet, 1996)

Soil characteristics are an important consideration when assessing conditions favorable for take-all infection. The soil pH range for take-all survival is between 5.5 and 8.5 (Hornby, 1981). Take-all disease has been shown to be greater at pH >5.4 because at pH < 5.4 *Gaeumannomyces graminis* is inhibited (Sivasithamparam and Parker, 1981). Soil temperature range for take-all infection is between 5.0 and 30.0°C with severe infection occurring between 12.0 and 20.0°C (Hornby, 1981). Soil that is conducive to root growth and has adequate moisture is unfavorable to take-all infection (Catt et al., 1986).

Deficiencies in both major and minor soil nutrients increase the risk for take-all infection (Hornby, 1998). Adequate nitrogen, phosporus, potassium, sulphur, chloride and magnesium decrease while calcium and potassium increases the risk of take-all disease (Huber, 1981, Reis et al., 1982). In addition, Catt et al., (1986) reported an increase in take-all in soils high in exchangeable potassium.

The form of nitrogen is also an important consideration when assessing the risk of take-all infection. When ammonium (NH_4^+-N) was used rather than nitrate (NO_3^--N), the risk of infection was decreased probably due to the decrease in rhizosphere pH (Trolldenier, 1985). Ammonium nitrogen is metabolized primarily into amino acids in the root whereas; nitrate nitrogen is translocated to the leaves where it is reduced to amino nitrogen and subsequent amino acids without affecting the rhizosphere.

Of all of the minor nutrients, manganese is the most important when examining the risk of take-all. Increasing manganese was shown to decrease take-all disease (Huber and McCay-Buis, 1993). Manganese is an activator, not a component of many plant enzyme reactions (deoxy-D-arabinoheptulosonate-7-phosphate synthase, phenylalanine

ammonia lyase, indoleacetic acid lyase, and lignin synthesis enzymes) (Huber and McCay-Buis, 1993). It affects nitrogen metabolism, respiration, photosynthesis and hormone metabolism, which subsequently effects root exudations and rhizosphere microorganisms (Hornby, 1998; Huber and McCay-Buis, 1993). Schulze et al. (1995) tested the hypothesis that Ggt reduces plant defense systems by catalyzing the oxidation of Mn^{2+}. Ggt was shown to oxidize Mn^{2+} (soluble), which is taken up by wheat roots to Mn^{4+} (insoluble), which is not utilized by wheat roots (Schulze et al., 1995). The hypothesis is that Ggt decreases plant defense response by oxidizing Mn^{2+} prior to infection of wheat roots (Schulze, 1995). This hypothesis is further supported by the synergistic relationship between manganese-oxidizing soil microbes and Ggt (Huber and McCay-Buis, 1993). In addition, copper, boron, and manganese are involved in the formation of phenolics that contain anti-fungal properties (Graham, 1983). Copper is also involved in formation of the plant disease resistant barrier lignin (Graham, 1983). Reis et al. (1982) also found that zinc and copper suppress take-all disease.

Hosts / Pathogen Interaction

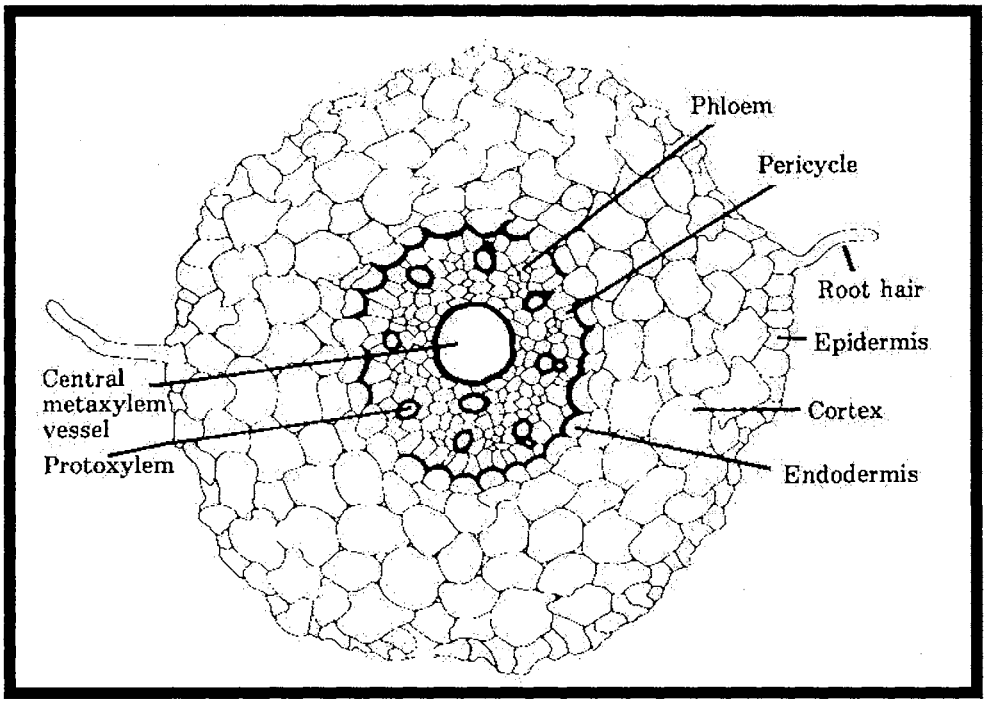
In a review of wheat worldwide, Briggle and Curtis (1987) describe wheat as being grown under minimum temperatures of 3°C to maximum of 32°C with the optimum at 25°C. They further go on to describe wheat growth conditions having a minimum average yearly rainfall between 250 and 1750 mm. Most of the global wheat is harvested between the months of April and September in temperate regions of the northern hemisphere. Wheat yields have increased while area harvested has remained relatively stable from 1961 to 1985 (Briggle and Curtis, 1987). During the 1984-85 crop year 514.5 Tg of wheat was produced, this is double the amount from 25 years earlier (Briggle and

Curtis, 1987). Developing countries during a ten-year period from 1971 to 1981, developing countries saw a 50% increase in wheat production compared to 35% in developed countries (Briggle and Curtis, 1987).

Although the yield and growth of the above ground parts of the plant have received much attention, relatively little attention has been given to the below ground parts (Hornby and Fitt, 1981). Reasons for this discrepancy may involve there being fewer wheat root diseases and root diseases are not as easily diagnosed as compared to the above ground diseases (Hornby and Fitt, 1981).

In order to follow the development of the wheat root diseases it is important to understand the root morphology (Figure 4).

Figure 4. Cross-Section Diagram Of A Wheat Root (Fahn, 1990)



A layer of epidermal cells that are slightly cutinized surrounds young wheat roots. Root hairs develop in the zone of maturation located behind the root tip. The layer of

cells located under the epidermis is the cortex. It consists of a seven to eight layers in seminal roots and fewer in crown roots. The innermost layer is the endodermis, which consists of a single layer of cells that surrounds the stele. The endodermis is a natural barrier to invasion by many root-invading fungi and this function increases with the age of the plant (Hornby and Fitt, 1981). This is due to the sclerotization of cells of the endodermis, pericycle, and parenchyma of the stele. The stele is where the pericycle and central cylinder of xylem vessels and phloem bundles are located. The pericycle is a single layer of cells where later roots originate. The pericycle of older roots consists of thicker cell walls as compared to younger pericycle cells. Older cells also have thicker endodermis cells walls. However, in wheat roots along with other cereals, there is no secondary thickening because there is no cambium present. Moreover, some winter wheat varieties do develop thick nodal roots with a resistant exodermis and sclerotinized outer cortex cells. Root morphology is affected by soil temperature, moisture, aeration, nutrients, structure and microflora (Hornby and Fitt, 1981). Changes in root morphology may not affect root function and wheat roots infected by soil fungi may not affect root growth (Hornby and Fitt, 1981).

The root disease take-all is regarded as the most damaging and important disease affecting wheat worldwide (Hornby and Fitt, 1981, McCay-Buis, 1993). The infection is caused by the soil-borne fungus *Gaeumannomyces graminis* var. *tritici* (Ggt) and is semi-host specific. Wheat is the most susceptible followed by triticale, barley and the relatively resistant rye (Scott, 1981, Wallwork, 1989). Ggt also affects wheat cultivars differently. Hard red winter wheat produced higher yields than the soft white winter wheat cultivars even-though they suffered from the same extent of take-all infection

(Huber and McCay-Buis, 1993). In a review by Huber and McCay-Buis (1993), it was suggested this difference in wheat cultivars was do to soft winter wheat having a higher nutrient requirement and a lower efficiency for partitioning vegetatively stored nutrients than hard red winter wheat. This implies that both market classes of wheat are susceptible, however hard red winter wheat are able to better tolerate the disease.

Initially, Ggt infects roots by secreting cell wall degrading enzymes that allow numerous dark hyphae to penetrate the cell wall (Sivasithamparam and Parker, 1981). These cell wall degrading enzymes may include pectic, cellulolytic, hemicellulolytic and proteolytic (Hornby and Fitt, 1981). Weste (1970) described Ggt as utilizing pectate, pectin, and cellulose as sole carbohydrate sources and that polygalacturonase, pectinmethylesterase and cellulases were produced respectively. Moreover, polygalacturonase was produced in advance of fungal hyphae growth shortly after inoculation; pectinmethylesterase was produced in about 5 days and cellulase in one week after inoculation.

Hyaline branches invade cortical cells and colonize the stele. The phloem is subsequently destroyed, decreasing the translocation of photosynthates from the shoot. The last stage of Ggt infection results in the colonization of the xylem where Ca^{2+} and H_2O uptake is affected. Moreover, in addition to the production of white-heads, the effect on shoots resembles water-stress. A time course of Ggt ascospore infection process in wheat roots has been described (Table 1) (Weste, 1972). However, since ascospore infection is not a problem in the field, this artificial condition was changed to study mycelium infection (Weste, 1975) which is how Ggt invades the tissue in field grown plants. Weste (1975) subsequently used mycelium and the time course of

infection was changed. However, the first time period used by Weste (1975) was 2 days. It is therefore possible that ascospores and mycelium effect at about the same rate. Our preliminary data agree to some of the time course infections described by Weste (1975) and disagree with most of those described by Weste (1972). However, without an earlier time point it is hard to compare the results of this preliminary data with that of Weste (1975). Moreover, without using mycelium (Weste, 1972) it is difficult to compare ascospores to mycelium-infected data.

Table 1. Time course for Ggt infection (ascospores) of wheat roots in culture (Weste, 1972)

Time after inoculation	Root Hairs	Epidermis	Cortex	Stele	Whole	Other Observations
4 hours	Collapsed at point of contact					
6 hours	Penetration, cell wall damaged, granular diffusate					
8 hours	Plasmolysed, bent, lignitubers					
10 hours		Penetration				
15 hours			First lesions, cells separated, walls torn, contents plasmolysed			
18 hours	Shrivelled					
21 hours			Lesions larger Lignitubers common			
1 day			Lesions extended into endodermis			
1 1/2 days			Heavily infected, cell contents granular, walls fractured			
2 days			Cell contents contracted to center			
6 days			Cell layers 4-6 of 1st seminal root penetrated			
8 days			Lesions common	Dark plugs in xylem, lignitubers		
11 days				Penetrated	Lesions large and numerous	Stem base rotted
15 days	Suppressed or shriveled		Eroded away		Some entirely black. Lesions extensive. Cell contents disorganized	
21 days					No autolysis of hyphae under gnotobiotic conditions	
63 days						Plant Dying

Take-all has been described to effect wheat, barley, rye, triticale, grasses, oats, and maize to differing extents. Although, each hosts is susceptible to different strains of *Gaeumannomyces graminis* (Hornby, 1998). In addition to Ggt, there are three other strains of *Gaeumannomyces graminis* that are host specific. *Gaeumannomyces graminis* var. *avenae* (Gga) specifically infects oats (*Avena sativa* L.) (Mathre, 1992), *Gaeumannomyces graminis* var. *graminis* (Ggg) infects turf grasses and other grass

species (Mathre, 1992) and *Gaeumannomyces graminis* var. *maydis* (Ggm) specifically infects maize (*Zea mays* L.) (Yao et al., 1992, Yao, 1993).

Oats are not infected by the wheat strain Ggt, however Gga infects them. It is believed that oats are not effected by Ggt do to the production of the root exudate avenacins (Asher, 1981). Avenacin is a tirterpenoglucosidic anti-fungal compound produced in the root cortex cells (Asher, 1981) of most oat species and localized in the root epidermal cells (Osbourn et al., 1994). The mechanism by which Gga effects oats is due to its ability to produce avenacinase which is an enzyme that is specific for avenacins produced by oat roots (Crombie et al., 1986; Osbourn et al., 1991). Bowyer et al., (1995) created mutants deficient in the saponin detoxifying enzyme avenacinase and found that Gga mutants did not infect oats. This suggests that the production of avenacinase is a determining factor for host range.

Another proposed mechanism for Ggt infection susceptibility in wheat and not oats was examined by looking at cortical cell death (Yeates and Parker, 1986). Oats have less cortical cell senescence than wheat in response to Ggt (Yeates and Parker, 1986). Therefore, penetrating fungal hyphae may acquire less resistance or fewer root cellular defense response in wheat than in oats.

Ggg is morphologically different in that it produces lobed hyphopodia whereas; Ggt and Gga produce simple hyphopodia (Walker, 1972). Ggg is the least pathogenic towards wheat (Mathre, 1992). This is do to Ggg being less able to cross the endodermal barrier in wheat than Ggt (Hornby and Fitt, 1981).

The maize take-all disease has recently been attributed to *Gaeumannomyces graminis* var. *maydis* (Yao, 1993).

There have been many studies into developing selective media for isolation and characterization of *Gaeumannomyces graminis* var. *tritici* from root tissue (Duffy and Weller, 1994). With the advancement of molecular techniques, DNA probes for mitochondrial DNA have been used for identification of *Gaeumannomyces graminis* species amongst other fungi and bacteria pathogenic in spring wheat (Henson, 1989). Henson (1992) used mitochondrial DNA primers from Ggt to identify all *Gaeumannomyces graminis* species. However, this study was not done in wheat root tissue. Thorton et al. (1997) characterized a monoclonal antibody (MAb) raised from a surface antigen from Ggt to differentiate between other fungi and Ggt. This surface antigen was characterized by exposing Ggt to phenolic compounds known to be released by wheat roots. This MAb only recognized the antigen produced by *Gaeumannomyces graminis* species and not other fungi. Polymerase Chain Reactions (PCR) has also been used in the detection of specific Ggt DNA segments from infected wheat root tissue (Schesser et al., 1991) and to identify *Gaeumannomyces graminis* species from other fungal species (Ward, 1995). Ribosomal DNA (rDNA) has also been used to classify Ggt, Gga, and Ggg by their phylogeny (Bryan et al., 1995). Based upon rDNA analysis, Ggt and Gga are more closely related (simple hyphopodia) while Ggg is more distant (lobed hyphopodia) (Bryan et al., 1995). Ribosomal DNA primers are also used to distinguish between Ggt and Gga in wheat root tissue (Bryan et al., 1995). Although there have been many reports of isolation and characterization of *Gaeumannomyces graminis* species there is no study on methods used to identify host gene expression after infection. The aim of this research is to characterize differential gene expression induced by Ggt infection of wheat.

Management

Control of take-all has been suggested to increase yields up to 10% to 50% (Heim, et al., 1986). However, managing take-all has been confounded because, there are no genetic resistant hosts or effective chemical controls (Huber and McCay-Buis, 1993). In order to implement management strategies for take-all it is important to know the high-risk conditions involved in its pathogenicity. The following table of high risk conditions was taken from Monsanto's Take-all website (1998) (table 2):

Table 2. High Risk Conditions for Take-all Infection

Soil	Previous Crop	Cultivation	Fertilization	Climate
Light aerated soils.	Wheat	Short plowing to drilling time.	Low nitrogen levels.	Autumn Wet
Compacted heavy soils.	Barley	Early sowing date.	Nitrate forms of nitrogen.	Winter Mild
Poor drainage / structure.	Pasture	High seedling rates.	Manganese deficiency.	Spring Cool / Moist
High organic matter content.	Set-aside	Loose seedbed conditions.	Low levels of P and K	Summer Hot / Dry
Alkaline soils.	Oilseed Rape	Poor weed control conditions.		

A more detailed table listing favorable and unfavorable conditions for the host, pathogen, biotic environment, and abiotic environment is given by Huber and McCay-Buis (1993).

Land Management

The best strategy for control of take-all is agronomic practice manipulation (crop husbandry). The term "crop husbandry" includes rotation, drainage, soil management, cultivation technique, seedbed preparation, straw incorporation, sowing date, herbicide use, and fertilizer practice (Yarham et al., 1989). Take-all can be

avoided by growing non-susceptible crops alternatively with susceptible crops (Hornby, 1998). Ggt is a soil invader, in that it cannot survive indefinitely without a susceptible host (Bockus, 1987, Yarham et al., 1989). Ggt can survive by parasitizing volunteer wheat or grassy weeds or by saprophytic infestation of wheat crop residue left on the soil (Bockus, 1987, Hornby, 1998). Take-all pathogens can survive up to three years in the soil without a susceptible host (Bockus, 1987). By following tillage practices to eliminate wheat crop residue and grassy weeds, the possibility for take-all infection can be decreased (Bockus, 1987). Monsanto's Take-all website (1998) suggested the following cultural practices to decrease take-all infection:

1. Avoid continuous cereal cropping practices.
2. Avoid early sowing of cereal crops.
3. Avoid loose seedbed conditions.
4. Reduce seedling rates, to avoid dense cropping
5. Avoid high nitrogen applications
6. Ensure high grass weed control in previous crops

Chemical

Chemical control of take-all is efficient but extremely costly. Fungicide spray has shown difficulty in targeting Ggt and soil fungicides have low activity soils that are more complex. Of all the chemical controls of take-all the most effective has been seed treatment with fungicide. Only a small amount of fungicide is needed (which makes it the best cost effective means of chemical control) with good accuracy (Hornby, 1998). Latitude is a new seed treatment that has recently been developed by Monsanto (Monsanto, 1998). This new treatment uses 2 L of Latitude per tonne of

seed (200 mL / 100 kg). Latitude moves off the seed rapidly but slowly through the soil allowing a barrier to be produced around the roots. Latitude affects the energy production in the cells of the fungus without harming beneficial soil microbes.

Biological

Biological control has shown great promise in decreasing take-all in wheat. The most recognized biological control is *Phialophora* spp. which complexes with *Gaeumannomyces graminis* and suppresses the onset of take-all (Hornby, 1998). Spores of *Phialophora* are known to exist in some species of *Gaeumannomyces* (Walker, 1972). *Phialophora radicicola* var. *graminicola* occurs on all wheat crops but is prominent in wheat crops grown after grass (Slope et al., 1978). The population of *Phialophora radiciola* var. *graminicola* decreases rapidly when wheat is grown consecutively without crop rotation practices (Slope et al., 1978).

Another example of a biological control agent (BCA's) is the rhizosphere bacteria *Pseudomonas fluorescens* that produces the antibiotic Phenazine, which helps control take-all in wheat (Thomashow and Weller, 1988). A mutant of *Pseudomonas fluorescens* deficient in the ability to produce Phenazine was not inhibitory to the take-all fungus *Gaeumannomyces graminis* var. *tritici* (Thomashow et al., 1990). For reviews of *Pseudomonas fluorescens* control of root diseases in wheat, see Thomashow et al., (1990) and Weller et al. (2002).

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Chapter II

Infection Time-Course in Wheat Roots in Response to the Take-All Fungus (*Gaeumannomyces graminis* var. *tritici)*

Take-all [*Gaeumannomyces graminis* var. *tritici* (Ggt)] is regarded as the most damaging root disease affecting wheat worldwide. This study was conducted to develop and implement procedures for examining take-all infection in wheat roots under controlled conditions.

A procedure for surface sterilization of seeds, growth conditions, and infection time course was developed. Seeds were sterilized by sonication in 1% $AgNO_3$ with Tween 20 (30s), rinsed with sterile-deionized water, and placed on sterile filter paper in a cold room (4.5°C) without light for 48 hours. Imbibed seeds were then aseptically transferred to 1/5X Potato Dextrose Agar (PDA) at 25°C without light for 48 hours. Seedlings with roots approximately 2.0 to 3.0 cm long were transferred to 1/5X PDA without Ggt (control), or with Ggt lawn and placed into a 25°C incubator without light for 12, 24, and 48 hours.

A time course for infection was determined with light microscopy. Results indicated that at 12 hours Ggt had colonized the root surface, at 24 hours root hairs were penetrated, and at 48 hours root hairs collapsed and the fungus penetrated the epidermis and cortex. Analysis of root length increase indicated that there was a 40.9%, 32.2%, and 61.5% decrease in growth with Ggt at 12, 24, 48 hours, respectively, as compared to the controls. The time-course of infection and the root tissue obtained from this analysis will be used in a subsequent investigation to study differential gene expression in wheat roots infected with Ggt.

Additional keywords: Ggt, wheat roots, infection, time course, microscopy.

The root disease Take-all [Gaeumannomyces graminis var. tritici (Ggt)] is regarded as the most damaging disease affecting wheat roots worldwide (Heim et al., 1986, Yarham et al., 1989). Control of take-all has been suggested to increase yield up to 10% to 50% (Heim, et al., 1986). Diseases that infect the above ground portion of the plant that affect yield and growth have received considerable attention with little attention being paid to the below ground root pathogens (Hornby and Fitt, 1981).

The study of root-rot diseases is complex due to soil type, nutrients, temperature, moisture, and pH affecting their pathogenicity. After being affected by the soil environment Ggt must reach the rhizosphere followed by the rhizoplane, epidermis, cortex, endodermis, and finally the vascular tissue. The Take-all fungus targets and eventually destroys the stelar elements (Sivasithamparam, 1998). There have been few studies into the microscopic examination of this infection process in roots (Schulz et al., 1995) and fewer still in looking at the whole infection process in young roots. The whole infection analysis being from colonization of the root surface to penetration of the stelar elements.

In order to investigate the infection process a system has to be developed that provides the best environment for growth of the pathogen and the host and is conducive to the highest degree of infection. Research into the infection process has utilized the growth media Potato Dextrose Agar (PDA) (Broadfoot, 1933; Davies, 1935; Rengal et al., 1994; Schulze et al., 1995; Speakman, 1982; Speakman and Lewis, 1978) for growth and maintenance of Ggt cultures and/or seedlings. Studies have also utilize different temperatures (Henry, 1932; Walker, 1972), wheat cultivars (Rengal et al., 1994), nutrients (Schulze et al., 1995), sterilized soil (Henry, 1932), times of infection

(Speakman and Lewis, 1978; Weste, 1972 and 1975), inoculum placement (Kabbage and Bockus, 2002), media (Broadfoot, 1933; Mathre, 1992), optimum pH (Broadfoot, 1933), and surface sterilization (Davies, 1935; Speakman, 1982; Mathre, 1992). Although it is important to develop and utilize a controlled environment to decrease the physical and biological factors of the soil environment (Hornby and Fitt, 1981), it is also important to remember in nature soil-borne diseases would consist of more than one microorganism (Broadfoot, 1933).

This research was performed to develop a controlled system for infecting wheat roots with the Take-all fungus and to examine, at critical stages, the whole infection process. An infection time-course was utilized to determine the critical infection stages and this data will be used in a subsequent paper to determine differential gene expression in wheat roots infected by the Take-all fungus.

RESULTS

Seeds of the hard red winter wheat cultivar "Jagger" were surface sterilized and aseptically grown on a lawn of Ggt as described below. Wheat roots placed on a lawn of Ggt (Figure 2 and 3) and grown under these conditions resulted in a significant ($P<0.05$) 40.9%, 32.2%, and 61.5% decrease in growth of infected roots compared to non-infected controls at 12, 24, and 48 h, respectively (Figure 1).

These time periods were chosen as the critical stages of infection for our system as a result of the microscopic analysis discussed later. In short, these time points represent the time in which the fungus is surrounding the root without penetration (12h), the fungus is penetrating root hairs (24), and the fungus penetrated the epidermis and colonized the cortex (48 h). The 72 and 96 hour time periods were used to take growth measurements for this study only and will not be used in the subsequent paper on differential gene expression. The Ggt infected roots had a 70% and 66% decrease in growth as compared to the controls (data not shown). At 72 hours the cortex was still being colonized however, the endodermis had not yet been penetrated. At 96 hours the endodermis had been penetrated and xylem vessels were colonized. No colonization or penetration of the phloem had occurred at 96 hours. A steady state growth rate was obtained beginning at 48 hours and continuing on through 96 hours for both the control and Ggt infected roots (data not shown).

Squash mounts, epidermal peels and longitudinal sections were taken at each time period as described in Figure 1. It is important that a microscopic analysis be performed under these growth conditions in-order to ascertain the time-course of infection within this system. Initial time points of 12, 24, 48, 72, and 96 hours were obtained from the

literature (Weste, 1972 and 1975). It should be noted that Weste (1972) used ascospores as means of infection and Weste (1975) used mycelium. In this proposal Ggt fungal mycelium was used to infect the wheat roots at all time periods as described in materials and methods. Microscopic examination at 12 hours reveled hyphae growth on the root surfaces with no penetration of the root surface (data not shown), although infected roots at this time period showed a significant decrease in growth (Figure 1). Figure 2 shows the petri dish and seedlings of the control and the Ggt infected roots after 24 hours growth. At 24 hours root hairs were penetrated and fungal hyphae were continuing to grow on the root surface (Figure 3). Although the roots hairs were penetrated at this time period the percentage decrease in root length was less than that at 12 hours as compared to the control (Figure 1). Figure 4 shows the arrangement on the petri dish of seedlings grown for 48 hours on 1/5X PDA inoculated with or without Ggt. At 48 hours root the cortex was colonized with fungal hyphae (Figure 5 and 7), hairs were collapsing (Figure 6 and 7) and hyphae penetrated the epidermis (Figure 7). At 96 hours, roots began to turn brown (Figure 8) and ectotrophic mycelium growth covered the seed and the crown tissue (Figure 9 and 11). Root tips were also turning brown (Figure 10) and this result is further supported by the 66% decrease in root growth at this time period (data not shown).

Tissue was also collected at the end of every experiment and placed on Ggt Selective media (SM-GGT3) (Juhnke et al., 1984). Figure 12 shows the characteristic branching pattern of Ggt coming from the root section taken from Ggt infected tissue and grown for 48 hours on SM-GGT3. This was done to ensure that the infection was due to Ggt and not attributed to any other contamination (Weste, 1975) and to maintain the isolates pathogenicity Naiki and Cook, 1983). The characteristic curling pattern of Ggt

hyphae can be clearly seen in the right had photograph of Figure 13. SM-GGT3 contains L-DOPA, which turns black in the presence of Ggt fungal hyphae (Figure 12 and 13).

In order to use the most pathogenic isolate of Ggt, (HV-92, Jo-8, and RL-4; provided by Dr. Bockus from Kansas State University and two isolates taken from an experimental field at Oklahoma State University – Stillwater) were tested for their pathogenicity (Figure 14). Although the above ground portion of the seedling infected with HV-92 did not look any different than the control and other isolates, below ground it produced the classic Take-All symptoms (Figure 15) and was determined to be the most pathogenic isolate tested. This isolate was used in all experiments described in this paper. The crown and foot of the Ggt infected is black with mycelium growth, characteristic of severe take-all infection.

This system also utilizes silver nitrate (AgNO3) as the wheat seed surface sterilization method instead of the traditional bleach method . Initially, experiments were performed in-order to determine the strength of bleach to use to surface sterilize seeds (Figure 16). This is important because contamination free seeds are needed for up to 8 days (2 days cold room, 2 days at 25°C, and 4 days growth) without a negative effect on root growth. The bleach concentration that gave the best median root length was 25% followed by 50%. According to these results 25% would be the best to use because it was not significantly different from 50% and it gave about the same % contamination. The two highest concentrations tested 75% and 100% were clearly to strong because of the decreased median root length and the lowest % contamination. A follow-up experiment was performed using 25% bleach to test this procedure for growth up to eight days on this media. After four days 50% of the seeds where contaminated using 25%

bleach as a surface sterilizer. This was unacceptable and upon personal communication with Dr. Singleton 1% silver nitrate was used.

To see how long silver nitrate treated seeds would grow on 1/5X PDA with out signs of contamination seeds were surface sterilized and left on a 1/5X PDA and checked every day for 11 days. Through 11 days no contamination of media or seed was seen. The silver nitrate sterilized seeds were then left on 1/5 PDA for up to 3 weeks with no sign of contamination (data not shown). This procedure of sterilizing, growing, and experimenting on wheat seedlings has proven successful and will be utilized in a subsequent paper on differential gene expression in wheat roots in response to infection by the Take-all fungus.

DISCUSSION

Infection System.

Ggt has been classified as a fungus whose effect on root function and shoot growth can be explained by stelar disruption (Hornby and Fitt, 1981). In order to investigate the infection process of Ggt, a system had to be designed that would allow the examination of one microorganism on its host without interference from other sources either physical or biological. Henry (1932) determined that a sterilized system is needed because more infections occur in sterilized versus unsterilized soil due to the antagonistic nature of the micro-organisms to Ggt in the unsterilized soil. This investigation has created a controlled system that allows for an examination of this interaction in an atmosphere that is conducive to the growth of the pathogen and the host. This infection system has been used in a subsequent experiments on differential gene expression in wheat roots in response to infection by the Take-all fungus (chapter III).

Jagger wheat was chosen as the host because in 2000, it was the most popular wheat grown in the wheat producing areas of Oklahoma and Kansas (personal communication Dr. Guenzi).

One of the most significant aspects of this infection system is the use of silver nitrate as a surface seed sterilizing agent. 1% AgNO3 was left for 11 days (Figure 17) up to three weeks on agar without contamination of the agar or the seedling (data not shown). In addition, diluted concentrations of AgNO3 have been described as not being toxic to *Ophiobolus graminis* (now named *Gaeumannomyces graminis*) (Davies, 1935).

The question of what type of fungal form to use to infect the host was described by Weste (1975). She used ascospores to infect wheat roots initially (Weste, 1972) and

later switched to mycelium (Weste, 1975) because in nature Ggt invades as mycelium (Weste, 1975). Mycelium from the HV-92 isolate of Ggt was chosen as the pathogen in the infection analysis because it was determined to be the most pathogenic isolate of all isolated tested (data not shown). This isolated produced the classic Take-all characteristic (Figure 15) such as, brown to black roots, black mycelium on the seed and base of stem (Massee, 1912).

Potato Dextrose Agar (PDA) was chosen for the infection analysis due to studies that describe wheat roots (Schulze et al,. 1995) and Ggt (Speakman and Lewis, 1978) grown on PDA. This allows for optimum conditions for growth of not only the pathogen but for the host.

Infecting wheat roots by placing the seedlings on a lawn of Ggt mycelium is a novel approach in that, instead of placing the fungus on the root via agar blocks (Rengel et al., 1994 and Weste 1975) the roots are being placed on the fungus. Wheat roots were placed directly on top of the Ggt fungal lawn, which caused decrease in growth as early as 12 hours (Figure 1). This is in agreement with Kabbag and Bockus (2002) whom found that the severity of infection by take-all is related to placement distance of the inoculum for the host. They found the most sever infection occurred when inoculum was placed at seed level in both greenhouse and field experiments. This system provides a way of infecting the whole root without regard for developmental regions of the root. In this way the whole root is treated equal.

The infection system's temperature of 25°C was chosen based upon the work of Henry (1932). He found that the optimum temperature for infection of wheat and Ggt was between 15 and 27°C.

Once the infection analysis is over, confirmation is needed that the infection was due to Ggt and not to another organism (Weste, 1972). This is accomplished by using Ggt Selective Media (SM-GGT3)(Juhnke et al., 1984). It is important to re-isolate the fungus from the infected root tissue to confirm that Ggt was the cause of the infection and to maintain the pathogenicity of Ggt. SM-GGT3 has been described as the best way in which to isolate Ggt from wheat roots (Mathre, 2000). When Ggt fungal hyphae come into close proximity with L-DOPA a black color is formed (Juhnke et al., 1984) and the characteristic mycelium pattern of curling of the edges and the branching (Walker, 1972) can clearly be seen (Figure 12 and 13). Naikia and Cook (1983) described Ggt's ability to lose its pathogenicity if it was not periodically passed through a host. In addition to confirming that the infection was do to Take-all, re-isolation of the fungus from the host then serves another important purpose of maintaining the pathogenicity of the pathogen (Speakman, 1982).

In summary, this system allows for optimum seed sterilization (Figure 17) and infection to occur between wheat roots and Ggt utilizing the optimum conditions for growth and infection of the host and pathogen. In addition, this system allows for confirmation that the infection is due to Ggt and to keep the pathogenicity Ggt throughout the experiments by isolation on Ggt selective media.

Infection Analysis.

There has been limited microscopic analysis of Ggt in wheat roots described in the literature (Clarkson, 1975; Hornby and Fitt, 1981; Massee; 1912; Mathre, 2000; Rengel et al., 1994; Schulz et al., 1995; Speakman and Lewis, 1978, Walker, 1972; Weste, 1972 and 1975). This is the first microscopic analysis time-course to be

performed with both control and infected tissue analysis under an optimized controlled environment for both the host and the pathogen. It was not in the scope of this research to do an exhaustive infection analysis but only to determine the approximate infection times for the fungus colonizing the root surface, penetration in to the root hairs and penetration of the epidermis and colonization of the cortex. With the enhancements to basic microscopy, such as, laser scanning confocal microscopy, immunocytohemical techniques, fluorescent probes, and green fluorescent protein (Gold et al., 2001) a more detailed time-course of Ggt infection into wheat roots should be pursued.

It was important for a precise time-course to be performed within the scope of this system. The tissue harvested from this time-course was used in a subsequent paper examining differential gene expression in wheat root in response to infection by the Take-all fungus, at 12, 24, and 48 hours. Although four critical time periods were determined utilizing this novel system (12h, 24h, 48h, and 96 h) only the first three time periods were used for differential gene expression analysis.

The early stages of growth of wheat has been described as when Ggt infection occurs (Massee, 1912). For a review of the components of the cereal root system structure and anatomy see Hornby and Fitt (1981). By infecting roots early our hope was to increase the severity of infection and to determine the time that we could see the roots surface colonized with fungal mycelium but with no penetration present.

With Jagger wheat and HV-92 isolate of Ggt, at 12 hours there was no penetration of root hairs or epidermis although the surface was colonized by fungal hyphae (data not shown). Hyphae grow along the surface of the root with out penetration has been described by Weste (1972) as being due to utilization of root exudates in the rhizosphere

as an energy source prior to penetration . The fungal hyphae colonizing the root surface were approximately 6.25 μm wide which is in close proximity to the 7 to 10 μm described by Walker (1972). Moreover, throughout the microscopic examination, at no point was there hyphopodia present or infection cushions present on the roots which also agrees with Walker (1972). This is also in agreement with Hornby and Fitt (1981) and Walker (1970) whom found that Ggt's penetration is not mechanical but is enzymatic.

Since 1912, Ggt has been described as initially entering the wheat roots through root hairs (Massee, 1912). At 24 hours, the Ggt entered the root through the root hairs with no penetration of the epidermis (Figure 3). Using ascospores, Weste (1972) described Ggt penetration at the root hairs and intact epidermis. She described hyphae penetration at many points along the root surface, which came from runner hyphae along the root surface. She described the penetration at the root hairs as plasmolyzed, bent, contracted, twisted, shriveled, and collapsed. Although in agreement with the time of root hair penetration, we found significant decreases in growth (Figure 1) whereas Weste, (1972) did not find any growth differences. Rengel et al. (1994) described Ggt as penetration the stele at 24 hours in the take-all sensitive wheat cultivar Bayonet but, not in the more resistant cultivar C8MM. Ggt did not penetrate the stele until 96 hours in the scope of the paper (Figure 8). Based upon this finding, Jagger wheat used in this experiment may have a higher level of resistance than Bayonet.

Weste (1975) used fungal mycelium and found that at 2 days lesions appeared, epidermis, cortex, and root hairs were penetrated. Moreover, Ggt entered the roots initially through the root hairs. This is in agreement with the results from this system in that at 48 hours root hairs, epidermis, and cortex were penetrated and colonized (Figure

4-7). Unlike no growth difference with ascospore infection (Weste, 1972), mycelium infection (Weste, 1975) caused a 50% decrease in growth at 2 days as compared to the controls. This is in agreement with the 61.3% decrease in growth at 48 hours in this system (Figure 1.) The roots were also covered with fungal hyphae at 48 hours and root growth may have decreased as a result of the collapsing root hairs (Figure 6 and 7). In addition, Speakman and Lewis (1978) also described Ggt as penetrating the epidermis and the cortex at 48 hours. Ggt will eventually erode the cortex and destroy the root system (Hornby and Fitt, 1981).

Clarkson et al. (1975) described rapid colonization of the cortex by fungal hyphae that caused dark brown to black discolorations. Moreover, the penetration into the stelar elements was slowed at the endodermis. Ggt hyphae eventually crossed the endodermis through plasmodesmata and rapidly colonized the stele. This is in agreement with the data presented here that the cortex was colonized by 48 hours (Figure 5) and it was not until 96 hours that the xylem was being colonized (Figure 8). Weste (1975) described black runner hyphae and brown stains on roots and embryonic areas of the seed were evident at 4 days as shown in this system (Figure 8 and 11). In comparing the results obtained in this research with those from Weste (1975), it is important to remember her first time point was 2 days. The time periods in this proposal are 12, 24, 48, 72, and 96 hours. The observations made before 48 hours in this system could therefore be in agreement with Weste (1975) observations at 2 days.

Differences in results from the previously described results from the literature could be attributed to Ggt being compatible with its host being dependent on the host and isolate of Ggt investigated (Weste, 1972). Research on soil-borne plant pathogens are

just now answering the question of how the disease is affecting the physiological processes of the root (Hornby and Fitt, 1981). This infection system and analysis was performed as a first step into understanding the infections process of Ggt and wheat roots. Subsequent analysis will be performed with tissue harvested from each time period on differential gene expression in wheat roots in response to infection by the take-all fungus in order to understand the molecular and physiological aspect of this infection process.

MATERIALS AND METHODS

Growth and Infection Time Course.

It is important to note that research on wheat roots due to fungal infection by *Gaeumannomyces graminis* var. *tritici* as well as other soil-borne fungi in the laboratory may not reflect the response of the plant in the field. This is due to the complexity of the soil environment versus that of an agar plate. Bateman and Kwasna (1999) described that there were approximately 107 species in 50 genera of fungi present on wheat roots in a continuous wheat crop field in the United Kingdom. It is not the purpose of this research to determine the effects of different fungi on the host pathogen interaction with wheat and Ggt. It is however, the purpose to determine host specific gene expression and characterization induce specifically by Ggt. It is for this reason that we will eliminate all influences from other fungi from our experimental system. In addition, all experiments contained three replications and were repeated 3 to 4 times unless otherwise indicated.

Isolate of Ggt, HV-92 (provided by Dr. William Bockus, Kansas State University) was stored as mycelial colonies on 1/5 strength Potato dextrose agar (PDA) plates incubated at 25.0+/-0.1°C. PDA (1/5X) is made by suspending 7.8 grams of PDA (Difco) and 12.0 grams of agar (Difco) in 1 Liter of deionized water (Milli-Q Water System, 18 MOhms). Boil this mixture with constant stirring to dissolve completely. Autoclave (Castle Steam Sterilizer M/C3522, MDT Biological Company) at 121-124°C at 15 psi for 20 minutes. Pour plates in the fume hood with the lid of the plates slightly off center to prevent condensation. Pour small plates with 25 mL each and large plates with 100 mL each of 1/5X PDA. The final pH should be 5.6 +/- 0.2.

Long-term storage of isolates was as 0.5 cm mycelium plugs in 20% autoclaved glycerol at –80°C (Mathre, 2000; Osbourn et al., 1991). Isolates removed from long term storage were grown by draining off the glycerol and plating the .5 cm diameter plug on 1/5 strength PDA and kept at 25°C. Seedlings of the hard red winter wheat cultivar Jagger were surfaced sterilized by means of a modified procedure described previously by Juhnke et al. (1984). Seeds were surfaced sterilized for 30 seconds in 1% silver nitrate (Sigma, ACS Reagent) and Tween 20 using a sonicator (la Sonic II, Model OCONX) and washed three consecutive times for 5 minutes each in autoclaved (Castle Steam Sterilizer M/C3522, MDT Biological Company) Milli-pore water (Milli-Q Water System, 18 MOhms) with continuous stirring (NUOVA II Stir Plate). Seeds were placed on autoclaved filter paper and saturated (10 mL/ plate) with autoclaved Milli-pore water. Plates were incubated in the dark for 48 h at 4.2 ± 0.1°C. Imbibed seeds were transferred crease-side down to 1/5 strength Potato Dextrose Agar (PDA, Difco) and incubated in the dark for 48 hours at 25.0 ± 0.1°C (Percival Scientific Growth Chamber, I-36LL) (Mojedhi et al., 1990). Bacterial and fungal free seeds with seminal roots of 2.5 ± 0.5 cm were placed on a lawn of *Gaeumannomyces graminis* var. *tritici* grown on 1/5 strength PDA or on fresh 1/5 strength PDA and grown at 25.0 ± 0.1°C for up to 4 days. Infected and non-infected wheat root tissue from all experiments were harvested after growth measurements were taken. Tissue was harvested by cutting roots into a small petri dish containing 10 mL of RNAlater (Ambion) (to inhibit endogenous RNases) and vacuumed infiltrated for 10 minutes. The root tissue was then blotted dry with autoclaved filter paper, placed into a 15 mL centrifuge tube, flash frozen in liquid nitrogen (to inhibit

endogenous RNases), and stored at –80°C until needed. Using these growth conditions, we elucidated the time-course of infection by Ggt in this experimental system.

Ggt Selective Media (SM-GGT3).

Ggt Selective media (SM-GGT3) (Juhnke et al., 1984) is comprised of 39 g PDA, 10 mg Dichloran, 10mg Metalaxyl, 25 mg HOE 00703 (1-(3,5-dichlorophenyl)-3-methoxymethyl-pyrrolidin-2,4-dion), 100 mg streptomycin sulfate, 500 mg L-DOPA (L-B-3,4-dihydroxylphenylalanine), and 1 L distilled water. PDA, L-DOPA, and distilled water are the basic ingredients with the others being anti-microbials (antibiotics and antifungal agents) to prevent the growth of competing organisms. Tissue from infected roots were surface sterilized as described previously for seeds and plated out on the Ggt selective media and grown at 25°C for 48 hours.

Microscopic Examination.

Microscopic analysis of infection by Ggt was performed utilizing light microscopy (Olympus BH-2), dissecting microscopy (Olympus SZH-RFL2 Coaxial Fluorescence Attachment), and a mounted digital camera (Kodak Microscopy Documentation System, MDS 290, with 1x universal adapter). At each time point three roots were picked at random and a squash mount, longitudinal section, and epidermal peel were taken and analyzed for fungal infection and penetration.

Pathogenicity Tests.

In order to use the most pathogenic strain of Ggt available, pathogenicity test were ran in Dr. Singleton's lab (modified from Dr. Bockus, personal communication). Briefly, 25 g of untreated whole oat grains (from Stillwater Mill) were placed in a 500 mL flask and 25 g of water was added. The flask was capped with a cotton-plugged lid,

shaken to moisten the oats and incubated 2-16 h to allow the oats to imbibe much of the water. The flasks were shaken again immediately prior to autoclaving and autoclaved for 60-90 minutes. The flask was then cooled in a laminar-flow hood to keep the lids sterile. The flasks were then inoculated with 3 to 4 cubes of agar cut from a fresh culture of Ggt growing on 1/5 PDA. Cubes were buried in the oats about 2 cm or more. The whole mixture was incubated at room temperature on a lab bench, shaking periodically (every 4 to 5 days) to help prevent clumping of kernels. After 2 to 3 weeks of incubation (when kernels are somewhat blackened from the fungus mycelium), the kernels were spread out on a shallow tray and air-dried in the lab. Air-dry inoculum will keep 3 to 6 months at 25°C before it begins to lose its effectiveness; at 4°C it will keep substantially longer.

Wheat seeds were planted on top of oats inoculated with different isolates of Ggt. As the roots of wheat seedlings grow they pass through the infected oats and become infected with Ggt. Wheat seedlings were grown for up to four weeks and assessed for fungal infection (Figure 14).

Bleach Treatment.

A growth response curve to bleach concentration was performed to determine the optimum bleach concentration to use that gives the best root growth increase and the least amount of contamination. Seeds were surface sterilized in 70% EtoH for 5 minutes followed by 0%, 25%, 50%, 75%, or 100% commercial bleach/Tween 20 for 15 minutes. Seeds were then rinsed in autoclaved Millipore water 10 times. 10 Seeds were plated on 1/5X PDA (3 plates per bleach treatment). Placed in the cold room for 48 hours followed by 48 hours growth at 25°C. Median growth measurements were calculated using a standard mm ruler.

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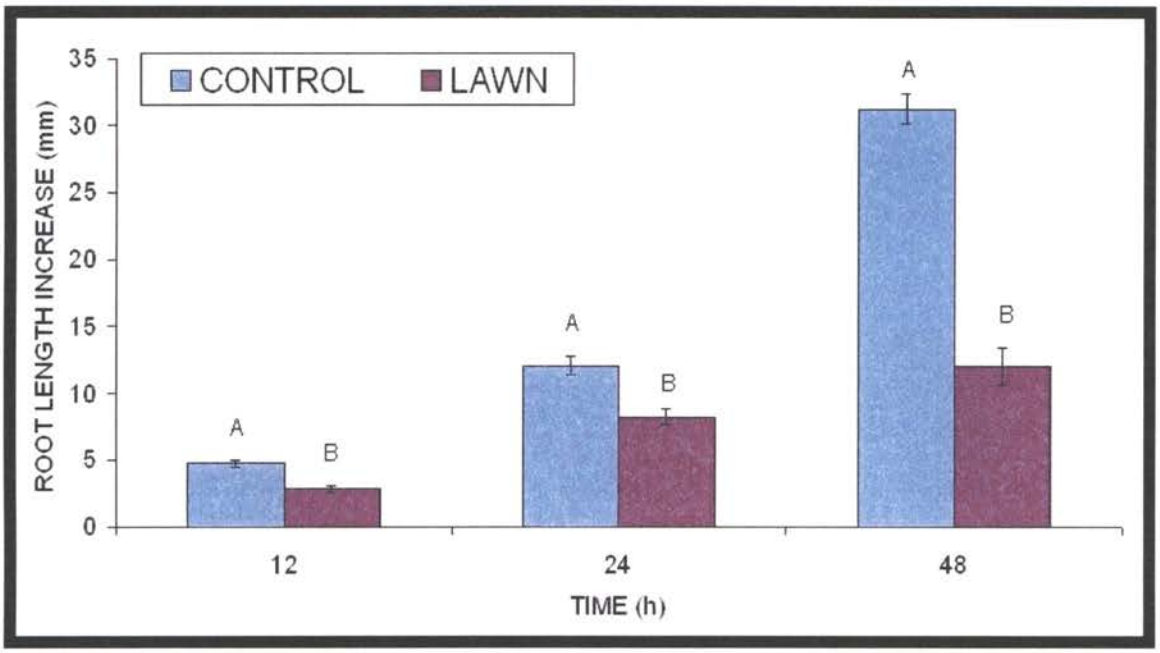

Figure 1. Wheat root length in control and Ggt infected (Lawn) seedlings at 12, 24, & 48 hours post-infection. Root length increase is determined by subtracting the initial length of the root from the final length. Letters represent significant differences (ANOVA: $\alpha 0.05$) at each time period.

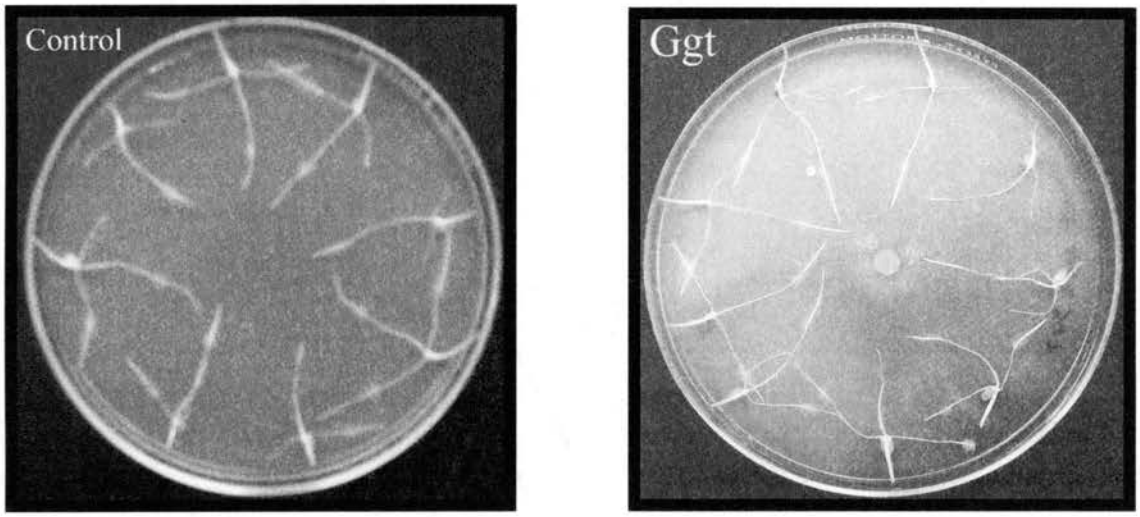

Figure 2. Control and Ggt infected roots after 24 hours as described in materials and methods.

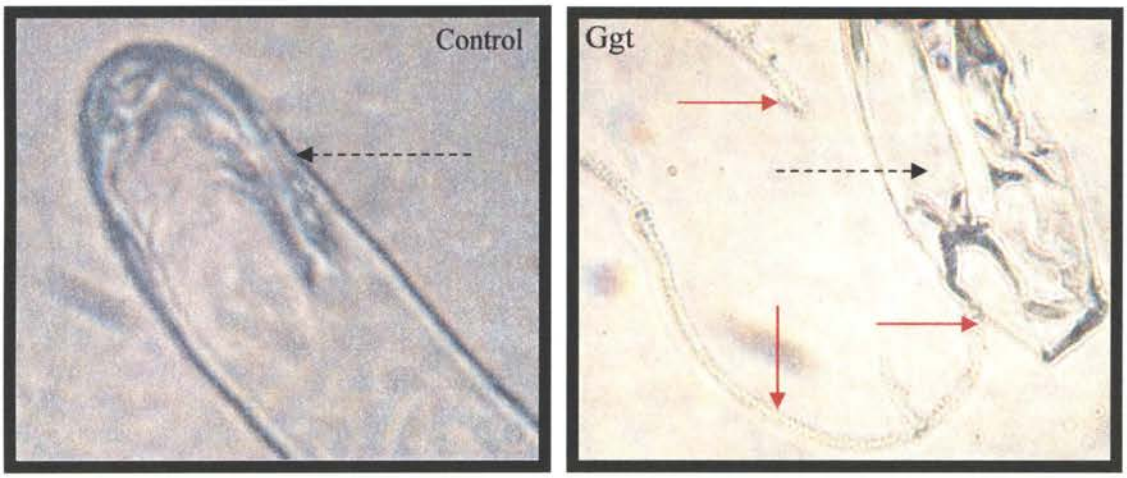

Figure 3. Microscopic analysis of control and Ggt infected root hairs after 24 hours as described in materials and methods. Black lines (dashed) indicate the root hair. Red lines (solid) indicate Ggt hyphae. Hyphae are approximately 6.25 μm wide. Root hairs are approximately 12.5 μm wide.

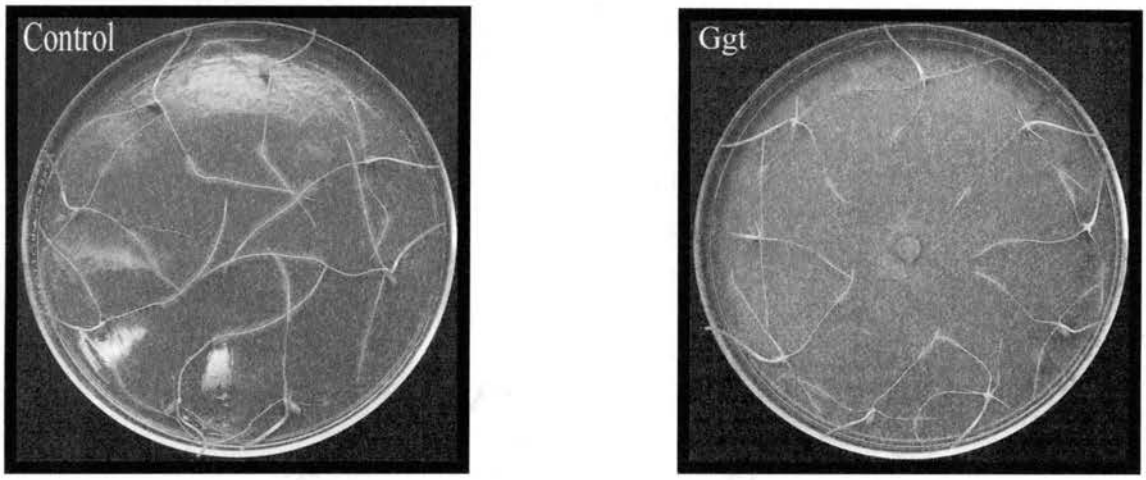

Figure 4. Control and Ggt infected roots after 48 hours as described in materials and methods.

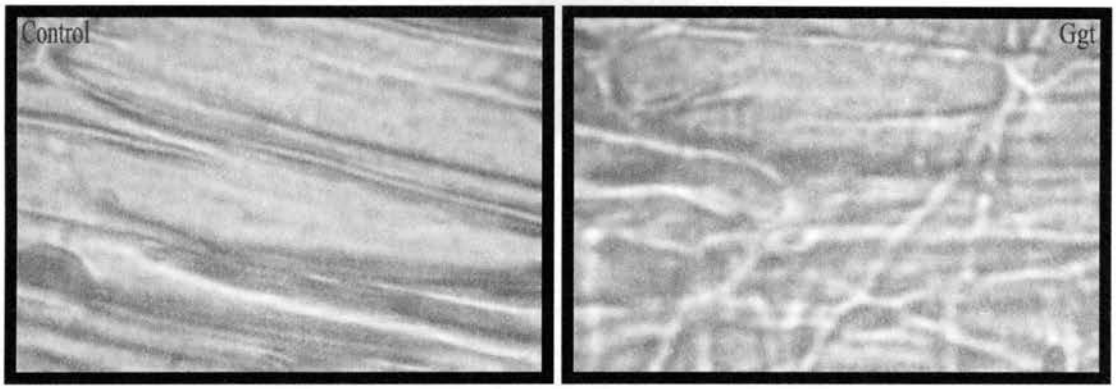

Figure 5. Microscopic analysis of control and Ggt infected cortical cells after 48 hours as described in materials and methods. Hyphae are approximately 6.25 mm wide. Root hairs are approximately 12.5 mm wide.

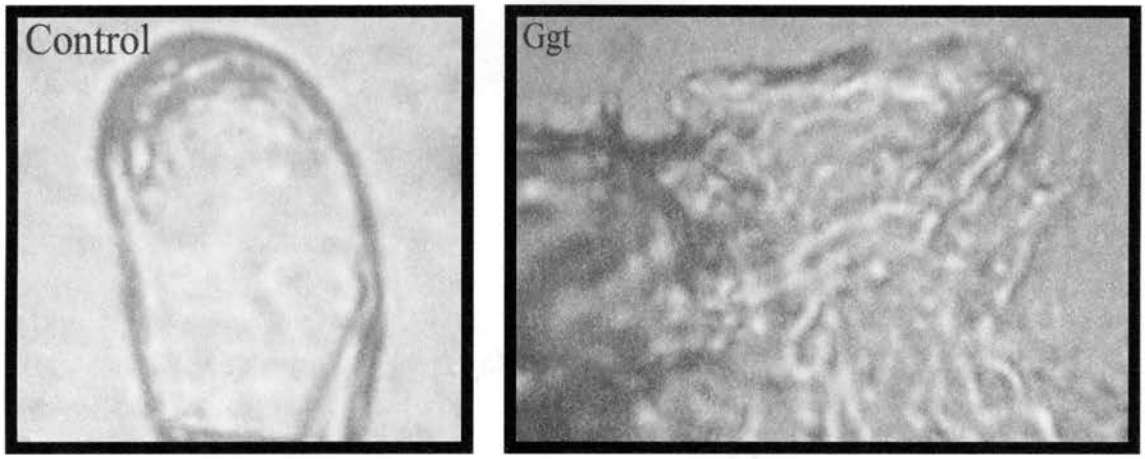

Figure 6. Microscopic analysis of control and Ggt infected root hairs after 48 hours as described in materials and methods.

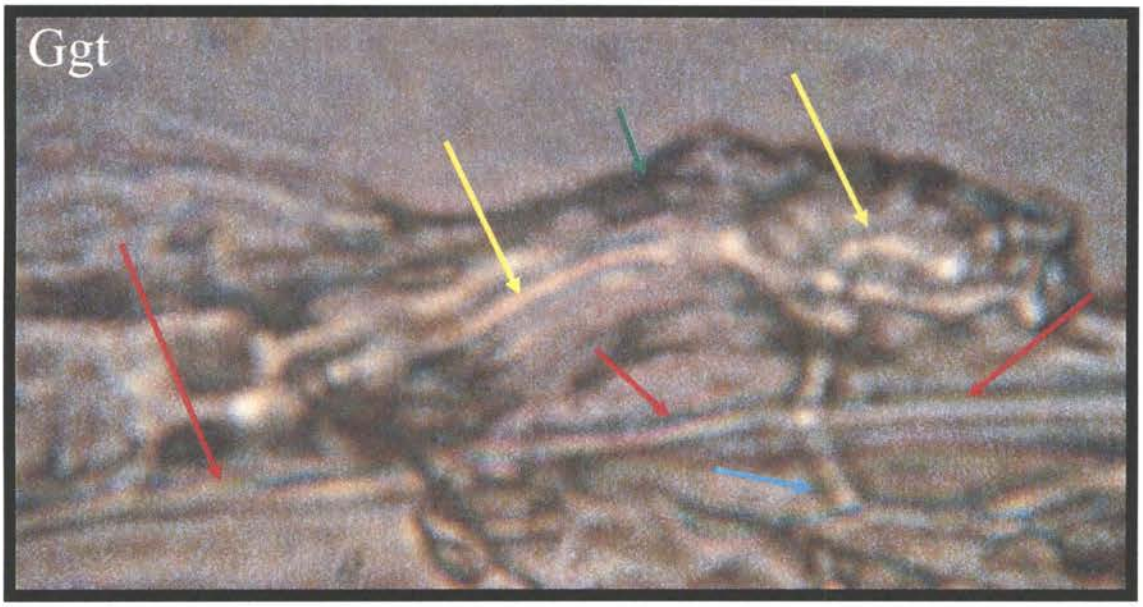

Figure 7. Microscopic analysis of Ggt infected root after 48 hours showing root hair collapse and hyphae growing along and into the epidermis. Root hair indicated with a green arrow. Yellow arrows show fungal hyphae in root hair, red arrows indicate runner hyphae along the epidermis and blue arrow indicated hyphae that penetrated the epidermis. Hyphae are approximately 6.25 μm wide. Root hairs re approximately 12.5 μm wide.

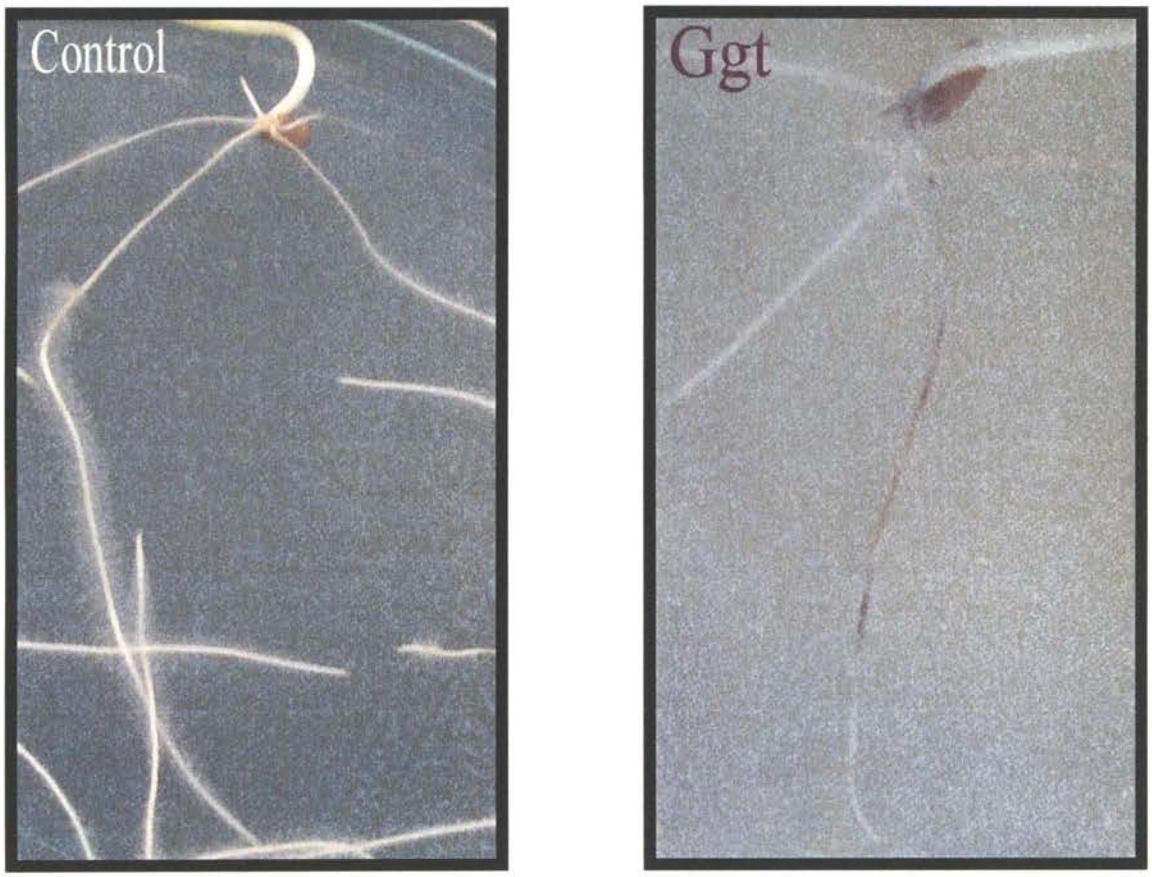

Figure 8. Control and Ggt infected seedlings after 96 hours as described in materials and methods.

Figure 9. Control and Ggt infected root hairs after 96 hours showing a close up view of the root hairs (control) and ectotrophic mycelium covering root (Ggt).

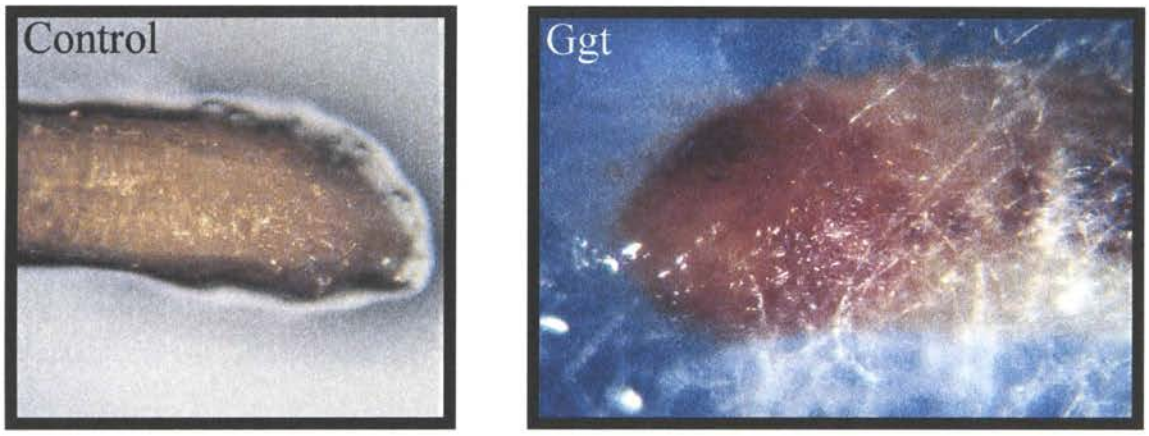

Figure 10. Control and Ggt infected root tips after 96 hours showing a close up view. Control view (left) taken with light from the bottom. Ggt infected root view (right) taken with light from the top only

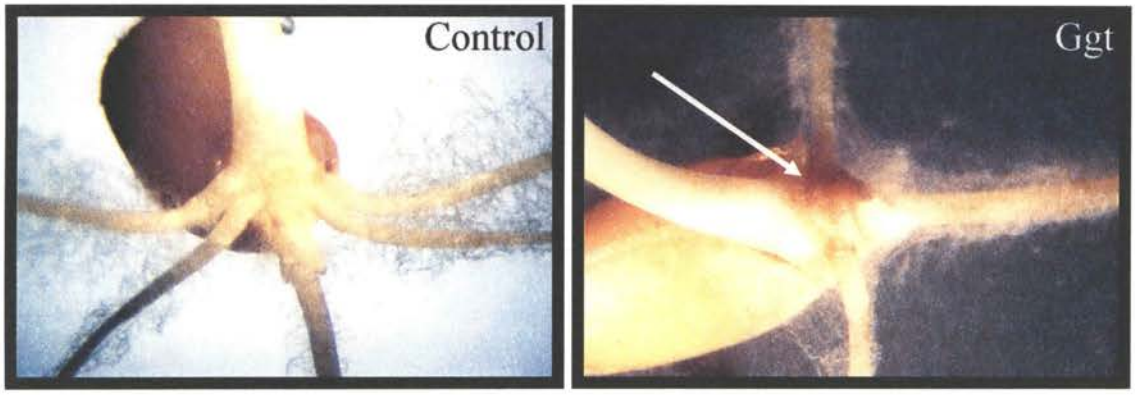

Figure 11. Control and Ggt infected crown tissue after 96 hours showing a close up view. Control root (left) showing numerous root hairs. Ggt infected view (right) shows ectotrophic mycelium covering the roots. Arrow indicated browning of foot tissue.

Figure 12. Ggt infected root tissue on Ggt selective media for 48 hours. Left photograph was taken with light from above and below. Right photograph was taken with light from the bottom only in order to show hyphae. Hyphae are approximately 6.25 μm wide. Root hairs are approximately 12.5 μm wide.

Figure 13. Ggt mycelium on Ggt selective media for 48 hours. Left photograph was taken with light from above and below. Right photograph was taken with light from the bottom only in order to show hyphae. Hyphae are approximately 6.25 μm wide. Root hairs are approximately 12.5 μm wide.

Figure 14. Pathogenicity test performed by "baiting" on different isolates to determine the most virulent isolate (Jo-8, Rl-4, and HV-92 provided by Dr. William Bockus, Kansas State University) (Ggt 1-4 isolated by Dr. Larry Singleton from the Plant Pathology Farm, Oklahoma State University).

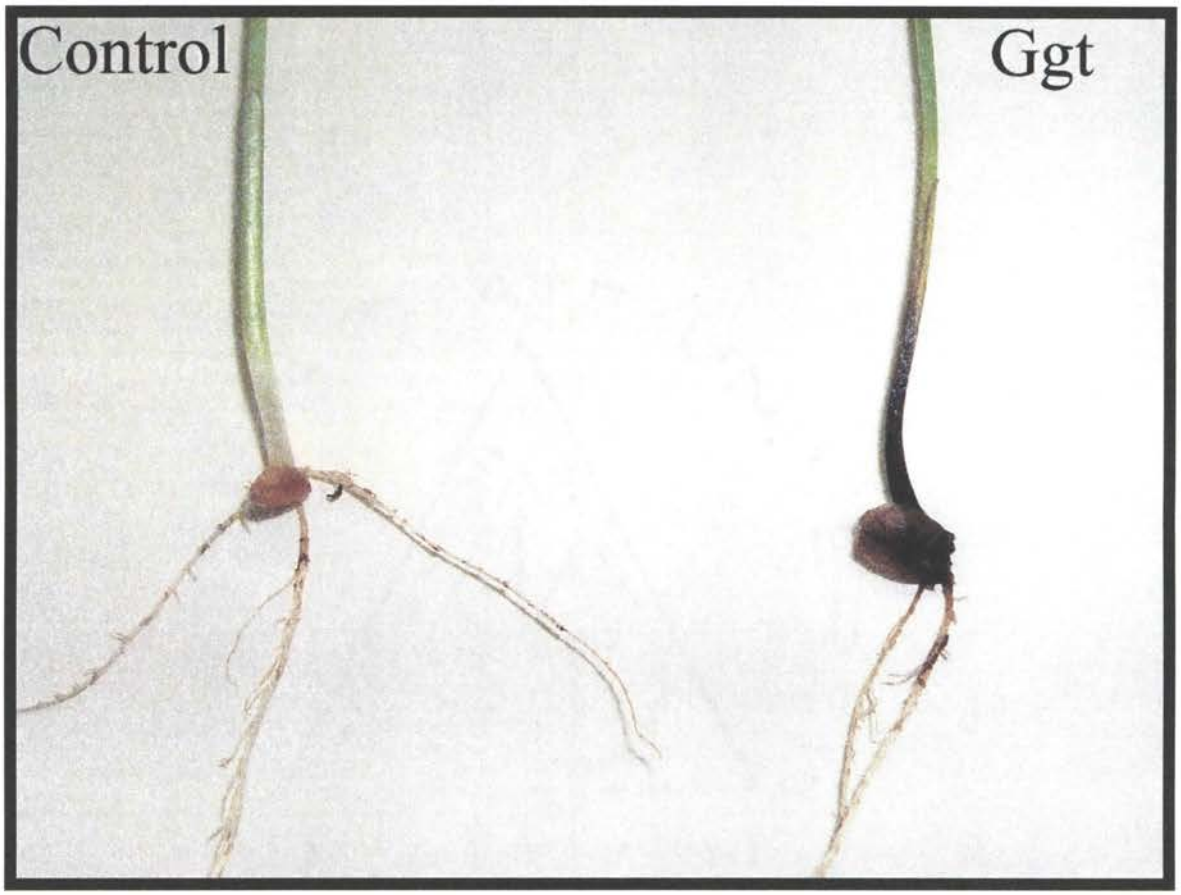

Figure 15. Four week phenotypic examination of HV-92 (Ggt, Right) reveled classic Take-All symptoms as compared to the non-infected control (Left). Symptoms include blackening of the stem up to the fist internode and blackening of the roots and black mycelium on the seed.

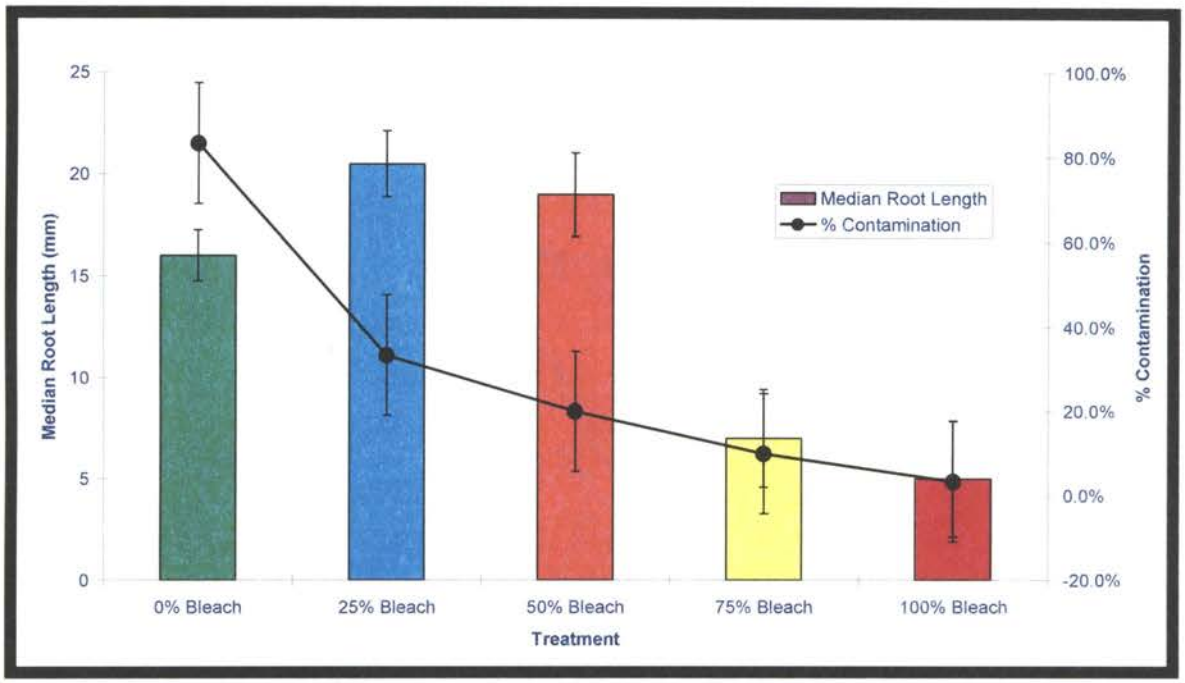

Figure 16. Median root length and percentage contamination at 48 hours after transfer to 1/5 PDA from the cold room as described in material and methods.

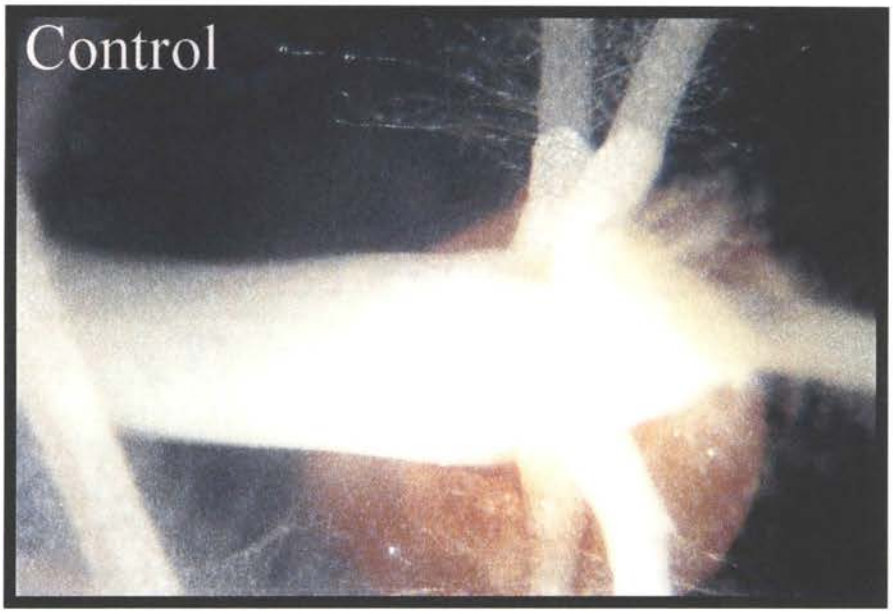

Figure 17. Eleven-day-old Jagger wheat seed surfaced sterilized for 30 seconds in 1% AgNO3 and grown for 11 days after transfer to growth chamber (25.0°C)

Chapter III

Differential Gene Expression in Wheat Roots In Response to Infection by The Take-All Fungus

Take-all [Gaeumannomyces graminis var. tritici (Ggt)], is regarded as the most damaging root disease affecting wheat worldwide. Previous microscopic analysis indicated that at 12 h Ggt had colonized the root surface, at 24 h root hairs were penetrated, and at 48 h root hairs collapsed and the fungus penetrated the epidermis and cortex.

At each time period, root tissue was sampled and forward and reverse normalized suppression-subtraction hybridization (SSH) cDNA libraries between infected and non-infected root tissues were constructed. A total of 802 colonies were picked at the 12 h time period (402 forward and 402 reverse), 750 colonies were picked at 24 h (356 forward and 394 reverse), and 938 colonies were picked at 48 h (475 forward and 463 reverse).

cDNA microarrays were utilized to determine significant up and down regulated genes at each time period. Results indicated that 84.9% of all the differentially expressed genes were unclassified, 10.8% were involved in intermediate metabolism and bioenergetics, 2.2% were involved in electron transport, 1.6% were involved in information pathways, and 0.5% were involved in signal transduction. These genes were grouped into five response profiles based on k-means clustering.

The differentially expressed genes obtained in this study provide insight into the infection process of this soilborne pathogen and its host roots .

Additional keywords: Ggt, Gaeumannomyces, Take-all, SSH, Microarray, wheat.

The cereal root disease take-all which, is caused by the soilborne fungus *Gaeumannomyces graminis* var. *tritici* (Ggt), is regarded as the most damaging disease affecting wheat (*Triticum aestivum* L.) worldwide (Yarham et al., 1989, Huber and McCay-Buis, 1993). According to Hornby (1998), different cereal species have differing resistance to Ggt, with wheat being the least resistance. In addition, although there are differing tolerance levels there is as yet no completely resistant cultivar of wheat to this root-rot pathogen. Therefore, a comparison of resistant to susceptible cultivars cannot be conducted. It is possible however to study the compatible interaction between Ggt and wheat.

Suppression subtractive hybridization (SSH) (Diatchenke et al., 1996) technology has been extensively used in conducting studies on plant pathogenic fungi and will lead to a greater understanding of the interactions between plant and fungus (Gold et. al., 2001). SSH cDNA libraries allow for the identification of differentially expressed genes and are based on the normalization and enrichment of differentially expressed genes in a single round of hybridization (Ji et al., 2002). A gene expression pattern is the first step in the collection of information in functional genomic analyses (Cushman, 1999).

Microarray analysis allows for the global monitoring of gene expression (Gold et. al., 2001; Leung and Cavalieri, 2003) and has been utilized in analyzing plant genes in developing seeds (Girke et al., 2000), brassinosteriod regulated genes in Arabidopsis (Goda et. al., 2002), mechanical wounding genes (Halitschke et. al., 2003), and genes regulated by jasmonic acid and wounding (Perez-Amador et. al., 2002).

Many studies have been conducted to investigate the interaction between phytopathogenic fungi and their plant hosts. These studies have focused on a few genes

at once rather than the thousands now possible with microarrays. Such studies have found lipoxygenases in plants important for fungal resistance (Oliw, 2002), plant genes for growth of powdery mildew (Vogel, 2002), and enzymes needed by Ggt to infect wheat roots such as polygalacturonase, pectin methyl esterases and cellulases (Weste, 1970a, 1970b, and 1978) [For a review of plant defense genes and DNA microarrays see Reymond (2001)].

The purpose of this study is to understand on a molecular basis the interactions of wheat to three different time stages of infection by Ggt: 1. Ggt mycelium covering the root without penetrations (12 h); 2. Ggt hyphae penetration into root hairs (24 h); 3. Ggt hyphae penetration and colonization of the epidermis and cortex of wheat roots (48 h) (Discussed in Chapter 2). SSH library construction and microarray analysis were used to investigate this interactions. Six SSH cDNA libraries were created consisting of a forward (induced genes) and reverse (suppressed genes) libraries at each time period. These libraries were examined by sequencing to check the quality of the libraries and by microarray analysis to determine truly differentially expressed genes. Although, there are many different genes involved in the physiological processes in plant-pathogen interactions, it is the goal of this research to elucidate some of the more important contributors.

RESULTS

RNA Isolation and Quantitation.

Tissue harvested as previously described (Chapter 2) from infected and non-infected wheat roots at 12, 24, and 48 h were homogenized in liquid nitrogen (to inhibit RNases) using a pre-frozen mortar and pestle treated with RNAzap (Ambion) (to decrease degradation by exogenous RNases). Total RNA was extracted using Fenozol™ (Active-motif). Fenozol consists of phenol, detergent, and chaotropic denaturants for isolation of total RNA in tissues that have polysaccharides, fatty acids, and proteins. Fenozol also protects against degradation of RNA by inhibiting RNases. Using this total RNA isolation method produced RNA purity readings on the spectrophotometer (A260/A280) of 2.0 to 2.1 for all three time periods in infected and non-infected tissue.

Isolation of mRNA from total RNA was accomplished by using mTRAP™ Total mRNA isolation kit (Active-Motif). This kit utilizes the Poly T PNA probe to capture the poly A mRNA. This probe is unique in that it captures mRNA with high secondary structures and shorter poly A tails. It also has a high affinity for mRNA, which allows decrease contamination by rRNA. The benefits of using Poly T PNA over oligo dT is that it allows hybridization to mRNA under low salt conditions, reduces rRNA and protein contamination, reduces genomic DNA contamination, and can hybridize to shorter poly A tails. Messenger RNA isolated by this method produced purities of 1.9 to 2.0 for all three time periods in infected and non-infected root tissue. Messenger RNA isolated from wheat roots were between 0.32% and 0.95% of the total RNA.

The total RNA and mRNA isolated were stored in RNA storage solution (Ambion) due to its ability to increase stability and integrity of RNA.

Suppression subtractive hybridization cDNA library.

At each time period (12, 24, and 48 h), root tissue was sampled and forward and reverse normalized suppression-subtraction hybridization (SSH) cDNA libraries between infected and non-infected root tissues were constructed for a total of 6 libraries. The forward libraries use non-infected root tissue as the "driver" and the infected root tissue as the "tester". The results obtained from these libraries will determine potential up-regulated (induced) genes. The reverse libraries use the non-infected root tissue as the "tester" and the infected root tissue as the "driver". The results of these libraries will determine potential down-regulated (suppressed) genes.

A total of 802 white colonies were picked from blue/white screening, at the 12 h time period (402 forward and 402 reverse), 750 colonies were picked at 24 h (356 forward and 394 reverse), and 938 colonies were picked at 48 h (475 forward and 463 reverse). To ensure the quality of these colonies, growth was analyzed again by checking the growth at 600nm wavelength. A total of 784 colonies grew, at the 12 h time period (397 forward and 387 reverse), 691 colonies grew at 24 h (348 forward and 343 reverse), and 938 colonies grew at 48 h (475 forward and 463 reverse). This resulted in a loss of 2.2%, 7.9%, and 0.0% colonies for 12, 24, and 48 h respectively as compared to the initial colony count.

To further check the quality of these libraries, colony PCR was performed to ensure that all clones that grew had a single insert. Clones that did not contain an insert or that had two or more inserts were discarded. Only those clones that contained a single insert was kept and used in further analysis. A total of 667 clones had a single insert, at the 12 h time period (325 forward and 242 reverse), 506 had a single insert at 24 h (263

forward and 243 reverse), and 796 had a single insert at 48 h (372 forward and 424 reverse). This resulted in a loss of 14.9%, 26.8%, and 15.1% clones for 12, 24, and 48 h respectively as compared to the number of colonies that grew at 600nm.

Sequencing of SSH library.

A total of 1869 clones were sent to the OSU core facility for plasmid preparation and then sent to KSU for sequence analysis. Out of the 1869 clones sent for sequencing a total of 1745 clones were sequenced. Moreover, 1006 were sequenced successfully which equates to 57.7% success rate of sequencing clones. All sequences were analyzed and their adapter sequences trimmed from 5' and 3" ends. Trimming reduced the number of clones from 1006 to 875 clones. This decrease was due to only finding one adapter sequence to trim, not finding any adapter sequences, and low quality sequencing reads. The 875 clones were processed based upon the libraries in which they came from (forward or reverse, 12, 24, or 48 h) and all together (for use with the microarray as discussed later). When blasted separately there were 442 contigs created however when blasted together there were 415 contigs were created. Meaning that the libraries taken all together had a 6.1% similarity in contigs. This indicates that there is not much redundancy in the number of contigs between libraries. In addition, 74 more sequences was added after the analysis of the libraries. These 74 additional sequences were trimmed and blasted with the 875 trimmed clones from above.

All sequences processed together resulted in 463 contigs and 376 (81%) of them were unclassified according to PipeOnline. Functional categories were assigned to the remaining 87 contigs. It should be noted that when describing functional categories some genes will be involved in more than one pathway. The breakdown of the functional

categories assigned to all libraries (not including unclassified contigs) were 12 % electron transport, 19% information pathway, 50% intermediate metabolism and bioenergetics, 9% signal transduction, 1% structure and function fo the cells, and 10% transmembrane transport.

Microarray analysis.

Quality assurance checks were conducted prior to microarray hybridizations. A Syto61 stain showed that all spots were similar in size which corresponded to approximately the same amount of DNA was applied per spot. An initial check hybridization was also conducted to ensure that there would be no cross-hybridizing with the control spikes from the Arabidopsis control set and to ensure that the proper amount of mRNA was used in the labeling reactions. This test included a self on self which was conducted using control (non-infected) mRNA split and labeled with the Alexafluor 647 and 543. The result was all yellow spots on the array showing that there was no difference in Alexafluors and that the right amount of mRNA was used. A total of 9 microarrays were printed containing 3 from each time period (12, 24, and 48) and each spot on the array was printed in triplicate. This results in 9 readings per spot on each array.

At 12 h, there were 161 significantly (α=0.05) differentially expressed genes of which, 107 were up-regulated (induced by the fungus) and 54 were down-regulated (suppressed by the fungus) (Figure 1A). The up-regulated genes are located above the top blue line (> 2 fold difference in expression) and the down regulated genes are located below the bottom blue line (< 2 fold difference in expression) (Figures 1 A-C). At 24 h, there were no significantly differentially expressed genes (Figure1B). All the gene are

located in between the two line which indicates that they are genes located in both the infected and non-infected root tissue. At 48 h there were 14 up-regulated genes and 3 down-regulated significantly differently expressed genes (Figure 1C). In total, there were 121 up-regulated differentially expressed genes and 57 down-regulated differentially expressed genes.

In order to make sure that the differentially expressed genes were not due to bias in the Alexafluor dyes, a dye swap experiment was conducted with 24 hour control (non-infected) mRNA labeled with Alexafluor 543 and 24 h Ggt (infected) mRNA labeled with Alexafluor 647 (Figure 2B). Normal conditions are with 24 h control (non-infected) mRNA labeled with Alexafluor 647 and 24 h Ggt (infected) mRNA labeled with Alexafluor 643 (Figure 2A). This dye swap identified the same differentially expressed genes at approximately the same intensities (Fig. 2 A-B).

Gene tree hierarchical cluster.

The gene tree shows the results of hierarchical clustering in the form of a phylogenetic tree (Figure 3). The log ratio (the ratio of the signal to the control, not their logs), are plotted logarithmically with the normal level of expression equal to 1 (yellow color). Those genes that are expressed 2 fold greater are labeled red and those that are two fold less are labeled green. This cluster shows 180 transcripts in different response to fungal infection for non-infected and infected wheat roots. Each gene is represented by a single row of colored boxes, and each time point is represented by a single column.

Gene tree non-hierarchical cluster.

K-means cluster analysis of normalized intensity (log scale) for non-infected and infected wheat roots were organized in 5 clusters (Figure 4). Cluster one shows the trend

of genes being suppressed at 12 h followed by no change at 24 h and no change to slightly suppressed at 48 h. Cluster two shows 37 genes that are significantly down-regulated at 12 h and up-regulated at 48 h. Cluster three shows 37 genes up-regulated at 12 h and are differentially expressed thereafter. Cluster 4 has 29 genes that are significantly up-regulated at 12 h but were slightly down-regulated at 48 h. The final cluster has 23 genes that are significantly up-regulated at 12 h, not differentially expressed at 24 h, and were up-regulated again at 48 h. The normalized ratio = 1 no change in expression, normalized ratio > 1 induced (up-regulated) genes, normalized ratio < 1 suppressed (down-regulated) genes.

Pipe Online (Ayoubi et. al., 2001) was used to determine contig name and functional group as well as, assigning gene name (Table 1). This table shows assigned functional group categories to all of the known classified sequenced clones and is sorted based upon cluster number.

An analysis of all 180 differentially expressed genes showing gene ID, normalized log ratio, HSP, the cluster number, GeneBank Index number, and the gene name associated with that number are listed (Table 2). The table is sorted based upon the cluster number as determined by k-means clustering (Figure 4) and analyzed by PipeOnline (Ayoubi et. al., 2001). Clones without a gene bank name have not been successfully sequenced. They will be re-sequenced at the OSU core facility from purified plasmid used to spot on the microarray.

DISCUSSION

RNA from plant tissue.

The RNA isolation method used for total and mRNA produced clean RNA based upon the A260/A280 ratios of between 1.9 to 2.1. This exceeds the purity found in *Phaseolus vulgaris* roots of 1.8 for total RNA (Kiefer et al., 2000). The percentage of total RNA that was mRNA ranged from .32 to .95% and was comparable to the 0.26% found in onion epidermal cell (Zhou et al., 2000). This is below the 1-5% that was described by most text books and also by the instructions to the M-Trap kit (Active Motif). In addition, Murillo et al., (1995) described mRNA percentages from plant tissue as being as low as 0.25% to as high as 1.53% in maize depending on the type of tissue used.

SSH Library.

To understand the molecular aspects of the interactions between Ggt and wheat roots, genes involved in regulating changes in the host are of most interest (Gold et. al., 2001). One problem associated with studying plant-fungal interaction is how to separate the fungal genes from the plant host genes. Gold et al. (2001) described that this separation is only important if the main focus of the study is on the fungus. The reason that this is not a problem when studying the host is because ratios of fungal mass to plant mass are low in infected tissue resulting in a very small amount of isolated mRNA will be fungal. Taken together with the knowledge that to be enriched by SSH PCR a given mRNA must be at least 0.1% of the total mRNA (Ji et. al., 2002). This also causes another problem in that low abundance transcripts will not be enriched that may play a vital role in regulating cell and stress related processes.

The Ggt SSH library was constructed using pooled samples of root tissue, total RNA and mRNA from the individual time periods to decrease error and bias of the samples. Pooling decreases the biological component of variation (Churchill, 2002).

From the PipeOnline analysis (Ayoubi et. al., 2001) the largest category from all time periods of this SSH library based upon standardized protein and enzyme name was NADH dehydrogenase with 9.73% of the classified clones (Table 1). NADH dehydrogenase is an important component of the electron transport chain. The next largest classification is h(+)-transporting ATP synthase and myo-inositol 4-o-methyltransferase each with 7.08% of the classified clones (Table 1). ATP is synthesized from ADP and phosphate by the enzyme h(+)-transporting ATP synthase. Inositol triphosphate is a common response to stress in plants (Souza et. al., 2001). There were 44 more categories of enzymes which all play a role in the cell maintenance or response to stress. There were 335 contigs that were created that had no classification in the PipeOnline system. The standard protein names / enzyme categories will not be discussed in detail here but rather they will be discussed in reference to significantly differentially expressed genes from the microarray analysis.

Microarray analysis.

Microarray analysis has been used for comparing differential expressed genes under an array of environmental or genetic conditions (Gold et. al., 2001). Churchill (2002) described 3 layers of variation that need to be addressed for a microarray experiment. These three layers are biological variation, technical variation, and measurement error. In preparing for the microarray analysis the biological variation was addressed above with the pooling of the samples. Technical variation was addressed by

using the same pool of mRNA for both the spotting and hybridization as well as using Arabidopsis control spikes. Dye swap experiment was performed to ensure that there was no dye bias in the hybridization of the array (Figure 2). Technical variation was also decreased due to a self on self experiment that showed equal intensity per spot and no cross-hybridization with control spots. The measurement error was addressed by using 2 internal house keeping genes (actin and GAPDH) and 3 Arabidopsis control spikes to normalize the data by as well as the dye swap mentioned earlier to check for dye bias. The other way experimental error was accounted for was by using a t-test to identify genes with significant ($P<0.05$) differential expression.

The largest number of up or down regulated genes were found at 12 h followed by 48 h (Figure 1). It is very interesting that at 24 h there were no differentially expressed genes. One reason for this my be that the absence of differentially expressed genes between the driver and tester during construction of the SSH library. As previously mentioned, enrichment of a given mRNA only occurs if it is at least 0.1% of the mRNA (Ji et. al., 2002). The differentially expressed genes at 12 h may be induced or suppressed in response to volatile compounds which were shown to decrease root growth significantly without coming in contact with the root itself (data not shown). At 12 h the fungus had colonized the root surface and did not penetrate the epidermis. A more intensive investigation at earlier time points could provide useful information about early responses which do not envolve contact of the fungus with the root. At 48 h the root epidermis was penetrated and the cortex colonized. The fact that at 24 h there are no differentially expressed genes and only 17 differentially expressed genes at 48 h may be due to the nature of the compatible interaction of the fungus with the host. Ggt has been

described as failing to elicit or suppress changes in wheat roots upon infection (Speakman and Lewis, 1978). Ggt infected roots showed the same amount of lignification as uninfected roots (Speakman and Lewis, 1978). This could explain why no differentially expressed genes were shown at 24 h. The evolution of this disease has resulted in wheat not recognizing Ggt as a pathogen which results in the lack of a defense response. Weste (1972) suggested that the slow growth of intercellular mycelium and the lack of extracellular toxins are responsible for a pseudo-symbiotic relationship present in the early stages of Ggt infection. This relationship allows Ggt to exist in equilibrium with the host roots.

Base upon the phylogenic tree (Figure 3) the majority of the differentially expressed genes at 12 h are up-regulated (induced) and these up-regulated genes are more similar based upon the phylogeny of the sample patterns over the time course. The majority of the genes suppressed at 12 h are induced at 48 h and again based upon the pattern are more similar (spaced closer together) on the tree.

Based upon the same data that created the tree, k-means cluster analysis were organized in 5 clusters with the majority of the significantly expressed genes located in cluster 3. These genes were induced at 12 h but then were not differentially expressed at 12 and 24 h. The smallest number of differentially expressed genes were found in cluster 1 which represents the genes that are suppressed at 12 h and show no differential expression thereafter (Figure 4). An interesting pattern occurred in cluster 2 with genes suppressed at 12 h and induce at 48 h.

The functional categories assigned to the k-means clusters (Table 1) showed that in cluster 1, 59% of the genes were unclassified, 6% were involved in electron transport,

6% were involved in information pathways, and 29% were involved in intermediate metabolism and bioenergetics. These genes include: a disulfide isomerase (contig 366) which plays a role in folding secretory proteins (Ciaffi et al., 2001); a DNA K-type molecular chaperone HSC71.0 (contig 119) which has been shown to be expressed during heat stress (DeRocher and Vierling, 1995); a 40S ribosomal protein S13 (contig 14) which is a cytoplasmic ribosomal protein found in growing tissues such as the root tip (Joanin et al., 1993); phosphoglucomutase (contig 20) which mutants in this enzyme have been shown to decrease starch synthesis (Davies et al., 2003); an O-methyltransferase (contig 323) which is responsible for methylation of phenolics, plays a role in lignin synthesis, chemical defense, & signaling and has also been shown to be involve in cold acclimation (NDong et al., 2003); and a glyoxalase I (contig 404) which has been shown to increase its polypeptide levels 2-3 fold in roots of NaCl, mannitol, and ABA treated and may be linked to increased demand for ATP in salt stressed plants (Espartero et al., 1995).

Cluster 2 showed 62% unclassified genes, 3% involved in electron transport, 5% were involved in information pathways, 27% were involved in intermediate metabolism and bioenergetics, and 3% were involved in signal transduction. These genes include: SNF-1 protein kinase (contig 11) (Hannappel et al., 1995) which plays a role in metabolic and transcriptional responses to nutritional and environmental stresses (Bradford et al., 2003) and is similar to Camodulin like protein kinase (Halford et al., 2003); UMP/CMP kinase A and B (contigs 18 and 292, respectively) which levels have been shown to increase in seedling development and catalyzes the phosphoryl transfer from ATP to either UMP or CMP to form ADP and UDP or CDP (Zhou et al., 1998); an O-

methyltransferase (contigs 19 and 81) that has been found in salt-tolerant barley roots but not in salt-sensitive roots and its expression increased 1.5 times by salt stress and it is also involved in lignin biosynthesis (Sugimoto et al., 2003); putative ubiquinol cytochrome C reductase (contig 35) which is found in the mitochondrial respiratory chain of plants also called alternative oxidase (Robson and Vanlerberghe, 2002); a guanine nucleotide binding protein beta subunit (contig 146) whose mRNA was found in rice plants particularly in roots (Iwasaki et al., 1995); a peroxidase (contig 223) which decreases lipid peroxidation in barley salt-stressed roots (Liang et al., 2003) and is involved in lignification during copper induced oxidative stress (Jouili and Ferjani, 2003); an alpha-tubulin 2 (contig 226) whose decreased gene expression in specifically roots caused swelling of root tip and lateral root expansion in Arabidopsis and is also involved in formation of phragmoblast in interphase of mitosis (Bao et al., 2001); an adenine nucleotide translocator (contig 405) that is responsible for taking ATP out and ADP in to mitochondria (Bathgate, 1989); a 40SrRibosomal protein S13 (contig 14) which functions as a cytoplasmic ribosomal protein found in growing tissues (Joanin et al., 1993).

In cluster 3, 95% of the genes were unclassified, 1 % were involved in electron transport, and 4 % were involved in intermediate metabolism and bioenergetics. These genes include a DNA K-type molecular chaperone HSC71.0 (contig 119) which has been shown to be expressed during heat stress (DeRocher and Vierling, 1995); an alpha-galactosidase (LacZ gene product) (contig 372) that converts lactose to galactose and glucose (unpublished); and a NADH dehydrogenase subunit 5 (contig 393) which is involved in the electron transport complex and fatty acid oxidation (Marschner, 1995).

All of the genes in cluster 4 were unclassified. In cluster 5, 92% were unclassified, 4% were involved in both electron transport and intermediate metabolism and bioenergetics. These genes include: ferredoxin precursor (Wheat) (contig 255) which was classified as both electron transport and intermediate metabolism and bioenergentics. It is involved in ferredoxin dependent glutamate synthase found in Maize roots in response to nitrate. Glutamate synthase plays a role in ammonia uptake, nitrate reduction, and nitrogen fixation which effects protein synthesis, nucleic acids, and other nitrogen containing compounds (Redinbaugh and Campbell, 1993).

The experimentation and analysis discussed in this paper have provided insight into the host-pathogen interaction between wheat roots and Ggt. A much more useful analysis into the infection process would be to compare a resistant and susceptible cultivar of wheat. However, at this time there is no known resistance in wheat to the take-all fungus. In addition, whole root tissue was utilized in this study but with the advent of laser capture systems that allow for analysis of single root cells, a more precise study should be conducted. This would allow for a cell by cell analysis of the penetration and differential gene expression from plant roots in response to infection.

MATERIALS AND METHODS

Infection System.

The conditions for growth and infection of Jagger wheat roots and Ggt were described in a previous paper (Chapter II). In short, seeds were sterilized by sonication in 1% AgNO3 with Tween 20, rinsed with sterile-deionized water, and placed on sterile filter paper in a cold room (4.5°C) without light for 48 h. Imbibed seeds were then aseptically transferred to 1/5X Potato Dextrose Agar (PDA) at 25°C without light for 48 h. Seedlings with roots approximately 2.0 to 3.0 cm long were transferred to 1/5X PDA without Ggt (control), or with a lawn of Ggt and placed into a 25°C incubator without light for 12, 24, and 48 h.

A time course for infection was determined with light microscopy. Analysis of root length increase indicated that there was a 40.9%, 32.2%, and 61.5% decrease in growth with Ggt at 12, 24, 48 h, respectively, as compared to the controls. Results indicated that at 12 h Ggt had colonized the root surface, at 24 h root hairs were penetrated, and at 48 h root hairs collapsed and the fungus penetrated the epidermis and cortex.

Root Tissue Harvest.

Infected and non-infected wheat root tissue from all experiments were harvested after growth measurements were taken. Tissue was harvested by cutting roots into a small petri dish containing 10 mL of RNA*later*™ (Ambion, Texas) (to inhibit endogenous RNases) and vacuumed infiltrated for 10 minutes. The root tissue was then blotted dry with autoclaved filter paper, placed into a 15 mL centrifuge tube, flash frozen in liquid nitrogen (to inhibit endogenous RNases), and stored at –80°C until needed. At

the end of each experiment, the tissue from all replicates (3 plates x 3 replicates per plate) within that experiment were combined prior to the flash freezing.

Total and mRNA Isolation.

Before beginning to extract RNA, it is important to ensure that all the precautions are taken to decrease degradation of the RNA by RNases (Ambion TechNotes (2002).

Prior to extracting total RNA, root tissue from each experiment was combined with the appropriate condition from each replicated experiment (3 to 4 replications per treatment). This was to insure that there was sufficient mixing of the samples both within and between experiments and to decrease bias of the samples.

Total RNA was isolated from wheat roots by first grinding the tissue (1 gram) in liquid nitrogen and then extracting the RNA with Fenozol^{TM} (Active Motif, California). The sample was incubated at 50°C (5 min), debris pelleted at 12,000 x g (10 min) at 4°C, and then chloroform was added to the supernatant and vortexed (20 sec). The supernatant was incubated at room temperature (3 min), centrifuged 12, 000 x g (10 min) at 4°C and the aqueous top phase was transferred to a fresh tube were cold iso-propanol was added. RNA was precipitated by incubating at room temperature (10 min), centrifuging 12,000 x g (15 min) at 4°C and then discarding the supernatant. The pellet was washed with 70% ethanol, centrifuged 12,000 x g (5 min) at 4°C, air dried and resuspended in 1X binding buffer (mTrapTM Total mRNA isolation kit, Active Motif, California) and stored at –80°C.

The mRNA was isolated using mTrap^{TM} Total mRNA isolation kit (Active Motif, California) per the manufactures instructions. Total RNA and mRNA concentrations and purities were determined by Beckman DU-65 spectrophotometer and 1%

Agarose/Ethidium Bromide gel analysis. After determining the mRNA concentration, the mRNA was concentrated to 0.5 ug/uL for use in the subsequent SSH step. The mRNA was concentrated by a second round of precipitation with TouchDown™ precipitation reagent supplied with the mTrap™ Total mRNA isolation kit (Active Motif, California). After the second round of precipitation the mRNA was resuspended in RNA Storage Solution (Ambion, Texas) and stored at –80°C.

Suppression Subtractive Hybridization (SSH).

The SSH was accomplished by using the CLONTECH PCR-Select™ cDNA Subtraction Kit (CLONTECH, California) per the manufactures instructions, with the exception of 32 cycles of primary and 21 cycles of secondary PCR (personal communication CLONTECH representative). Each time period (12, 24, and 48 h) and the kit control (skeletal muscle cDNA provided by the manufacture) were utilized to create both forward and reverse subtractions. Two micrograms of root mRNA from each time period was used to begin the cDNA subtraction kit. Additional polymerase and positive/negative controls for PCR were used per the manufacture instructions in the Advantage™ cDNA PCR Kit & Polymerase Mix (CLONTECH, California).

Cloning, Cryoplate Preparation, and Archive Storage of Subtracted PCR Products.

In order to provide the most precise measurement of PCR product concentration from the subtraction described above, PicoGreen® dsDNA Quantitation Reagent and Kit (Molecular Probes, Oregon), a dsDNA quantitative fluorescent nucleic acid stain was utilized per the manufactures instructions. An initial volume of 5 uL duplicated (10 uL total) of PCR product used in the quantitation of dsDNA.

The subtracted and quantified cDNA populations were cloned and screened (blue/white) using QIAGEN PCR Cloning^{plus} Kit (QIAGEN, California) per the manufactures instructions. The initial amount of PCR products needed to use in the ligation reaction of this kit were calculated (per manufactures recommendations) based upon and average insert size of 400 bp.

Cloned cells were picked by blue/white screening (per QIAGEN PCR Cloning^{plus} Kit recommendations) with sterile toothpicks and placed in cryoplates containing 1 mL of Terrific Broth (47.6 g Terrific Broth, 4 mL Glycerol, and 996 mL sterile water) with 100 μg/mL ampicillin in a 96 well culture block. Plates were covered with aluminum tape and incubated for 22 h at 37°C with shaking (250 rpm). Glycerol stocks were made by placing 90 μL of overnight culture in 90 μL of 30% glycerol (long term storage) or 90 μL 80% glycerol (working stock) and storing them at –80°C and 4°C, respectively. In addition to the stock solutions, 180 uL of the overnight culture was analyzed for growth at 600 nm wavelength using a Bio-Tek microplate reader (OSU Core Facility).

Colony PCR.

Colony PCR was performed to insure that each clone that showed growth at 600 nm as described previously, did in fact contain an insert. Colony PCR was performed by a modified cDNA array instructions (PCR-Select Differential Screening Kit Users Manual, CLONTECH, California). In summary, 1 μL of the overnight culture was added to 19 μL of PCR master mix [2.0 μL 10X PCR reaction buffer (100 mM Tris, pH 8.0, 500 mM KCl, 25 mM MgCl2, 1% Triton X-100), 0.6 μL each of Primer 1 and Primer 2R (10 μM; provided in CLONTECH PCR-Select^{TM} cDNA Subtraction Kit, CLONTECH, California), 0.4 uL dNTP (2.5 mM/dNTP; provided in CLONTECH PCR-Select^{TM}

cDNA Subtraction Kit, CLONTECH, California), 0.4 μL *Taq* Polymerase, and 15.0 μL Milli-Pore water] for a total volume of 20 μL. The following PCR parameters were utilized for each plate: denature 94°C (30 sec) followed by 28 cycles of denature 95°C (30 sec) and annealing 68°C (3 minutes). The final PCR products were stored at –20°C. A 2% agarose/ethidium bromide gel in 1X TAE was ran using 5 uL of each PCR product. The gel was analyzed by exposing it to ultraviolet light and looking for a single insert. Those clones that contained one insert were then isolated and consolidated from the other clones that contained no insert or multiple inserts, for use in plasmid preparation and purification.

Plasmid Preparation.

Bacterial transformed colonies from –80°C stock corresponding to those colonies with single inserts only were grown in cryoplates as described previously (SEE Cloning, Cryoplate Preparation, and Achieve Storage of Subtracted PCR Products). For preparation of plasmids used for microarray analysis LB Broth was used in the cryoplates whereas for plasmid isolation for sequencing, TB broth was used. Plasmid preparation for DNA sequencing of clones (described below) was accomplished by in house alkaline lysis protocol by the Oklahoma State University Recombinant DNA/Protein Resource Facility (Janet Rogers). Plasmid preparation for microarray analysis (described below) was performed in collaboration with the Samuel Roberts Noble Foundation Plant Biology Division (Dr. Gonzales). In short, bacterial cryoplate solution (10 uL) was placed in 1 mL LB broth and incubated at 37°C for 22 h. This solution was pelleted, the supernatant removed, and the pellets frozen at –80°C and transported on blue ice bags to the Noble Foundation, Ardmore OK. Plasmids were isolated by an in house automated protocol

using Biomek 2000 robots (http://www.noble.org/plantbio/Genomics/ProtocolBiomek.htm). The final plasmid preparation was resuspended in 100 μL ddH2O.

DNA Sequencing.

Plasmids isolated and purified from the Oklahoma State University Recombinant DNA/Protein Resource Facility (described above) were packaged on dry ice and transported to the DNA Sequencing and Genotyping Facility, Department of Plant Pathology, Kansas State University, Manhattan, KS (Dr. John Fellers). DNA sequencing was performed using the ABI 3700 (Perkin-Elmer) and an in house protocol (http://www.oznet.ksu.edu/pr_dnas/services.htm). DNA sequenced PCR samples were generated from plasmids using standard M13R (CAGGAAACAGCTATGACC) and/or SP6 (GATTTAGGTGACACTATAG) primers (provided by Dr. John Fellers).

Sequence data obtained from Kansas State University was analyzed by Blastx and functional groups using PipeOnline 2.0 (Ayoubi et al., 2002).

Microarray Printing.

Purified PCR products were prepared from plasmids isolated from the Noble Foundation, Ardmore OK by an in house protocol. In short, 2 mL of resuspended plasmid preparation (described previously) was placed in 200 mL of TE followed by 2 mL of plasmid/TE being placed in 200 mL of filtered (0.22 mm) autoclaved ddH2O. One mL of diluted plasmid was placed in 24 mL PCR mix which contained 10X buffer and Yiledase poly HotStart Taq from Stratagene (California) and dNTPs from Idaho Technology (Idaho). M13R (TCACACAGGAAACAGCTATGAC, MW 6,721.4, TM

56.2°C) and M13F (TGTAAAACGACGGCCAGT, MW 5,532.7, Tm 55.2°C) were designed in house. PCR parameters were as follows:

1. 92°C for 2:00 min
2. 10 cycles at:
    a. 95°C for 20 sec
    b. 52°C for 20 sec
    c. 72°C for 1:45 min
3. 25 cycles at:
    a. 95°C for 20 sec
    b. 49°C for 20 sec
    c. 72°C for 5:20 min
4. 72°C for 7:00 min

PCR products were purified by ArrayIt™ Brand 96-Well PCR Purification Kit (Telechem International, Inc.) according to the manufactures instructions. Purified PCR products were dried O/N in a speedvac and transported back to Oklahoma State University on blue ice until microarray printing proceeded.

Dried PCR products were resuspended in 15 μL filtered (0.22 μm) autoclaved nanopure H20 and shaken at room temperature for 2 h at 100 rpm to make sure pellet was dissolved. An additional 15 μ L of ArrayItTM Brand Products Micro Spotting Plus 2X Solution was add and mixed. Ten μ L of this 1X solution was transferred to Genetix 384 well plates and placed at –20°C. The remainder of the 1X PCR products mix were archived in 384 well plates at –80°C.

Printing of the microarray was accomplished by using the following parameters:

1. Printing of the microarrays were accomplished using Arrayer (Cartesiam Technology, CA) software and Generation III Array Spotter (Amersham Biosciences Corp.).
2. Seventy-five Corning GAPS II Coated (amino-silane) Slides (25 x 75 mm) (Corning Inc, New York) were printed due to their uniform spot morphology and their ability to retain maximum signal strength.
3. Each spot was replicated 3 times per slide. There were 4608 (2304 spots for Ggt only) individual spots printed 3 times on each slide for a total of 13, 824 (6,912 for Ggt only) spots per slide. There were 48 sub arrays containing 288 (144 for Ggt only) spots each with a 17 x 17 spot configuration using a 4 x 4 pin configuration.
4. Printing was performed using Arrayer Software and according to Corning GAPS II slides protocol.
5. AFGC Microarray Control Set [Distributed by the Nottingham Arabidopsis Stock Centre (http://arabidopsis.org.uk)] was used according to the manufactures instructions. In short, 8 transgene controls were used as negative controls, all 10 spiking controls were used as negative controls and 3 (Sp1, 5, and 9) were used as positive control spikes. Amplification control products were purified according to the manufacture protocol and utilizing QIAquick^{R}PCR purification kit (Qiagen), Riboprobe^{R} System – T3 in vitro transcription kit (Promega),transcription clean-up by Rneasy^{R} mini kit (Qiagen), and quantification measured by A260. A total of 3.5 ug each of the

spike controls used were obtained by this method (only 1 ng per hybridization is needed).

4. Wheat actin (provided by Dr. Mike Anderson, OSU) and a wheat GAPDH (provided by Sathyanarayana Elavarthi, OSU) primers were used to create positive controls for normalizing the data on the microarray. Using unsubtracted cDNA from 24 hour control and infected root tissue, the following PCR conditions were used to isolate the actin and GAPDH cDNA. M13R and M13F primers were used under the following conditions:
    a. 92°C for 2:00 min
    b. 10 cycles at:
        i. 95°C for 20 sec
        ii. 52°C for 20 sec
        iii. 72°C for 1:45 min
    c. 25 cycles at:
        i. 95°C for 20 sec
        ii. 49°C for 20 sec
        iii. 72°C for 5:20 min
    d. 72°C for 7:00 min
6. After drying O/N at 80°C a SYTO-61 stain was performed to check the print quality according to the manufactures instructions (Molecular Probes, CA).

Microarray Hybridization.

Microarray hybridizations were performed using Genisphere 3DNA Array 350 Expression Array Detection Kit for Microarrays utilizing Alexa Fluor 546 and 647 kit

(Genisphere, Inc. Pennsylvania) and LifterSlips™ (25 x 60 mm) (Erie Scientific, New Hampshire) according to the manufactures instructions. Poly(A)+RNA (200 ng/hybridization) was used to prepare the cDNA for hybridization according to the manufactures instructions. The cDNA was concentrated by using Millipore Microcon® YM-30 Centrifugal Filter according to the manufactures instructions with the exception that 7 μL of 1X TE buffer was used to elute cDNA off of membrane in the finale step.

The following exceptions were used based upon the protocols used at Oklahoma State University Recombinant DNA/Protein Resource Facility and Genesphere, Inc.. The 2X formamide based hybridization buffer was heated at 65°C prior to use. Volume of hybridization mixture was adjusted to 81 μL due to LifterSlipsTM (25 x 60 mm) being used. All hybridizations were performed at 42°C and post hybridization washes were done at room temperature. Glass cover slips (25 x 60 mm) were used in 3 DNA hybridizations. The 3DNA hybridization mix was incubated at 75-80°C and then at 42°C prior to hybridization. A volume of 30 μL of hybridization mix per hybridization was used for the 25 x 60 glass slides. 3DNA hybridizations were performed at 42 °C. Post 3DNA hybridization washes occurred at room temperature.

The control (non-infected) cDNA was labeled with the green or 546 Alexafluor and the Ggt (infected) cDNA was labeled with the red or 647 Alexafluor. As discussed in the materials and methods infection system three time points (12, 24, and 48 h) were chosen for analysis by microarray. At each time period there were three microarrays printed in triplicate for a total of 27 replicate spots per PCR product printed. For quality assurance a self on self (no spike) hybridization was performed to check for cross hybridization with Arabidopsis controls discussed previously. This hybridization was

performed on 24 hour control (non-infected) cDNA labeled with Alexafluor 546 and 647. This should produce all yellow spots corresponding to no bias in dye and should not hybridize to controls. A dye swap (with spike) experiment was performed to ensure no bias in dye labeling and to ensure no cross hybridization with spikes to other control spots. This experiment was performed by labeling 24 h control (non-infected) cDNA with Alexafluor 546 and Ggt (infected) cDNA with Alexafluor 647 and hybridizing to the array and at the same time under the same conditions switch the dyes and hybridize to another array. The dye swap should produce the same yellow color with the same intensity at the same location as the initial experiment and the green and red colors should be switched in the dye swap as compared to the initial experiment.

Microarray Analysis.

Scanning of the microarrays was conducted using ScanArray Scanner and ScanArray Express Software. Signal extraction and spot quantification were performed by GenePix Pro 4.0 (Axon Instruments, CA). Initial normalization was based on 2 house keeping genes Actin and GAPDH and 3 Arabidopsis control spikes (discussed previously). By utilizing 5 different controls the two Alexaflours were brought into equal intensities which produced a yellow color.

Pre-normalized microarray data was analyzed by GeneSpring version 6 (GeneSpring Inc., CA). The following parameters were conducted in order to analyze the microarrays:

Gene lists were created initially to include all PCR products on the microarray (All genes). This was further reduced to include only the Ggt genes that had pre-normalize values greater than 150 (Gaeumannomyces Raw Data 150). Further reduction

was conducted by a resulting t-test p-value 0.05 with the addition of a multiple testing correction (Benjamini and Hochberg False Discovery Rate) which is less stringent and will decrease the occurrence of false positives (Gaeumannomyces t-test p-value less that 0.05 with default multiple test to decrease false positives). The number of genes were furthered filtered into individual gene list for Gaeumannomyces that had a t-test p-value less than 0.05 and were 2 fold higher or lower (up and down regulated genes) base on the natural log ratio of control signal to raw (infected) signal at 12, 24, and 48 h.

Experiments were created using Genesphere and using a normalization per spot / per chip Lowess which is the same as per pin and per block Lowess. Normalization by this method performs an intensity dependent (Lowess) normalization and divides the signal channel by the control channel. It uses 20% of the data for smoothing. Since we have in our data files (.gpr) the block, row, and column that each spot came from this normalization will be the same as doing a pin to pin and block to block normalization.

K-means clustering was conducted using the natural log normalized gene list with t-test p-value <0.05 with multi test (Benjamini and Hochberg False Discovery Rate), with a raw value higher than 150 and with a greater or less than two fold change from the log ratio of infected to control values. The minimum cluster size was 10 with minimum correlation of 0.95. These constraints will use only the significant up and down regulated genes with a raw score of at least 150 and the log ratio of the raw and control to make the cluster tree. Five trees were constructed using these parameters.

List of genes fitting the above criteria were constructed and the sequence data were matched with their corresponding gene list and analyzed and interpreted using PipeOnline (Ayoubi et al., 2002) into functional groups.

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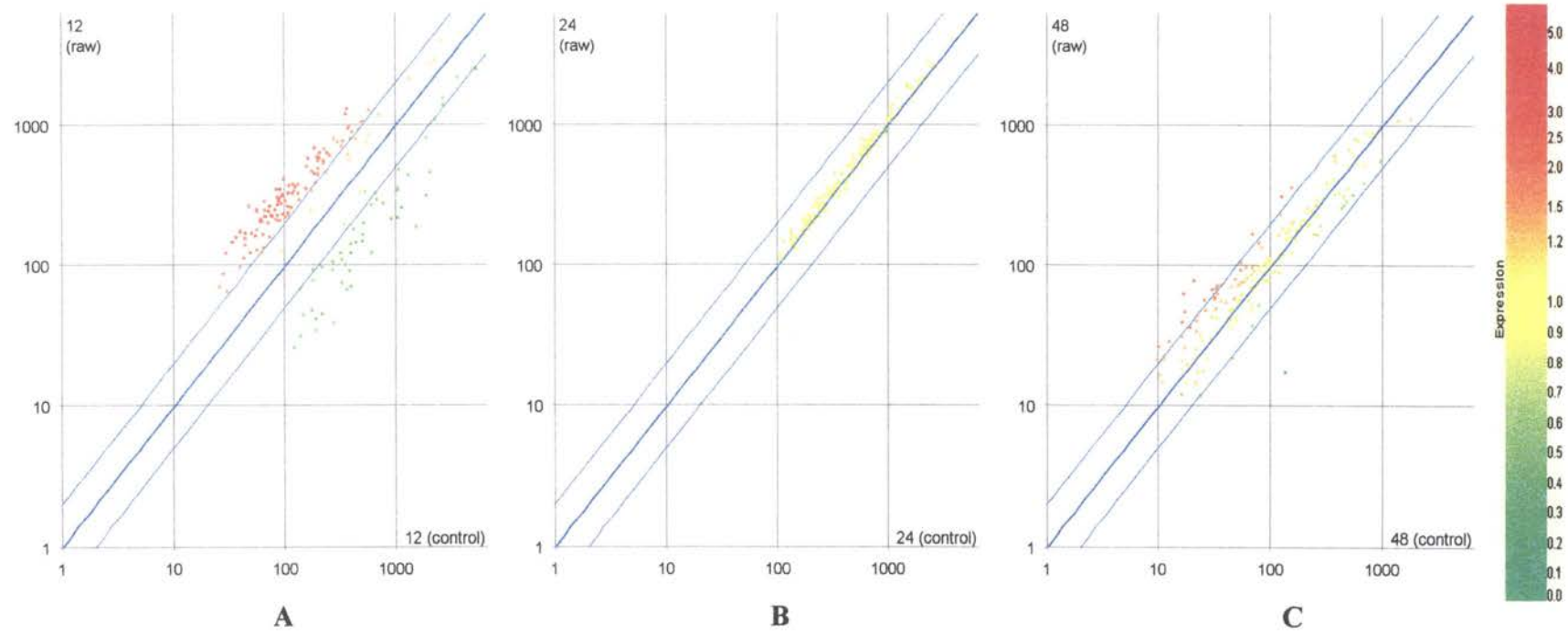


Fig. 1. Scatter plot comparing the spot intensities in hybridizations with probes from non-infected (control) (x axis) and infected (raw) (y axis) wheat root tissue. Data from images of both alexafluor dyes log of ratio mode graphs normalized values (the ratio of the signal to the control, not their logs), and spaces them logarithmically. The color bar on the right indicates the level of expression with the normal expression level of 1 equal to yellow. Blue lines indicate two fold difference levels (above the top blue line is equal to >2 fold difference and below the bottom blue line is equal to <2 fold difference in expression .

(A) Scatter plot of signal intensities of 12 hour non-infected and infected wheat roots.

(B) Scatter plot of signal intensities of 24 hour non-infected and infected wheat roots.

(C) Scatter plot of signal intensities of 48 hour non-infected and infected wheat roots.

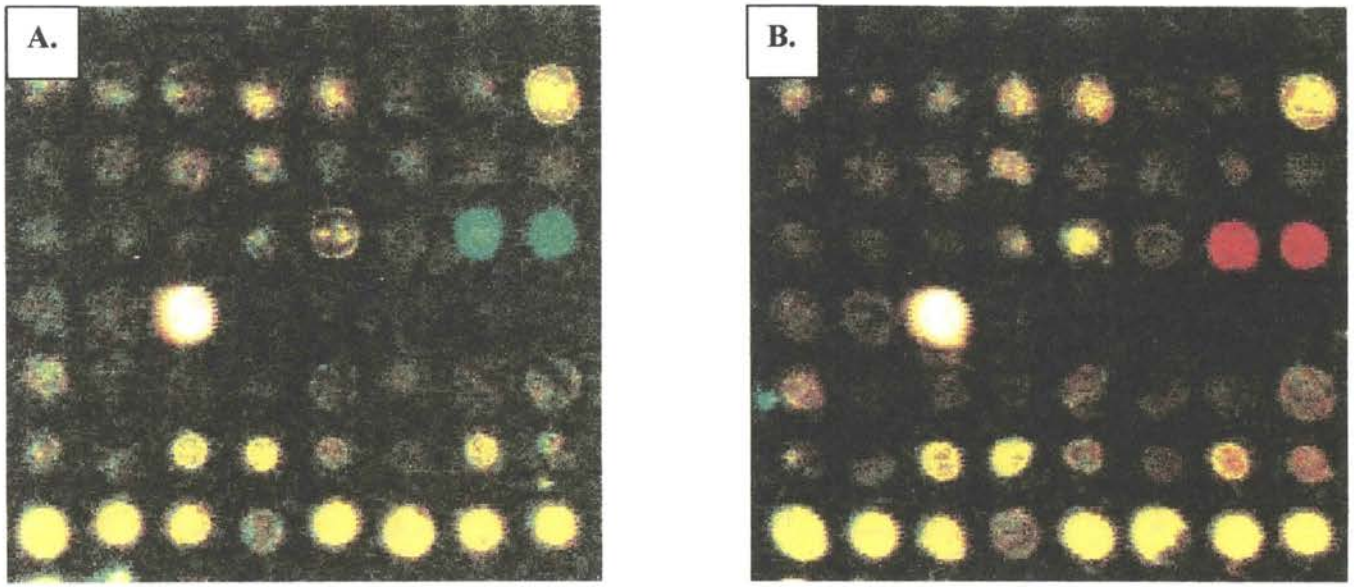


Figure 2. Dye swap experiment performed on 24 hour rep 2 and 3 shows no bias due to the dye used and shows the same intensity of spots between microarrays. A. Shows control cDNA labeled with Alexafluor 543 (green) and Ggt cDNA labeled with Alexafluor 647 (red). Yellow color is in response to equal expression of control and Ggt labeled cDNA. B. Shows control cDNA labeled with Alexafluor 647 (red) and Ggt cDNA labeled with Alexafluor 543 (green).

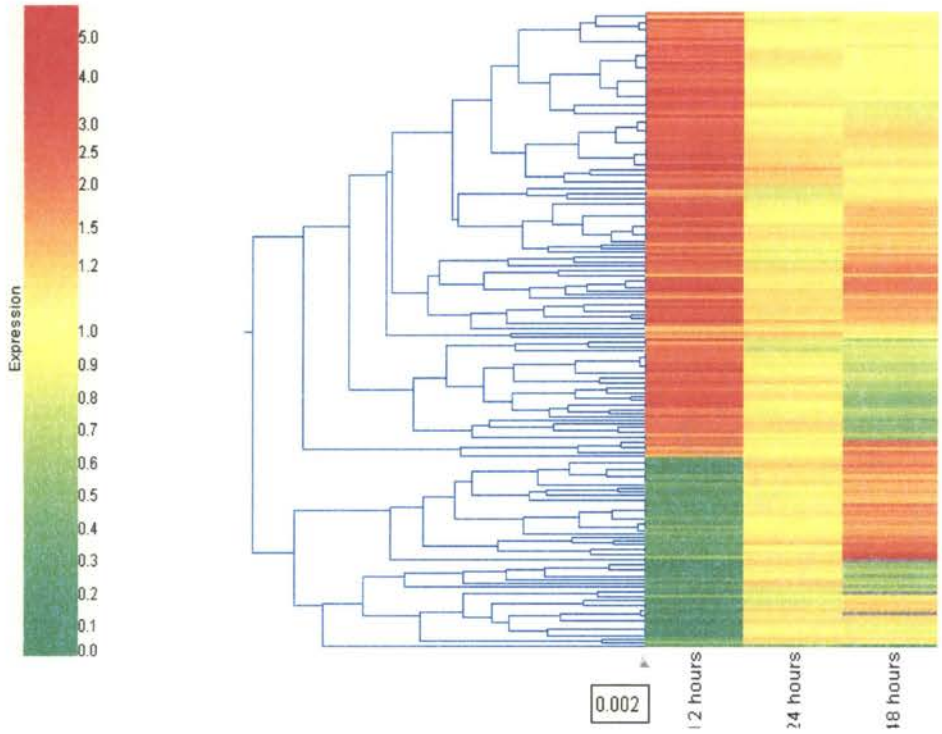


Fig 3. The gene tree shows the results of hierarchical clustering in the form of a phylogenetic tree. Genes having similar expression patterns are clustered together. Genes shown have a t-test p-value less than 0.05 and the expression level log of ratio graphs normalized values (the ratio of the signal to the control, not their logs), and spaces them logarithmically. The color bar on the left indicates the level of expression with the normal expression level of 1 equal to yellow. The parameters used: Similarity Measure Standard Correlation, Only annotate with standard lists, on 180 transcripts in different response to fungal infection for non-infected and infected wheat roots. The color saturation reflects the magnitude of the log expression ratio (infected/non-infected) for each transcript. Due to space limitation the clone name, GenBank ID, and annotation are described in Table 2. Transcripts are grouped into patterns according to their expression profiles of induced (red) and suppressed (green) at different time points. Each gene is represented by a single row of colored boxes, and each time point is represented by a single column.

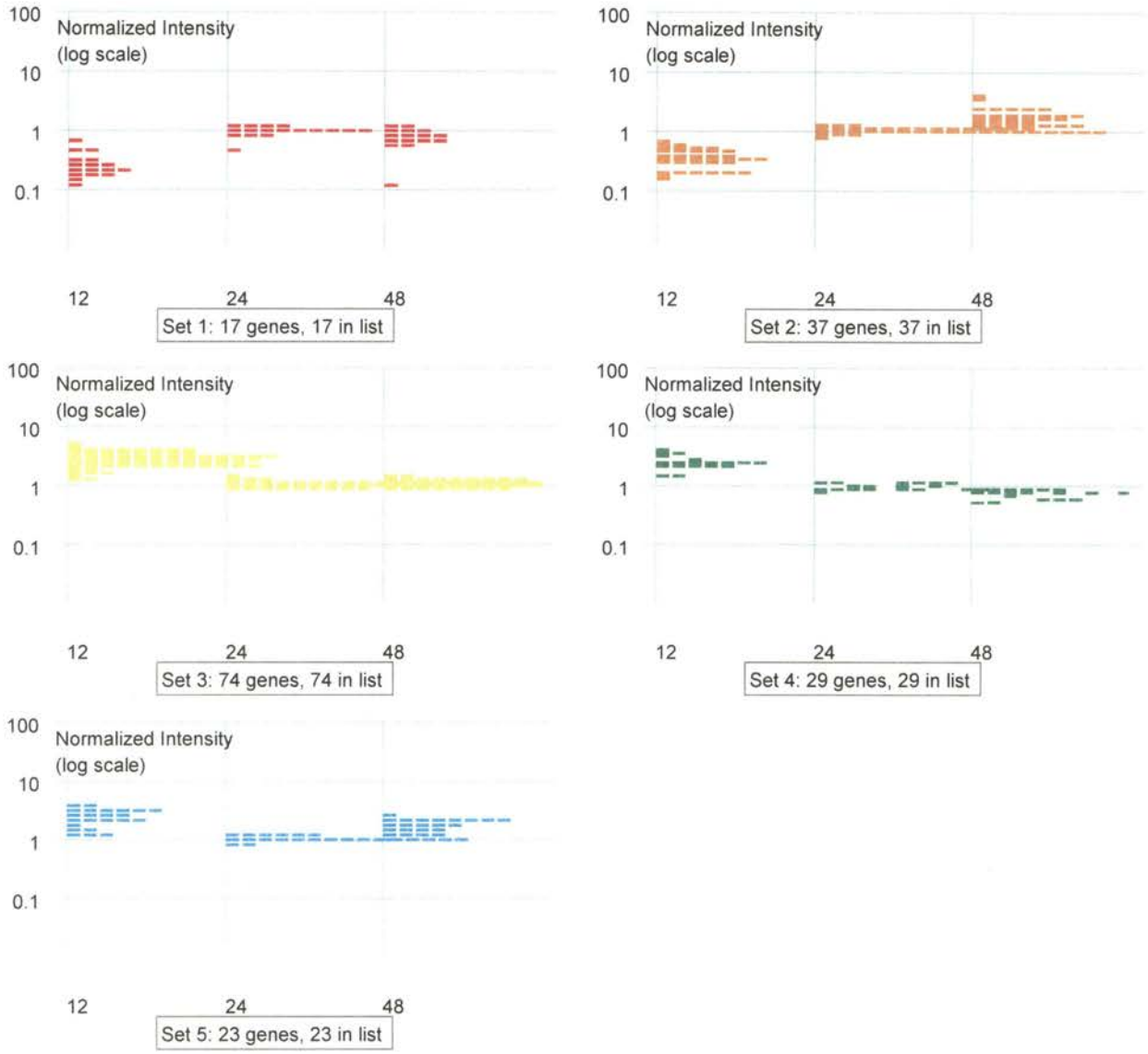


Fig. 4. Normalized intensity (log scale) of cluster analysis by k-mean for non-infected and infected wheat roots. The normalized intensity value (y-axis) represents the normalized log ratio of expression of all genes present in each cluster. The expression ratios (y axis) of transcripts for differentially expressed genes are presented by bars for each time period and are colored according to cluster number. Normalized ratio = 1 no change in expression, normalized ratio > 1 induced genes, normalized ratio < 1 suppressed genes.

Table 1. Gene name, k-means cluster, pipe-online contig name, functional group classification, and gene name of differentially expressed genes from in infected and non-infected roots at 12, 24, and 48 h found in the *Gaeumannomyces graminis* var. *tritici* microarray analysis.

Gene Bank Index	Name	Cluster	ContigName	Functional Group	GeneName
gi|12056117	G2F2A5	1	366	electron transport	disulfide isomerase [Triticum turgidum subsp. durum]
gi|27777630	G1F1D5	1	323	intermediate metabolism and bioenergetics	[Secale cereale]
gi|629641	G2F1G12	1	119	information pathways	dnaK-type molecular chaperone HSC71.0 - garden pea
gi|18076790	G1F2F11	1	20	intermediate metabolism and bioenergetics	[Triticum aestivum]
gi|18076790	G1F2F7	1	20	intermediate metabolism and bioenergetics	[Triticum aestivum]
gi|4126809	G2R1G4	1	404	intermediate metabolism and bioenergetics	[Oryza sativa (japonica cultivar-group)]
gi|464705	G1F3G9	1	14	intermediate metabolism and bioenergetics	40S RIBOSOMAL PROTEIN S13 [Zea mays]
gi|575292	G1F3C4	2	11	signal transduction	protein kinase [Hordeum vulgare subsp. vulgare]
gi|21554004	G2R3B4	2	35	electron transport	ubiquinol--cytochrome-c reductase [Arabidopsis thaliana]
gi|21554004	G2R3B4	2	35	intermediate metabolism and bioenergetics	ubiquinol--cytochrome-c reductase [Arabidopsis thaliana]
gi|464705	G2R1C11	2	14	intermediate metabolism and bioenergetics	40S RIBOSOMAL PROTEIN S13 [Zea mays]
gi|1346109	G2F3C4	2	146	information pathways	Guanine nucleotide-binding protein beta subunit-like protein (GPB-LR) (RWD)
gi|1346109	G2F3C4	2	146	intermediate metabolism and bioenergetics	Guanine nucleotide-binding protein beta subunit-like protein (GPB-LR) (RWD)
gi|6683811	G1F2A11	2	18	information pathways	UMP/CMP kinase a [Oryza sativa]
gi|6683811	G1F2A11	2	18	intermediate metabolism and bioenergetics	UMP/CMP kinase a [Oryza sativa]
gi|27777630	G2R2F8	2	19	intermediate metabolism and bioenergetics	[Secale cereale]

gi|22168	G2R1F5	2	405	intermediate metabolism and bioenerg	nucleotide translocator [Zea mays]
gi|23452335	G2R2H9	2	226	intermediate metabolism and bioenergetics	[Gossypium hirsutum]
gi|27777630	G2R3B2	2	19	intermediate metabolism and bioenergetics	[Secale cereale]
gi|6683813	G2R2F1	2	292	information pathways	UMP/CMP kinase b [Oryza sativa]
gi|6683813	G2R2F1	2	292	intermediate metabolism and bioenergetics	UMP/CMP kinase b [Oryza sativa]
gi|27777630	G2R3D7	2	81	intermediate metabolism and bioenergetics	[Secale cereale]
gi|27261094	G2R3A4	2	223	intermediate metabolism and bioenergetics	peroxidase [Oryza sativa (japonica cultivar-group)]
gi|629641	G2F1C4	3	119	intermediate metabolism and bioenergetics	dnaK-type molecular chaperone HSC71.0 [Pisum sativum]
gi|1899163	G1R2G4	3	372	intermediate metabolism and bioenergetics	gene product [unidentified cloning vector]
gi|5835703	G3F3D11	3	393	electron transport	NP_008518.1|ND5_15045 NADH dehydrogenase subunit 5 [Rhipicephalus sanguineus]
gi|5835703	G3F3D11	3	393	intermediate metabolism and bioenergetics	NP_008518.1|ND5_15045 NADH dehydrogenase subunit 5 [Rhipicephalus sanguineus]
gi|19569591	G2R3D3	5	255	electron transport	ferredoxin precursor [Triticum aestivum]
gi|19569591	G2R3D3	5	255	intermediate metabolism and bioenergetics	ferredoxin precursor [Triticum aestivum]

Table 2. Cluster analysis of infected and non-infected wheat roots based upon k-means cluster in Figure 4. Normalized log ratio is calculated by infected/non-infected normalized ratio and plotted on a log scale. The HSP is the alignment score as determined by Pipe Online (Ayoubi et. al., 2001). The cluster number was determined by GeneSpring software. In addition the contig name the gene name associated with that number are listed. The table is sorted based upon the cluster number and on the lowest normalized log ratio for each cluster at 12 hours.

	Normalized Log Ratio						
Name	12 Hour	24 Hour	48 Hour	Cluster	ContigName	HSP	GeneName
G2F2A5	0.126	0.934	1.057	1	366	921	disulfide isomerase [Triticum turgidum subsp. durum]
G1F1B6	0.135	1.149	1.127	1	447		No homology
G1F1D5	0.176	1.025	0.894	1	323	389	[Secale cereale]
G2F1G12	0.178	1.071	0.884	1	119	172	dnaK-type molecular chaperone HSC71.0 - [Pisum sativum]
G1FB8	0.186	1.018	0.521	1	92	394	[Oryza sativa (japonica cultivar-group)]
G1F2F11	0.206	0.84	NO DATA	1	20	194	[Triticum aestivum]
G1F2F4	0.209	1.207	0.632	1	22		No homology
G2R2H3	0.233	1.079	0.64	1	90	664	synthetase [Triticum monococcum]
G1F2F7	0.238	0.872	1.137	1	20	194	[Triticum aestivum]
G2R1G4	0.263	1.303	0.768	1	404	409	[Oryza sativa (japonica cultivar-group)]
G1F3H2	0.287	1.028	0.938	1	16	270	[Oryza sativa (japonica cultivar-group)]
G2F2D10	0.29	0.491	0.131	1	365		No homology
G1F3G9	0.307	0.874	0.77	1	14	263	40S RIBOSOMAL PROTEIN S13 [Zea mays]
G1F2H1	0.33	1.068	0.722	1	47	255	synthetase [Triticum monococcum]
G2F2C5	0.476	1.104	0.689	1	7		No homology
G1F3H9	0.488	1.188	0.54	1	438	401	AU032852(S15362),AU070591(S5037) correspond to a region of the predicted gene [Oryza sativa (japonica cultivar-group)]
G1F3A6	0.628	0.97	0.936	1	335	206	60S RIBOSOMAL PROTEIN L36 [Oryza sativa (japonica cultivar-group)]
G1F1H1	0.179	1.312	2.46	2	334	680	translationally controlled tumor protein [Triticum aestivum]
G2R3D5	0.198	1.082	1.952	2	90	664	synthetase [Triticum monococcum]
G2R2G8	0.209	1.186	1.311	2	61	417	protein [Triticum aestivum]

G1F3D12	0.216	1.235	NO DATA	2	92	394	[Oryza sativa (japonica cultivar-group)]
G1F2C6	0.222	0.831	1.292	2	353		No homology
G1F3C4	0.223	1.136	1.891	2	11	765	protein kinase [Hordeum vulgare subsp. vulgare]
G2R3B4	0.225	1.051	2.398	2	35	319	ubiquinol--cytochrome-c reductase [Arabidopsis thaliana]
G1F2C3	0.228	1.099	NO DATA	2	407	442	[Oryza sativa (japonica cultivar-group)]
G2R1C9	0.315	1.162	1.828	2	382	183	protein L19 [Triticum aestivum]
G2F2D11	0.318	0.961	1.677	2	364		No homology
G1F4C7	0.33	1.154	1.635	2	16	270	[Oryza sativa (japonica cultivar-group)]
G2R1C11	0.331	1.085	1.945	2	14	263	40S RIBOSOMAL PROTEIN S13 [Zea mays]
G2F3C4	0.337	0.93	1.407	2	146	838	Guanine nucleotide-binding protein beta subunit-like protein (GPB-LR) (RWD)
G2F3D6	0.346	1.274	2.612	2	360		No homology
G1F1B8	0.347	1.082	1.908	2	332		No homology
G1F2A11	0.348	0.971	1.789	2	18	86	UMP/CMP kinase a [Oryza sativa]
G2R2F8	0.356	1.12	3.505	2	19	162	[Secale cereale]
G2R1F5	0.376	1.121	1.665	2	405	108	nucleotide translocator [Zea mays]
G2R2H9	0.379	1.228	1.157	2	226	178	[Gossypium hirsutum]
G1F3F2	0.382	1.028	1.296	2	55	348	AAD26879.1~gene_id:T30G6.9~strong similarity to unknown protein [Arabidopsis thaliana]
G3F2G11	0.417	1.054	1.157	2	395	203	40S ribosomal protein [Triticum aestivum]
G2R2E8	0.424	1.004	1.408	2	295		No homology
G2R3B2	0.426	1.077	2.043	2	19	162	[Secale cereale]
G2F2D12	0.433	1.026	1.637	2	363		No homology
G2R2F1	0.435	1.011	2.573	2	292	813	UMP/CMP kinase b [Oryza sativa]
G1F1F6	0.442	0.933	1.146	2	446	363	carboxylase/oxygenase large subunit [Hordeum marinum subsp. marinum]
G2F1D4	0.443	1.058	1.265	2	368	179	cytoplasmic ribosomal protein L18 [Oryza sativa]
G2R3D7	0.449	1.025	2.354	2	81	176	[Secale cereale]
G2R3B3	0.457	0.992	1.609	2	57		No homology
G2R3A4	0.468	1.138	1.864	2	223	102	peroxidase [Oryza sativa (japonica cultivar-group)]

G2R1C3	0.502	1.038	1.432	2	359		No homology
G2F1C10	0.509	1.064	1.309	2	182		No homology
G1F4C4	0.515	1.177	1.359	2	414		No homology
G1F4C9	0.556	0.944	1.509	2	82		No homology
G2R2F11	0.573	0.969	1.369	2	287		No homology
G2F3D11	0.61	1.13	1.111	2	45		No homology
G2R3D10	0.668	1.059	3.958	2	400	136	40S RIBOSOMAL PROTEIN S8 [Oryza sativa (japonica cultivar-group)]
G3R1G12	1.325	0.99	0.994	3	105		No homology
G2F1C4	1.336	0.929	1.021	3	119	172	dnaK-type molecular chaperone HSC71.0 - [Pisum sativum]
G1F2C11	1.454	1.184	0.996	3	347		No homology
G2F2G8	1.598	0.949	1.14	3	N.D.		
G2R3C7	1.599	0.903	1.028	3	22		No homology
G1F2F5	1.769	1.337	1.095	3	92	394	[Oryza sativa (japonica cultivar-group)]
G2R1H9	1.975	0.899	1.157	3	403		No homology
G3R4H12	2.015	1.077	1.178	3	105		No homology
G1R2B8	2.067	0.935	1.053	3	99		No homology
G1R2C2	2.099	1.009	0.913	3	99		No homology
G3R4H10	2.099	1.145	1.077	3	105		No homology
G3R4F3	2.112	0.999	1.03	3	101		No homology
G3F4C7	2.131	1.129	0.999	3	N.D.		
G3F2B5	2.16	1.146	1.201	3	398		No homology
G1R2B9	2.168	0.956	1.052	3	89		No homology
G2R1F7	2.232	1.005	1.229	3	68	188	NP_181994.1| ALG6, ALG8 glycosyltransferase family [Arabidopsis thaliana]
G1R3B9	2.244	1.007	0.983	3	N.D.		
G1F1D11	2.265	1.032	1.09	3	321		No homology
G2F2F7	2.271	0.993	1.041	3	362		No homology
G3R2F11	2.3	1.087	0.995	3	101		No homology
G3F1D8	2.359	0.956	1.012	3	27		No homology
G1R2H4	2.36	1.099	1.084	3	N.D.		

G1R3B3	2.362	0.957	1.039	3	N.D.		
G1R3B12	2.393	1.019	0.998	3	N.D.		
G2R3B9	2.44	0.958	1.166	3	214		No homology
G2R1A5	2.47	0.879	1.069	3	23		No homology
G1R2G4	2.498	1.064	1.002	3	372	81	gene product [unidentified cloning vector]
G3R4C8	2.517	1.087	1.131	3	101		No homology
G2R2F4	2.534	1.091	0.966	3	401		No homology
G1R2D11	2.599	0.993	0.983	3	89		No homology
G3F2B12	2.607	1.073	1.093	3	97		No homology
G3R2E2	2.743	0.956	1.355	3	103	477	NP_040703.1| rf replication, viral strand synthesis protein [Coliphage phiX174]
G3R3C7	2.75	1.041	1.138	3	101		No homology
G1R2A10	2.761	1.066	1.411	3	374		No homology
G2R1C10	2.768	1.048	1.219	3	276		No homology
G1F3H5	2.853	1.116	1.237	3	435		No homology
G3R2D8	2.857	1.119	1.381	3	96	294	NP_040748.1| gene E protein [Enterobacteria phage S13]
G3F2G6	2.859	1.062	0.97	3	104		No homology
G3F4C12	2.883	1.242	1.071	3	391		No homology
G3F3D9	2.912	1.126	1.048	3	27		No homology
G3R4C12	2.915	1.125	1.053	3	103	477	NP_040703.1| rf replication, viral strand synthesis protein [Coliphage phiX174]
G2R1B4	2.975	1.119	1.18	3	24		No homology
G3R1H10	2.98	1.018	1.292	3	N.D.		
G1R2D4	3.028	1.206	1.034	3	4		No homology
G1R2C10	3.058	1.134	0.968	3	171		No homology
G3F2C12	3.076	1.057	1.16	3	397		No homology
G1R2G2	3.127	0.998	0.875	3	94		No homology
G3F4A11	3.158	0.977	1.307	3	104		No homology
G3R2B1	3.178	1.056	1.194	3	101		No homology
G2F2F12	3.19	0.85	0.944	3	2		No homology
G3F2H4	3.203	1.012	0.971	3	97		No homology

G3F2B4	3.256	0.942	1.221	3	103	477	NP_040703.1| rf replication, viral strand synthesis protein [Coliphage phiX174]
G3F2G5	3.278	1.105	0.96	3	394		No homology
G1R2B4	3.286	1.127	1.169	3	373	117	XP_236501.2| similar to CG9346-PA [Rattus norvegicus]
G1R2D5	3.312	1.101	1.068	3	4		No homology
G3R1E12	3.336	0.971	1.342	3	101		No homology
G3F4D7	3.455	1	1.041	3	104		No homology
G2R1H4	3.549	1.078	1.038	3	N.D.		
G1R1H5	3.577	1.01	1.1	3	376		No homology
G1F1D4	3.619	1.136	0.882	3	85		No homology
G2F2A12	3.674	0.988	1.343	3	113		No homology
G2F1E10	3.68	1.075	0.94	3	182		No homology
G1R1H9	3.682	1.029	0.995	3	375		No homology
G2R1B6	3.79	1.093	1.394	3	280		No homology
G3F3E4	3.958	0.982	0.88	3	392		No homology
G2F1A7	3.983	0.869	1.043	3	15		No homology
G3R1D12	4.072	1.091	0.866	3	N.D.		
G1R3B10	4.123	1.17	1.041	3	371		No homology
G3F4D6	4.273	1.18	1.429	3	388		No homology
G1R2D6	4.36	1.206	1.118	3	N.D.		
G3F2A2	4.377	1.108	0.95	3	103	477	NP_040703.1| rf replication, viral strand synthesis protein [Coliphage phiX174]
G3F3D11	4.397	1.327	1.392	3	393	192	NP_008518.1|ND5_15045 NADH dehydrogenase subunit 5 [Rhipicephalus sanguineus]
G3F4D5	4.443	1.051	1.047	3	389		No homology
G1R1H12	5.529	1.486	0.961	3	377		No homology
G2F2H6	1.473	0.936	0.88	4	N.D.		
G2R2B5	1.552	0.972	0.754	4	402		No homology
G1R2C3	1.887	0.945	0.778	4	99		No homology
G1R1B1	1.917	0.928	0.738	4	420		No homology
G2F1H9	2.102	1.087	0.556	4	367		No homology
G2R1G7	2.171	0.849	0.81	4	24		No homology

G2F2H11	2.184	1.005	0.844	4	361		No homology
G1R2H1	2.201	1.022	0.757	4	310		No homology
G3F1H9	2.222	0.928	0.842	4	N.D.		
G1R2B10	2.23	0.946	0.865	4	99		No homology
G1R3D10	2.427	1.23	0.639	4	369		No homology
G1R1H11	2.439	0.907	0.573	4	378		No homology
G2R2F3	2.448	0.925	0.835	4	N.D.		
G1R3C7	2.456	1.136	0.568	4	N.D.		
G2F2B7	2.475	0.785	0.858	4	111	218	NP_604475.1| Putative RNA dependent RNA polymerase [Atkinsonella hypoxylon partitivirus]
G1R1D3	2.494	1.154	0.603	4	380		No homology
G3F2D10	2.556	1.095	0.839	4	396		No homology
G1R1D7	2.682	1.042	0.692	4	N.D.		
G3F4C5	2.696	0.957	0.794	4	390		No homology
G1R1D1	2.93	1.115	0.672	4	445		No homology
G1R3C9	3.062	1.155	0.614	4	370		No homology
G3R3B12	3.189	0.936	0.772	4	96	294	NP_040748.1| gene E protein [Enterobacteria phage S13]
G1R1G10	3.222	1.094	0.585	4	379		No homology
G1R1B8	3.236	0.993	0.635	4	381		No homology
G2R2F6	3.268	0.935	0.693	4	290		No homology
G1R3B7	3.484	0.979	0.581	4	181		No homology
G3F2D7	3.544	0.847	0.675	4	104		No homology
G3F3D5	3.572	1.208	0.738	4	N.D.		
G2R1E6	3.992	1.041	0.763	4	406		No homology
G3R4F6	1.182	1.062	1.078	5	105		No homology
G3R3D6	1.262	1.062	1.214	5	105		No homology
G2R3D3	1.346	0.999	1.353	5	255	275	ferredoxin precursor [Triticum aestivum]
G2R1F11	1.404	0.978	1.962	5	9		No homology
G3R2D6	1.582	1.113	1.285	5	101		No homology
G2R3C2	1.95	1.002	2.314	5	210		No homology

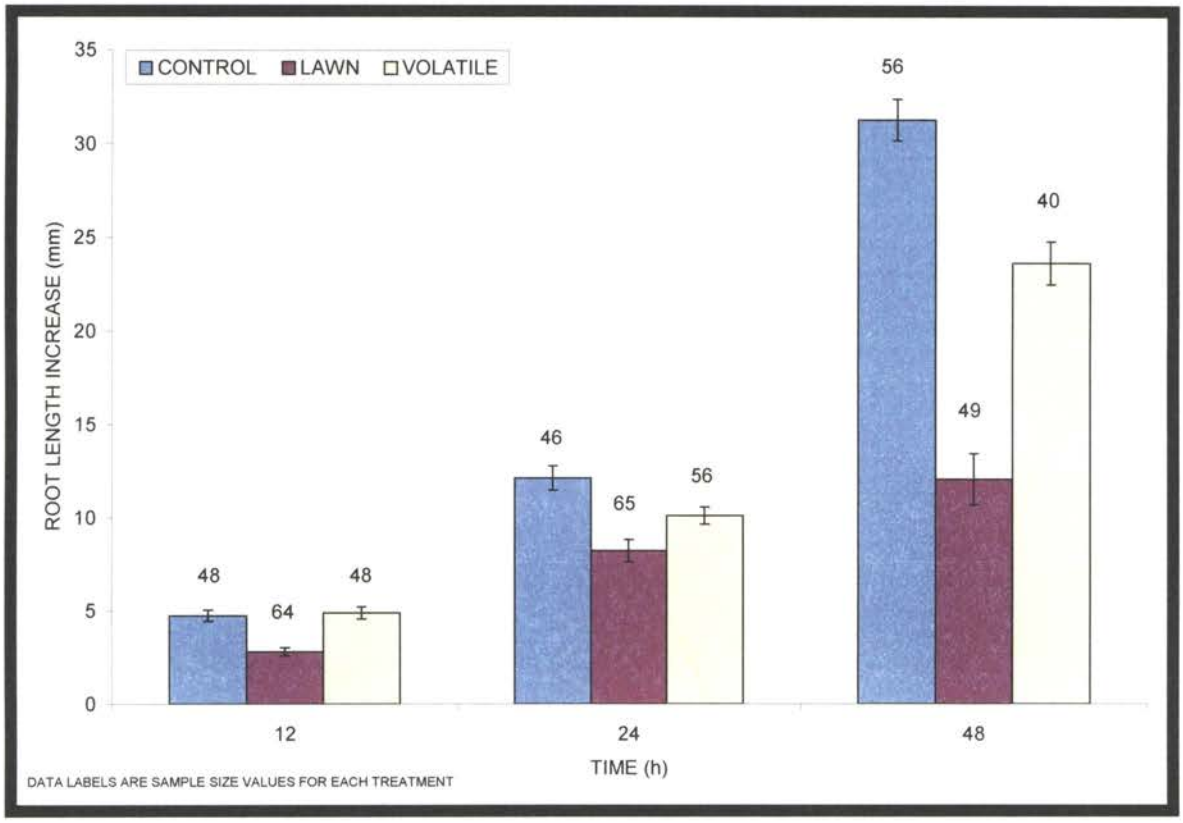
G2F2A2	2.053	1.121	1.621	5	6	176	[Arabidopsis thaliana]
G3R4F7	2.054	0.947	1.351	5	96	294	NP_040748.1| gene E protein [Enterobacteria phage S13]
G2R3D9	2.119	1.044	2.341	5	399		No homology
G2R3D4	2.178	1.136	2.103	5	17		No homology
G2R2E6	2.244	1.12	2.051	5	296	416	[Oryza sativa (japonica cultivar-group)]
G2R2H5	2.45	1.096	1.429	5	N.D.		
G2F2E3	2.458	1.176	1.502	5	53		No homology
G3F1B10	2.496	1.046	2.593	5	N.D.		
G3R3G9	2.704	0.92	1.855	5	105		No homology
G3R3A7	3.031	0.854	1.608	5	105		No homology
G3R2F9	3.055	1.042	2.098	5	N.D.		
G3R5A8	3.246	1.06	2.162	5	N.D.		
G3R4B5	3.378	0.968	1.657	5	101		No homology
G3R2F2	3.397	1.043	2.047	5	101		No homology
G2R2H12	3.513	1.147	1.71	5	N.D.		
G3F1H1	3.565	1.106	1.658	5	104		No homology
G3R1H2	4.212	1.17	2.273	5	N.D.		

APPENDIX

APPENDIX I. Volatile Compounds

In additon, these growth conditions were utilized to examine the possible effects of volatile compounds coming from the fungus that may effect root growth without actual physical contact between the root and the fungus. This was accomplished by plating out seedlings as previously described, with the exception that the seedlings would not be placed on a lawn of Ggt. A small petri dish was placed in the center of the larger petri dish and 1/5X PDA was added to the same height in both plates. The agar was allowed to solidify and a 1 cm plug of Ggt was inoculated in the smaller plate at the center of the larger plate. Once the Ggt covered the small plate, seedlings were placed around the outside of the small petri dish so that the roots would not be impeded in their growth and not come in contact with the fungus. The petri dishes were wrapped with parafilm and the roots were allowed to grow for 12, 24, and 48 hours. The results in figure 1 showed no significant growth difference at 12 hours between control and volatile roots. At 24 hours, the volatile experimental conditions resulted in a significant 16.5% decrease in growth as compared to the controls. At 48 hours, this difference grew to 24.4% decrease in growth in the volatile experimental conditions as compared to the controls.

Figure 1. Growth analysis of control (non-infected), Ggt lawn, and volatile experiments.



The root tissue from these volatile experiments were harvested as previously described for future analysis. The observed growth inhibition under the volatile experimental conditions was further investigated to answer the question as to whether or not volatile compounds were being released from the fungus that could effect root growth indirectly. That is, by plating the fungus in a small petri dish in the center of a larger petri dish where the seedlings were grown we could physically separate the direct contact of the fungus to the wheat roots and thus measure growth as an effect of possible volatile compounds. Ethylene is a known root growth inhibitor and it has been shown under abiotic stress conditions that ethylene does cause a decrease in root growth. It is not known if Ggt produces ethylene or even if it does is it enough to decrease root growth.

Ethylene measurements where taken from Ggt cultures grown on 1/5X agar slates in 30 mL glass test tubes. A 1 cm plug of Ggt was placed on the agar and allowed to grow across the surface of the slant. The tubes were then sealed and two head space samples were taken at 4 and 24 hours. The samples were taken using a 1 mL syringe and a 26 gauge needle which was inserted into the cap and a 1 mL sample was drawn from the 20 mL of head space in the test tube. This sample was then injected into the gas chromatograph (GC) which consists of a 1/8" x 5" stainless steel column packed with activated alumina 60/80 mesh, 90°C isothermal, 100°C injector, 150°C flame ionization detector (FID), and 30 mL / min flow rate of helium at 52 psi pressure. Prior to measure ethylene from Ggt cultures, a standard curve was established using ethylene concentrations of 0, 2.5, 5, 7.5, and 10 ppm ethylene, with a R^2 value of 0.989. In addition, control tubes were utilized to insure that no ethelyne was being evolved from the agar or the cap. Measurements indicated no ethylene evolution in the control tubes or the Ggt culture tubes at 4 and 24 hours. It is possible although not checked that the wheat roots themselves produced ethylene and inhibited root growth due to another undetermined volatile compound coming from the fungus. This experiment was repeated three times with the same results.

APPENDIX II. Fenozol Total RNA Isolation Protocol
For 1.5 grams FW / tube

Purpose: To extract total RNA from infected (Ggt) and non-infected (Control) wheat root tissue.

TODAYS DATE: _____

CONTROL TISSUE: _____ FW / TUBE (g): _____

INFECTED TISSUE: _____ FW / TUBE (g): _____

Ctrl	Ggt	
____	____	Get liquid nitrogen
____	____	Prepare 0.1% diethyl pyrocarbonate (DEPC)
		Add 1 mL DEPC (located in white fridge Dr. Guenzi's lab in brown bottle) to 1 Liter Millipore water (18Mohm) and stir under the fume hood for 1 hr. Use CMS magnetic stirrer (CMS#244-793) speed set @ middle of second dash line.
____	____	Prepare Poly T PNA
		Add 200 uL HPLC Water (provided with kit) in bottle supplied in kit. Mix and store at –20oC
____	____	Prepare Touchdown Precipitant Reagent
		Add 20 mL of 100% Ethanol (located in white freezer Dr. Guenzi's lab) to bottle supplied with kit. Mix and store at –20oC.
____	____	Soak all plasticware overnight in DEPC treated water and autoclave poor off water.
____	____	Root tissue (1.5 g) treated with RNA later and frozen in liquid nitrogen stored at –80oC
____	____	Mortar and pestle wrapped in aluminum foil and frozen at –20oC

____ ____ Turn on water-bath to 50oC (Dr. Guenzi's lab)

____ ____ Grind tissue in mortar and pestle with liquid nitrogen

____ ____ New nygen 40 mL centrifuge tubes

____ ____ Write label for new tubes (Waste #1)

____ ____ Place homogenized tissue into waste #1 tube *Place tube in liquid nitrogen if many samples are being homogenized*

____ ____ Add 24.0 mL Fenozol located in glass fridge in metal tin with tap on it.

____ ____ Incubate centrifuge tube containing Fenozol in water-bath set @ 50oC for 5 minutes

____ ____ Turn water-bath temperature to 65oC (Dr. Guenzi's lab)

____ ____ Centrifuge both tubes in Sorval Superspeed RX2-B automatic refrigerated centrifuge located in Dr. Anderson's lab set @ 12,000 x g, 4oC for 10 minutes

____ ____ New nygen 40 mL centrifuge tubes

____ ____ Write label for new tubes (Waste #2)

____ ____ Pour supernatant into waste #2 tube

____ ____ Set waste tube #1aside

____ ____ Add 6.0 mL chloroform to remove lipids (located in fume hood Dr. Guenzi's lab) to supernatant tube

____ ____ Vortex waste tube #2 for 20 seconds using Thermolyne Maxi-MixII vortex set @ 10 with continuous shake

____ ____ Styrofoam 40 mL test-tube rack

____ ____ Incubate @ room temperature (approx. 20oC) for 3 minutes

____ ____ Centrifuge both tubes in Sorval Superspeed RX2-B automatic refrigerated centrifuge located in Dr. Anderson's lab set @ 12,000 x g, 4oC for 10 minutes

____ ____ New nygen 40 mL centrifuge tubes

____ ____ Write label for new tubes (Final RNA 740 uL)

____ ____ Pour supernatant (top aqueous phase) into Final RNA 740 uL tube

____ ____ Set waste tube #2 aside

____ ____ Add 7.5 mL Isopropanol (2-propanol) to top aqueous phase located in white freezer in Dr. Guenzi's lab

____ ____ Incubate @ room temperature (approx. 20oC) for 10 minutes

____ ____ Mix tubes gently on vortex set @#7 for 30 sec (Dr. Guenzi's lab)

____ ____ Centrifuge both tubes in Sorval Superspeed RX2-B automatic refrigerated centrifuge located in Dr. Anderson's lab set @ 12,000 x g, 4oC for 15 minutes

____ ____ 2 new 15 mL centrifuge tubes treated with RNase Zap (Ambion)

____ ____ Write label for new tubes (Waste #3a and #3b)

____ ____ Pour supernatant !!Carefully!! into 15 mL waste tube #3a and #3b

____ ____ Set waste tube #3a and #3b aside

____ ____ Add 21 mL 70% Ethanol (Make up 100 mL 70% Ethanol by adding 70 mL HPLC grade 100% pure Ethanol located under the fume hood in Dr. Guenzi's lab add 30 mL DEPC treated water) to pellet located in white freezer in Dr. Guenzi's lab

____ ____ Centrifuge tubes in Sorval Superspeed RX2-B automatic refrigerated centrifuge located in Dr. Anderson's lab set @ 12,000 x g, 4oC for 5 minutes

____ ____ 2 new 15 mL centrifuge tubes treated with RNase Zap (Ambion)

____ ____ Write label for new tubes (Waste #4a and #4b)

____ ____ Remove solution by pouring off slowly and place in waste tubes #4a and #4b)

____ ____ Set waste tubes #4a and #4b aside

____ ____ Air dry pellet (pellet contains total RNA)

____ ____ Dilute 2X buffer to 1X (1 mL 2X buffer:1 mL DEPC water)

____ ____ Add 1000 uL of 1X Binding Buffer to resuspend pellet

____ ____ Vortex briefly (30 sec) set on #7 (Dr. Guenzi's lab)

____ ____ Incubate @ 65oC in water-bath for 10 minutes

____ ____ Vortex briefly (30 sec) set on #7 (Dr. Guenzi's lab)

____ ____ **If mixing tubes, do so now after vortexing**

____ ____ 100 uL quartz micro cuvette

____ ____ Place 10 uL resuspended pellet solution in spectrophotometer cuvette and add 90 uL 1X Binding Buffer (dilution D=10)

____ ____ Place 100 uL 1X Binding Buffer for blank

____ ____ Beckman DU-65 Spectrophotometer located in Dr. Dillwith's lab

____Control Tube A260_____ A280_____;A260/A280_____

____ Ggt Tube A260_____ ;A280_____;A260/A280_____

____ ____ **The purity of RNA should be between 1.9 and 2.1**

____ Ctrl Tube RNA concentration by (A260)(0.04ug/uL)D = _____ug/uL

____ Ggt Tube RNA concentration by (A260)(0.04ug/uL)D = _____ug/uL

____ ____ **NOTE that the A260 should be greater than 0.05 to give an accurate RNA concentration**

____ Control RNA yield (concentration)(volume of RNA solution is equal to 1000 uL) (_____ug/uL)(1000 uL) = _____ug Total RNA

____ Ggt RNA yield (concentration)(volume of RNA solution is equal to 1000 uL) (_____ug/uL)(1000 uL) = _____ug Total RNA

____ Corrected Control RNA yield (concentration)(volume 990 uL due to –10 uL for spec) (_____ug/uL)(990 uL) = _____ug Total RNA

____ Corrected Ggt RNA yield (concentration)(volume 990 uL due to –10 uL for spec) (_____ug/uL)(990 uL) = _____ug Total RNA

____ ____ **Tubes can be stored here @-80oC until mRNA Isolation**

APPENDIX III. M-TRAP TOTAL mRNA Isolation Protocol

Purpose: To isolate mRNA from Total RNA obtained with the Fenozol extraction protocol.

TODAYS DATE: _____

CONTROL TISSUE: _____ Total RNA A260/A280:_____

INFECTED TISSUE: _____ Total RNA A260/A280:_____

Ctrl Ggt

____ ____ **M-TRAP TOTAL mRNA Kit**

____ ____ Turn on water-bath to 70oC (Dr. Guenzi's lab)

____ ____ Turn on small water-bath to 95oC (Dr. Guenzi's lab)

____ ____ Get 2 RNAse-free 1.7 mL tubes from kit

____ ____ Label new tubes (initial RNA)

____ Add Control Total RNA_____ug =_____uL [No more than 500 ug total RNA and no more than 1.5 mL total solution (Total RNA + Binding Buffer)]

____ Add Ggt Total RNA _____ ug =_____uL [No more than 500 ug total RNA and no more than 1.5 mL total solution (Total RNA + Binding Buffer)]

____ ____ Add 15 uL of Poly T PNA mix to each tube (mix located in –20oC in clear box label Tim S.)

____ ____ Vortex briefly (30 seconds) set @ #7 Dr. Guenzi's lab

____ ____ Incubate tubes at 70oC in water-bath for 5 minutes

____ ____ Turn temperature of water-bath to 75oC

____ ____ Centrifuge samples for 5 seconds using Jovan Centrifuge set @ 14,000 located in Dr. Guenzi's lab use slots #7 (Ctrl) and 17 (Ggt) with front of cap down

____ ____ Incubate @ RT for 15 minutes with gentle shaking (rocking platform Lab-line instruments Junior Orbit Shaker set at 1500 rpms SN#0184-0129 located in Dr. Singleton's lab)

____ ____ Add 60 uL steptavidin beads to each tube (beads located in 4oC glass fridge in bag labeled Tim S.)

____ ____ Incubate @ room temperature (approx. 20oC) for 45 minutes on rocking platform Lab-line instruments Junior Orbit Shaker set at 1500 rpms SN#0184-0129 located in Dr. Singleton's lab

SKIP SKIP Centrifuge both tubes with Jovan centrifuge located in Dr. Guenzi's lab set @ 14,000 for 5 minutes @ RT

____ ____ !!!Use magnet only, Do not use centrifuge with beads!! When using magnet, set on magnet until solution is clear, this is approximately one minute.

____ ____ Set tubes in magnet (Magnetight Separation Stand, Novagen)

____ ____ New 2.0 mL micro-centrifuge tubes

____ ____ Label tubes (Waste #1)

____ ____ Remove hybridization buffer with 1 mL pipette !!!KEEP TUBES ON MAGNET!!! MOST DANGER IN LOSING YIELD!!!! and place in waste tube #1 set waste tube #1 aside

____ ____ Resuspend the steptavidin beads in 750 uL wash buffer supplied with kit by vortexing gently @ setting #7 for 30 sec.

SKIP SKIP Centrifuge both tubes with Jovan centrifuge located in Dr. Guenzi's lab set @ 14,000 for 5 minutes @ RT

____ ____ Set tubes in magnet (Magnetight Separation Stand, Novagen)

____ ____ New 2.0 mL micro-centrifuge tubes

____ ____ Label tubes (Waste #2)

____ ____ Remove wash buffer !!carefully!! with 1 mL pipette and place in waste tube #2 and set waste tube #2 aside

____ ____ Resuspend the steptavidin beads in 100 uL wash buffer supplied with kit

____ ____ Add 2 units or 2 uL [1 unit/uL; concentration of DNASE in stock] RNase-free DNase (DNase I Amplification Grade, 20oC in purple box labeled DNA marker

____ ____ Incubate @ room temperature (approx. 20oC) for 10 minutes

SKIP SKIP Centrifuge both tubes with Jovan centrifuge located in Dr. Guenzi's lab set @ 14,000 for 3 minutes

____ ____ Set tubes in magnet (Magnetight Separation Stand, Novagen)

____ ____ New 2.0 mL micro-centrifuge tubes

____ ____ Label tubes (Waste #3)

____ ____ Remove solution with 200 uL pipette set @ 100 uL and place in waste tube #3 and set waste tube #3 aside

____ ____ Resuspend the steptavidin beads in 750 uL wash buffer supplied with kit

SKIP SKIP Centrifuge both tubes with Jovan centrifuge located in Dr. Guenzi's lab set @ 14,000 for 5 minutes

____ ____ Set tubes in magnet (Magnetight Separation Stand, Novagen)

____ ____ New 2.0 mL micro-centrifuge tubes

____ ____ Label tubes (Waste #4)

____ ____ Remove wash buffer !!carefully!! with 1 mL and place in waste tube #4 and set waste tube #4 aside

____ ____ Resuspend the steptavidin beads in 750 uL wash buffer supplied with kit

SKIP SKIP Centrifuge both tubes with Jovan centrifuge located in Dr. Guenzi's lab set @ 14,000 for 5 minutes

____ ____ Set tubes in magnet (Magnetight Separation Stand, Novagen)

____ ____ Remove wash buffer !!carefully!! and place in waste tube#4 and set waste tube #4 aside

____ ____ ** FIRST ELUTION OF mRNA**

____ ____ Resuspend the steptavidin beads in 75 uL DEPC-H2O and ensure that beads are evenly distributed.

____ ____ Incubate @ 75oC for 2 minutes in water-bath (this step is to elute of the mRNA from the Poly T PNA Probe)

SKIP SKIP Centrifuge tubes with Jovan centrifuge located in Dr. Guenzi's lab set @ 14,000 for 5 minutes @ RT

____ ____ Set tubes in magnet (Magnetight Separation Stand, Novagen)

____ ____ ****mRNA****NOW IN SUPERNATANT****

____ ____ New 1.7 mL micro-centrifuge tubes

____ ____ Label tubes (Final mRNA)

____ ____ Carefully transfer supernatant to the sterile 1.7 mL microcentrifuge tube (This is the mRNA sample!!!).

____ ____ ** SECOND ELUTION OF mRNA**

____ ____ Add 75 uL DEPC-H2O to the steptavidin beads for second time

____ ____ Incubate @ 75oC for 2 minutes in water-bath

____ ____ Turn temperature of water-bath to 70oC

SKIP SKIP Centrifuge tubes with Jovan centrifuge located in Dr. Guenzi's lab set @ 14,000 for 5 minutes @ RT

____ ____ Set tubes in magnet (Magnetight Separation Stand, Novagen)

____ ____ Carefully remove supernatant and place in the same 1.7 mL microcentrifuge tube with the supernatant previously removed (tube labeled Final mRNA) (This is the mRNA sample!!!).

____ ____ Place 1.7 mL microcentrifuge tube with steptavidin beads in it aside (tube labeled initial RNA)

____ ____ Tube should contain 150 uL solution containing mRNA (combining both supernatants) (75 +75 = 150) (Visual Check).

____ ____ Heat @ 95oC for 3 minutes in water-bath to inactivate DNase

____ ____ Turn off 95oC water-bath after this step

____ ____ Add 5 volumes or 750 uL (150x5=750uL) of Touchdown Precipitation Reagent supplied with kit

SKIP SKIP ***Special circumstances sometimes will arise that produce an expected yield less than 1.0 ug mRNA. If this is suspected then add 10 volumes of TD precip reagent instead of 5 volumes add vortex and then place @ -80oC instead of 4oC for 20 minutes***

____ ____ Vortex Inverted for 10 seconds using Thermolyne vortex set on 10 and continuous located in Dr. Guenzi's lab

____ ____ Place tubes in 4oC fridge (Glass fridge in Dr. Guenzi's lab) for 20 minutes

____ ____ Centrifuge tubes @14,000 x g for 20 minutes @4oC (located in cold room) (should see pellet, very small) may need to use black background

____ ____ New 2.0 mL micro-centrifuge tubes

____ ____ Label tubes (Waste #5)

____ ____ Remove as much Touchdown Precipitation Solution as possible (place in waste tube#5, and set waste tube #5 aside) leaving pellet and approx. 10 uL solution in tube

____ ____ Add to each tube 200 uL pure-Ethanol located in freezer in Dr. Guenzi's lab in box labeled Tim S.

____ ____ Vortex briefly 30 sec using Thermolyne vortex set on 7 located in Dr. Guenzi's lab (should see pellet floating around)

____ ____ Start spectrophotometer located in Dr. Dillwith's lab

____ ____ Centrifuge both tubes @14,000 x g for 20 minutes @4oC (located in cold room)

____ ____ New 2.0 mL micro-centrifuge tubes

____ ____ Label tubes (Waste #6)

____ ____ Remove 200 uL pure-Ethanol and place in waste tube #6, and set waste tube #6 aside, leaving pellet and approx. 10 uL solution in tube

____ ____ Air-Dry pellet for 10 minutes or until ethanol evaporates

____ ____ Resuspend pellet in 30 uL of RNA Storage Solution; Ambion

____ ____ **DO NOT FORGET** Heat @ 70oC for 10 minutes in water-bath (Dr. Guenzi's lab)

____ ____ Vortex briefly (30 Seconds) using Thermolyne vortex set on 7 located in Dr. Guenzi's lab

____ ____ **If mixing tubes, do so now after vortexing**

____ ____ 100 uL quartz micro cuvette

____ ____ Place 10 uL resuspended pellet solution in spectrophotometer cuvette and add 90 uL RNA storage solution, Ambion (dilution D=10)

____ ____ ***NOTE: if NOT mixing tubes, use 5 uL of resuspended pellet in 95 uL RNA storage solution, Ambion (D=25) ***

____ ____ Place 100 uL RNA storage solution, Ambion for blank

____ ____ Beckman DU-65 Spectrophotometer located in Dr. Dillwith's lab

____ Control Tube A260_____ ;A280_____; A260/A280_____

____ Ggt Tube A260_____ ;A280_____; A260/A280_____

____ ____ **The purity of RNA should be between 1.9 and 2.1**

____ Ctrl Tube mRNA concentration by (A260)(0.04ug/uL)D = _____ug/uL

____ Ggt Tube mRNA concentration by (A260)(0.04ug/uL)D = _____ug/uL

____ ____ **NOTE that the A260 should be greater than 0.05 to give an accurate RNA concentration**

____ ____ **NOTE: for the calculations below, the uL used depends on whether or not tubes were mixed together after resuspension of mRNA. If tubes were not mixed this value is equaled to 30 uL**

____ Control RNA yield (concentration)(volume of RNA solution is equal to ___ uL)

(_____ug/uL)(__ uL) = _____ug mRNA RNA

____ Ggt RNA yield (concentration)(volume of RNA solution is equal to ___ uL)

(_____ug/uL)(__ uL) = _____ug mRNA RNA

____ Corrected Control RNA yield (concentration)(volume ___ uL due to –___ uL for spec) (_____ug/uL)(___uL) = _____ug mRNA RNA

____ Corrected Ggt RNA yield (concentration)(volume ___ uL due to –___ uL for spec) (_____ug/uL)(___uL) = _____ug mRNA RNA

____ ____ After determining the concentration of mRNA in the sample, the concentration of the mRNA must be concentrated down to 0.5 ug/uL for use in the next step of cDNA synthesis. To do this, the mRNA must be reprecipitated with TD precipitation reagent.

____ ____ **Also, If adding mRNA from previous experiments to concentrate all mRNA do so at this step and readjust the amount of TD precipitation reagent needs**

____ ____ Add 5 volumes of Touchdown Precipitation Reagent supplied with kit. ***This amount depends on what the volume of mRNA used to resuspend previously minus the amount of solution used for the spec reading. For example, if 30 uL was resuspended and 5 uL were used for spec. Then you would add 5 volumes or 125 uL (25 uL x 5 volumes = 125 uL) to the 25 uL for a total solution amount of 150 uL)***

____ ____ Vortex Inverted for 10 seconds using Thermolyne vortex set on 10 and continuous located in Dr. Guenzi's lab

____ ____ Place tubes in 4oC fridge (Glass fridge in Dr. Guenzi's lab) for 20 minutes

____ ____ Centrifuge tubes @14,000 x g for 20 minutes @4oC (located in cold room) (should see pellet, very small) may need to use black background

____ ____ New 2.0 mL micro-centrifuge tubes

____ ____ Label tubes (Waste #7)

____ ____ Remove as much Touchdown Precipitation Solution as possible (place in waste tube #7, and set waste tube #7 aside) leaving pellet and approx. 10 uL solution in tube

____ ____ Add to each tube 200 uL pure-Ethanol located in freezer in Dr. Guenzi's lab in box labeled Tim S.

____ ____ Vortex briefly 30 sec using Thermolyne vortex set on 7 located in Dr. Guenzi's lab (should see pellet floating around)

____ ____ Centrifuge both tubes @14,000 x g for 20 minutes @4oC (located in cold room)

____ ____ New 2.0 mL micro-centrifuge tubes

____ ____ Label tubes (Waste #8)

____ ____ Remove 200 uL pure-Ethanol and place in waste tube#8, and set waste tube #8 aside, leaving pellet and approx. 10 uL solution in tube

____ ____ Air-Dry pellet for 10 minutes or until ethanol evaporates

____ ____ Resuspend pellet in appropriate amount of RNA storage solution, Ambion. ***This amount depends on what concentrations were obtained with the first round of precipitation. The final concentration needs to be 0.5 ug/uL***

CALCULATIONS:

Control

_____ug mRNA * 1.0 uL / 0.5 ug = _____uL RNA storage sol.

Ggt

_____ug mRNA * 1.0 uL / 0.5 ug = _____uL RNA storage sol.

____ ____ **DO NOT FORGET** Heat @ 70oC for 10 minutes in water-bath (Dr. Guenzi's lab)

____ ____ Vortex briefly (30 Seconds) using Thermolyne vortex set on 7 located in Dr. Guenzi's lab

____ ____ mRNA can be stored @-80oC at this point until cDNA synthesis is performed.

Finished mRNA isolation and concentration

____ Run Agarose Gel Analysis of Total RNA **Should see a smear**

PROCEED WITH CLONETECH SSH KIT - cDNA synthesis reaction

APPENDIX IV. CLONTECH PCR-Select cDNA Synthesis Protocol

Purpose: To produce ds cDNA from mRNA obtained from M-Trap Isolation previously described.

First Strand cDNA Synthesis:

Date: _____

Tester_____ Concentration (ug mRNA/ uL)_____ A260/A280 _____

Driver_____ Concentration (ug mRNA/ uL)_____ A260/A280 _____

Kit Ctrl_____ Concentration (ug mRNA/ uL)_____ A260/A280 N/A

Test	Driver	Ctrl	Instructions are per tube
____	_____	____	Get **Ice**
____	_____	____	New **0.5 mL microcentrifuge tubes**
____	_____	____	New tube labels (Waste #1)
____	_____	____	Add 0.5 to 2 ug **poly A^+ RNA** (No more than 2 to 4 uL) (**Human skeletal muscle poly A^+ RNA** control supplied in kit and kept @ -80°C) (green tube)
____	_____	____	Add 1 uL **cDNA synthesis primer** (10 uM) (in kit, green tube)
____	_____	____	Total Volume Calculation (uL)
____	_____	____	Qs with **sterile water** (in kit, clear tube) to 5 uL
____	_____	____	Mix contents by briefly vortexing (30 sec) set @ # 7 Dr. Guenzi's lab
____	_____	____	Spin briefly (5 sec) in microcentrifuge in Dr. Guenzi's lab

____ _____ ____ Incubate tubes @ 70°C for 2 minutes in a thermal cycler (MJ Research Inc. PTC-100^{tm}, located in Dr. Guenzi's lab)

____ _____ ____ Cool tubes on **ice** for 2 minutes

____ _____ ____ Spin briefly (5 sec) in microcentrifuge in Dr. Guenzi's lab

____ _____ ____ Add 2 uL **5X first-strand buffer** (in kit, green tube)

____ _____ ____ Add 1 uL **dNTP** (10 mM each) (in kit, clear tube)

____ _____ ____ Add 1 uL **sterile water** (in kit, clear tube)

____ _____ ____ Add 1 uL **AMV reverse transcriptase** (20 units / uL) (in kit, green tube)

____ _____ ____ Check total volume should be 10 uL (visual)

____ _____ ____ Gently vortex briefly (5 sec) (setting #7, Dr. Guenzi's lab)

____ _____ ____ Spin briefly (5 sec) in microcentrifuge in Dr. Guenzi's lab

____ _____ ____ Incubate tubes @ 42°C for 1.5 h (90 min) in an air incubator (located on 3rd floor plant path). DO NOT USE WATER BATH OR THERMAL CYCLER DO TO EVAPORATION!!

____ _____ ____ Place tubes on **ice** to terminate first-strand cDNA synthesis and proceed immediately to second-strand cDNA synthesis.

Second-Strand cDNA Synthesis:

Test	Driver	Ctrl	Instructions are per tube
____	_____	____	Add 48.4 uL **Sterile-Water** (in kit, clear tube)
____	_____	____	Add 16.0 uL **5X Second-Strand Buffer** (in kit, pink tube)
____	_____	____	Add 1.6 uL **dNTP (10 mM)** (in kit, clear tube)
____	_____	____	Add 4.0 uL **20X Second-Strand Enzyme Cocktail** (in kit)
____	_____	____	Mix contents by vortex (30 sec) set @ #7 (Dr. Guenzi's lab)
____	_____	____	Spin briefly (5 sec) in microcentrifuge in Dr. Guenzi's lab
____	_____	____	Check the total volume and it should be 80 uL (visual)
____	_____	____	Incubate tubes @ 16°C for 2 hours in thermal cycler (water bath in core facility)
____	_____	____	Add 2 uL (6 units) **T4 DNA polymerase** (in kit, pink tube)
____	_____	____	Mix contents by vortex (30 sec) set @ #7 (Dr. Guenzi's lab)
____	_____	____	Incubate tubes @ 16°C for 30 minutes in thermal cycler (water bath in core facility)
____	_____	____	Add 4 uL of **20X EDTA / glycogen** (in kit, clear tube)
____	_____	____	Mix contents to terminate second-strand synthesis by vortex (30 sec) set @ #7 (Dr. Guenzi's lab)
____	_____	____	Add 100 uL of **phenol:chloroform:isoamyl alcohol** (25:24:1) (located in glass fridge 4oC, Dr. Guenzi's lab)
____	_____	____	Thoroughly vortex (1 minute) (setting #7, Dr. Guenzi's lab)
____	_____	____	Centrifuge @ 14,000 rpm for 10 minutes @ room temperature (microcentrifuge located in Dr. Guenzi's lab)

____ _____ ____ New **0.5 mL microcentrifuge tubes**

____ _____ ____ New tube labels (Waste #2)

____ _____ ____ Carefully remove the top aqueous layer (little less than 100 uL) and place in a clean (sterile) **0.5 mL microcentrifuge tube** (Waste #2)

____ _____ ____ Store the interphase and lower phase (Waste #1) at –20oC

____ _____ ____ Add 100 uL of **chloroform:isoamyl alcohol** (24:1) to the aqueous layer (located under fume hood in Dr. Guenzi's lab)

____ _____ ____ Thoroughly vortex (1 minute) (setting #7, Dr. Guenzi's lab)

____ _____ ____ Centrifuge @ 14,000 rpm for 10 minutes @ room temperature (microcentrifuge located in Dr. Guenzi's lab)

____ _____ ____ New **0.5 mL microcentrifuge tubes**

____ _____ ____ New tube labels (Final ds cDNA 44 uL)

____ _____ ____ Carefully remove the top aqueous layer and place in a clean (sterile) **0.5 mL microcentrifuge tube** (Final ds cDNA 44 uL)

____ _____ ____ Store the interphase and lower phase (Waste #2) at –20oC

____ _____ ____ Add 40 uL of **4 M NH4Oac** (clear tube in kit)

____ _____ ____ Add 300 uL of **95% ethanol** (Make up 1 mL; 950 uL HPLC grade pure Ethanol + 50 uL autoclaved milli-pore water)

____ _____ ____ **Precede immediately with precipitation. DO NOT store tubes at –20°C do to prolonged exposure to this temperature can precipitate unwanted salts.

____ _____ ____ Thoroughly vortex (1 minute) (setting #7, Dr. Guenzi's lab)

____ _____ ____ Centrifuge @ 14,000 rpm for 20 minutes @ room temperature (microcentrifuge located in Dr. Guenzi's lab)

____ _____ ____ New **0.5 mL microcentrifuge tubes**

____ _____ ____ New tube labels (Waste #3)

____ _____ ____ Remove supernatant carefully and place in waste tube #3 and store waste tube at –20°C ****Pellet is ds cDNA**** Use black background to visualize the pellet

____ _____ ____ Overlay the pellet with 500 uL **80% ethanol** (Make up 2 mL; 1600 uL HPLC grade pure Ethanol + 400 uL autoclaved milli-pore water)

____ _____ ____ Centrifuge @ 14,000 rpm for 10 minutes @ room temperature (microcentrifuge located in Dr. Guenzi's lab)

____ _____ ____ New **0.5 mL microcentrifuge tubes**

____ _____ ____ New tube labels (Waste #4)

____ _____ ____ Remove supernatant carefully and place in waste tube and store waste tube at –20°C ****Pellet is ds cDNA**** Use black background to visualize the pellet

____ _____ ____ Air dry the pellet for approx. 10 minutes to evaporate residual ethanol *****Pellet is ds cDNA***** Use black background to visualize the pellet

____ _____ ____ Dissolve pellet in 50 uL **sterile-water** (clear tube in kit)

____ _____ ____ Vortex for 30 seconds set @ #7 Dr. Guenzi's lab ****No pellet should be visible****

___ ___ ___ New **0.5 mL microcentrifuge tubes**

___ ___ ___ New tube labels (Final ds cDNA 6 uL)

___ ___ ___ Transfer 6 uL of dissolved precipitate to a fresh **0.5 mL microcentrifuge tube** (Final ds cDNA 6 uL) and store this tube @ -20°C until after ***Rsa* I** digestion for **agarose gel electrophoresis** to estimate yield and size range of ds cDNA products synthesized

___ ___ ___ Be sure this label is on final tube (Final 44 uL)

___ ___ ___ Store the **ds cDNA** @ -20°C until ready to proceed with ***Rsa* I** digestion

APPENDIX V. Rsa I Digestion

Purpose: To create blunt-ended ds cDNA fragments, necessary for adaptor ligation.

Rsa I is a four base cutting restriction enzyme.

Date: _____

Driver Tester Kit

____ ____ ____ Name of tissue used (Ctrl, Ggt and 12, 24, 48 h)

____ ____ ____ List Tube Labels Here (Name of Final 44 uL ds cDNA tube)

____ ____ ____ Turn on incubator to 37°C in soils lab

____ ____ ____ New **0.5 mL microcentrifuge tubes**

____ ____ ____ List Tube Labels Here (Waste #1)

____ ____ ____ Add 43.5 uL **ds cDNA** (Taken from 44 uL ds cDNA leaving 0.5 uL stored @ -20°C)

____ ____ ____ Add 5.0 uL **10X Rsa I restriction buffer** (Pink tube in kit @-20°C)

____ ____ ____ Add 1.5 uL **Rsa I** (10 units / uL) equal to 15 units (Pink tube in kit @ -20°C)

____ ____ ____ Total volume should be 50.0 uL (visual check)

____ ____ ____ Mix by vortexing set @#7 for 30 seconds

____ ____ ____ Centrifuge briefly (5 sec) to spin down tube contents

____ ____ ____ Incubate @37°C for 1.5 hours (Air incubator in soils lab)

____ ____ ____ New **0.5 mL microcentrifuge tubes**

____ ____ ____ List Tube Labels Here (Rsa I digest 5.0 uL)

____ ____ ____ Place 5.0 uL of **reaction mixture** in new tube to analyze efficiency of Rsa I digestion

____ ____ ____ Store the 5.0 uL @-20°C until needed for analysis

____ ____ ____ Total volume should be 45.0 uL (visual check)

____ ____ ____ Add 2.5 uL of **20X EDTA / Glycogen mix** to terminate reaction (Clear tube in kit @ -20°C)

____ ____ ____ Total volume should be 47.5 uL (visual check)

____ ____ ____ Add 50 uL **phenol:chloroform:isoamyl alcohol (25:24:1)** (In clear fridge @ 4°C)

____ ____ ____ Total volume should be 97.5 uL (visual check)

____ ____ ____ Vortex thoroughly for 1 minute set @#7

____ ____ ____ Centrifuge tubes @ 14,000 rpm @ RT for 10 minutes to separate phases (Dr. Guenzi's lab)

____ ____ ____ New **0.5 mL microcentrifuge tubes**

____ ____ ____ List Tube Labels Here (Waste #2)

____ ____ ____ Place **top phase** in new centrifuge tube (Waste #2) and set aside **bottom and interphase waste** tube (Waste #1)

____ ____ ____ Add 50.0 uL **chloroform:isoamyl alcohol (24:1)** (In flammable cabinet under fume hood) to top phase solution that was placed in the new tube in the previous step.

____ ____ ____ Vortex thoroughly for 1 minute set @#7

____ ____ ____ Centrifuge tubes @ 14,000 rpm @ RT for 10 minutes to separate phases (Dr. Guenzi's lab)

____ ____ ____ New **0.5 mL microcentrifuge tubes**

____ ____ ____ List Tube Labels Here (Final Rsa I Digest 5.5 uL)

____ ____ ____ Place **top aqueous phase** in new centrifuge tube (Final Rsa I Digest 5.5 uL) and set aside **bottom and interphase waste** tube (Waste #2)

____ ____ ____ Add 25 uL **4M NH_4OAc** (Clear tube in kit –20°C)

____ ____ ____ Add 187.5 uL of **95% Ethanol** (Make 1000 uL by adding 950 uL 200 proof Ethanol and 50 uL sterile autoclaved milli-pore water)

____ ____ ____ *NOTE PRECEED IMEDIATELY WITH PRECEPITATION. DO NOT STORE TUBES @ -20°C DUE TO PRECIPITATION OF SALTS*

____ ____ ____ Vortex thoroughly for 1 minute set @#7

____ ____ ____ Centrifuge tubes @ 14,000 rpm @ RT for 20 minutes to separate phases (Dr. Guenzi's lab)

____ ____ ____ New **2.0 mL microcentrifuge tubes** for waste

____ ____ ____ List Waste Tube Labels Here (Waste #3)

____ ____ ____ Remove supernatant carefully and place in waste tube #3 and set waste tube #3 aside (should see **small white pellet**, may need to use black background)****PELLET is ds FRAGMENTED cDNA****

____ ____ ____ Add 200 uL **80% Ethanol** overlaid on pellet (Make 1000 uL by adding 800 uL 200 proof Ethanol and 200 uL sterile autoclaved milli-pore water)

____ ____ ____ Centrifuge tubes @ 14,000 rpm @ RT for 5 minutes (Dr. Guenzi's lab)

____ ____ ____ New 2.0 mL microcentrifuge tubes for waste

____ ____ ____ List Waste Tube Labels Here (Waste #4)

____ ____ ____ Remove supernatant carefully and place in waste tube #4 and set waste tube #4 aside (should see **small white pellet**, may need to use black background)****PELLET IS ds FRAGMENTED cDNA****

____ ____ ____ Air dry pellet for 10 minutes

____ ____ ____ Dissolve pellet in 5.5 uL of **sterile water** (clear tube supplied with kit)

____ ____ ____ Store tubes @-20°C **Called Clontech and they confirmed storing the samples at this stage and said it was a good stopping point**

____ ____ ____ These 5.5 uL samples will serve as **experimental driver cDNA** and **control skeletal muscle driver cDNA**

____ ____ ____ Check 5.0 uL of **Rsa I digested cDNA** (using the 5.0 uL held back in earlier steps in the Rsa I digestion protocol

____ ____ ____ Using **1% agarose / ethidium bromide in 1X TAE buffer gel electrophoresis**, described in section V.B of the Clontech manual.

____ ____ ____ Check 2.5 uL of **non-digested cDNA** yield and size range of ds cDNA products synthesized (This is from the 6.0 uL held back in the cDNA synthesis protocol)

____ ____ ____ Run **1% Agarose / Ethidium Bromide** Gel Analysis of ds cDNA

____ ____ ____ ****PROCEED TO ADAPTOR LIGATION****

APPENDIX VI. Adaptor Ligation

Purpose: To provide 2 adaptors for use by the PCR primer once the recessed ends have been filled. Two tester populations are created with different adaptors, but the driver cDNA has no adapter.

Date:_____

Driver Tester

_____ _____ Name of **experimental condition tissue** used (Ctrl, Ggt, Kit, 12h, 24h, 48h)

_____ _____ ****Adaptors will not be ligated to the driver cDNA****

Preparing Diluted Tester cDNA

_____ _____ New **0.5 mL microcentrifuge tubes**

_____ _____ Labels for new tubes

_____ _____ Add 1.0 uL of **Rsa I-digested experimental cDNA** (from 5.5 uL Rsa I fragmented ds cDNA leaving 4.5 uL)

_____ _____ Add 5.0 uL **sterile water** (located in kit)

_____ _____ Total volume should be 6.0 uL

Preparation of master ligation mix

_____ _____ New **0.5 mL microcentrifuge tube**

_____ _____ Labels for new tubes

_____ _____ Add 45.0 uL / 15 reactions (3.0 uL / reaction) **sterile water** (supplied with kit)

_____ _____ Add 30.0 uL / 15 reactions (2.0 uL / reaction) **5X Ligation Buffer** (supplied with kit @ -20°C in light blue colored tube)

_____ _____ Add 15.0 uL / 15 reactions (1.0 uL / reaction) **T4 DNA Ligase** (400 units / uL) (Supplied with kit @ -20°C in light blue colored tube)

_____ _____ **This is the **master ligation mix** for 15 reactions, This mix will be used for Kit Ctrl, Ctrl 12h, Ggt 12h, Ctrl 24h, Ggt 24h, Ctrl 48h, Ggt 48h, and One Additional Reaction. Kit Ctrl, Ctrl 48h, Ggt 48h are covered in this protocol. See Additional sheets for other experimental conditions**

Preparation of adaptor-ligated tester cDNA

_____ _____ Set-up the ligation reaction by the following table:

	Non-Infected 12 hours	**Non-Infected 12 hours**	**Ggt 12 hours**	**Ggt 12 hours**
Component	**T-2-1**	**T-2-2**	**T-3-1**	**T-3-2**
Diluted Tester cDNA	2.0 uL	2.0 uL	2.0 uL	2.0 uL
Adaptor 1 (10 uM)	2.0 uL		2.0 uL	
Adaptor 2R (10 uM)		2.0 uL		2.0 uL
Master Mix	6.0 uL	6.0 uL	6.0 uL	6.0 uL
Total Volume	**10.0 uL**	**10.0 uL**	**10.0 uL**	**10.0 uL**

_____ _____ **Diluted tester cDNA** comes from 6 .0 uL (prepared earlier in this protocol) leaving 2.0 uL per tube of diluted tester cDNA

_____ _____ Vortex thoroughly set @#7 for 1 minutes for all 6 tubes (Dr. Guenzi's lab)

_____ N/A New **0.5 mL microcentrifuge tube**

_____ N/A Labels for new tubes

_____ N/A Add 2.0 uL of **T-2-1** to new tube (leaving 8.0 uL)

_____ N/A Add 2.0 uL of **T-2-2** to same tube (leaving 8.0 uL)

_____ N/A ****After ligation is complete this is the unsubtracted tester control label T-2-C (Total volume 4.0 uL)****

N/A _____ New **0.5 mL microcentrifuge tube**

N/A _____ Labels for new tubes

N/A _____ Add 2.0 uL of **T-3-1** to new tube (leaving 8.0 uL)

N/A _____ Add 2.0 uL of **T-3-2** to same tube (leaving 8.0 uL)

N/A _____ ****After ligation is complete this is the unsubtracted tester control label T-3-C (Total volume 4.0 uL)****

_____ _____ **After ligation, approximately 1/3 (33%) of the cDNA molecules in each unsubtracted tester control tube will have 2 different adaptors**

_____ _____ ****Should have the following tubes and amounts according to this table**:**

Label	**T-2-1**	**T-2-2**	**T-2-C**	**T-3-1**	**T-3-2**	**T-3-C**
uL	8.0	8.0	4.0	8.0	8.0	4.0

_____ _____ Centrifuge briefly (5 seconds) all 9 tubes (Dr. Guenzi's lab)

_____ _____ Incubate in 16°C water bath (Core Facility) overnight (12 h?)

_____ _____ Start water bath @ 72°C (Dr. Guenzi's lab)

_____ _____ Add 1.0 uL **20X EDTA / Glycogen mix** to each tube to stop ligation reaction (provided with kit in clear tube)

_____ _____ Vortex briefly (30 seconds) set @#7 (Dr. Guenzi's lab)

_____ _____ Heat all **tubes** in water bath set @ 72°C earlier for 5 minutes to inactivate the **ligase** (Dr. Guenzi's lab)

_____ _____ Centrifuge briefly (5 seconds) all tubes (Dr. Guenzi's lab)

_____ _____ ****Adapter-Ligated Tester cDNAs (T-2-1, T-2-2, T-3-1, T-3-2) and Unsubtracted Tester Controls (T-2-C, T-3-C) are now complete****

_____ N/A New **1.7 mL microcentrifuge tube**

_____ N/A Labels for new tubes

_____ N/A Add 1.0 uL **T-2-C** to new tube (leaving 4.0 uL)

_____ N/A Add 1000.0 uL **sterile water** (provided with kit)

_____ N/A This tube is for PCR, store @ -20°C until after 2nd hybridization step

N/A _____ New **1.7 mL microcentrifuge tube**

N/A _____ Labels for new tubes

N/A _____ Add 1.0 uL **T-3-C** to new tube (leaving 4.0 uL)

N/A _____ Add 1000.0 uL **sterile water** (provided with kit)

N/A _____ This tube is for PCR, store @ -20°C until after 2nd hybridization step

_____ _____ Store all **tubes** @ -20°C

_____ _____ ****Proceed when ready to Ligation Efficiency Analysis****

APPENDIX VII. Analysis of Adaptor Ligation

Purpose: To determine if at least 25% of the cDNAs have adapters on both ends.

Date:_____

Ctrl 12h Ggt 12h Kit Ctrl

___ ___ ___ ___ ___ ___ Labels of **Ligated cDNA** to be used (should be 9uL per tube in each)

___ ___ ___ ___ ___ ___ 6 new **0.5 mL microcentrifuge tubes**

___ ___ ___ ___ ___ ___ Labels for new tubes

___ ___ ___ ___ ___ ___ Place 200 uL **autoclaved Millipore water** in each tube

___ ___ ___ ___ ___ ___ Place 1 uL of **Ligated cDNA** into appropriate tube (eg. T-1-1 into D-1-1)

___ ___ ___ ___ ___ ___ Total volume should be 201 uL (visual check)

___ ___ ___ ___ ___ ___ 12 new **0.5 mL microcentrifuge tu**bes

___ ___ ___ ___ ___ ___

___ ___ ___ ___ ___ ___ Labels for new tubes

___ ___ ___ ___ ___ ___

___ ___ ___ ___ ___ ___ Add the following **reagents** in order to appropriate tube:

___ ___ ___ ___ ___ ___

Reagent	C1	C2	C3	C4	G1	G2	G3	G4	K1	K2	K3	K4
Diluted Ligated Adapter 1 (D-2-1, D-3-1, D-1-1)	1	1			1	1			1	1		
Diluted Ligated Adapter 2R (D-2-2, D-3-2, D-1-2)			1	1			1	1			1	1
Primer G3PDH 3' (10uM) Clear Tube SSH Kit									1	1	1	1
Primer G3PDH 5' (10uM) Clear Tube SSH Kit										1		1

Primer Actin 3' (2uM)	1	1	1	1	1	1	1	1				
Primer Actin 5' (2uM)		1		1		1		1				
PCR Primer 1 (10uM) Yellow Tube SSH Kit	1		1		1		1		1		1	
Total Volume (uL)	3	3	3	3	3	3	3	3	3	3	3	3

Actin Primer 3' – 21bp – Tm 66.9 – GC 43% From Dr. Anderson

Actin Primer 5' – 33 bp – Tm 83.4 – GC 54% From Dr. Anderson

_____ 1 new **0.5 mL microcentrifuge tube**

_____ Label for new tube

_____ Prepare **master mix** for each reaction (12) plus 1 additional reaction (Total 13) as follows:

Reagent	1 Reaction (uL)	13 Reactions (uL)
Sterile-water Not Autoclaved ONLY Milli-pore	18.5	240.5
10X PCR Reaction Buffer Lt. Blue Tube Advantage Kit	2.5	32.5
dNTP mix (10mM) Pink Tube Advantage Kit	0.5	6.5
50X Advantage cDNA Polymerase Mix Green Tube Advantage Kit	0.5	6.5
Total Volume (uL)	22	286

_____ Vortex **Master Mix** (1 min) set @ #7 Dr. Guenzi's lab

_____ Briefly centrifuge (5 sec) Dr. Guenzi's lab

___ ___ ___ ___ ___ ___ Add 22 uL **Master Mix** to each reaction tube (eg. C1-C4, G1-G4, K1-K4)

___ ___ ___ ___ ___ ___

___ ___ ___ ___ ___ ___ Vortex each tube (1 min) set @ #7 Dr. Guenzi's lab

___ ___ ___ ___ ___ ___

___ ___ ___ ___ ___ ___ Briefly centrifuge (5 sec) Dr. Guenzi's lab

___ ___ ___ ___ ___ ___

___ ___ ___ ___ ___ ___ DO NOT overlay each tube with **mineral oil**. (Use Hot Bonnet, on Thermal Cycler)

___ ___ ___ ___ ___ ___

___ ___ ___ ___ ___ ___ Incubate all 12 tubes in thermal cycler (PT-100) Dr. Guenzi's lab set @ 75°C for 5 minutes to extend adaptors.

___ ___ ___ ___ ___ ___

___ ___ ___ ___ ___ ___ ****DO NOT REMOVE SAMPLE FROM THERMAL CYCLER****

___ ___ ___ ___ ___ ___

___ ___ ___ ___ ___ ___ ****The 75°C incubation "fills in" the missing strand of the adaptors, thus creating binding sites for PCR primers****

___ ___ ___ ___ ___ ___ Immediately commence the following thermal cycler schedule:

___ ___ ___ ___ ___ ___ Program Name on Thermal Cycler = LIGSSH

20 Cycles	Temperature	Time
Step 1	94.0 °C	30 sec
Step 2	65.0 °C	30 sec
Step 3	68.0 °C	2.5 min

___ ___ ___ ___ ___ ___ Analyze 5 uL from each reaction on a **2.0% Agarose/Ethidium Bromide gel** run in **1% TAE buffer** as follows:

Should see a bands identical in intensity of within 25% intensity with each reaction tube (eg. C1 & C2 should be the same or 25 % less of each other same with tube pairs C3 & C4 etc....)

VITA

Timmy Don Samuels

Candidate for the Degree of

Doctor of Philosophy

Thesis: DIFFERENTIAL GENE EXPRESSION IN WHEAT ROOTS IN RESPONSE TO INFECTION BY THE 'TAKE-ALL' FUNGUS (*Gaeumannomyces graminis* var. *tritici*)

Major Field: Plant Science

Biographical:

EDUCATION:
Oklahoma State University, Ph.D. Plant Science 2003
University of Missouri, ABD. Plant Physiology 1999
Midwestern State University, M.S. Biology 1996
Midwestern State University, B.S. Biology (Cum Laude) 1994
Vernon Regional Junior College, A.O.T. Management, 1991
Community College of the Air Force, A.A.S. Pharmacy Technology, 1991
Seminole Junior College, General Studies, 1986-1987

PROFESSIONAL EXPERIENCE:

2000-Present	Doctoral Fellowship, Oklahoma State University, Stillwater, Oklahoma
1996 - 1999	Research Assistant, University of Missouri, Columbia, Missouri
1996	Botany Instructor, Dept. of Biology, Midwestern State University, Wichita Falls, Texas
1994 - 1996	Graduate Assistant, Midwestern State University, Wichita Falls, Texas
1995	General Botany Lab Instructor, Midwestern State University, Wichita Falls, Texas

1994 - 1995	Plant Physiology Research Assistant, Midwestern State University, Wichita Falls, Texas
1992 - 1994	Microbiology / Bacteriology Media Lab Technician, Midwestern State University, Wichita Falls, Texas
1992	Comparative Anatomy of Vertebrates Lab Assistant, Midwestern State University, Wichita Falls, Texas
1987 - 1991	Pharmacy Technician, United States Air Force, Sheppard Air Force Base, Wichita Falls, Texas

ORGANIZATIONS:
Phi Kappa Phi National Honor Society
Alpha Chi National Honor Society
Beta Beta Beta Biology Honor Society
Great Plains Consortium of Plant Biotechnology
American Society of Plant Biologists
American Phytopathological Society
Phytochemical Society of North America
Disabled American Veterans

HONORS AND AWARDS:
Military:
- Air Force Good Conduct Medal (1991)
- Air Force Longevity Service Award Ribbon (1991)
- National Defense Service Medal (1991)
- Air Force Achievement Medal (1990)
- Air Force Organizational Excellence Award (1990)
- Nominated For Airman Of The Quarter (1990)
- Small Arms Expert Marksmanship Ribbon (1987)
- Air Force Training Ribbon (1987)
- Distinguished Graduate Pharmacy Technician School (1987)

College/University:
- USDA Fellowship Oklahoma State University (2000-present)
- Agronomy Tuition Waiver Oklahoma State University (2000-present)
- Deans Tuition Waiver Oklahoma State University (2000-present)
- Early Start Scholarship Oklahoma State University (2000)
- Travel Grant to Pullman Washington for Plant Biochemistry (1999)

Graduate Research Assistantship University of Missouri-Columbia (1996-1999)
American Society of Plant Physiologists Travel Grant to Cocoyo, Mexico (1995)
Who's Who Among Students in American Universities and Colleges (1995)
Top Five Finalist Graduate Student of the Year Midwestern State University (1995)
Graduate Assistantship Midwestern State University (1994 - 1996)
National Science Foundation Scholarship Midwestern State University (1994 - 1996)
Outstanding Biology Student Midwestern State University (1994)
Outstanding Senior Man Midwestern State University (1994)
Honor Roll Midwestern State University (1991 - 1996)
Honor Roll Vernon Regional Junior College (1989 - 1991)
Honor Roll Seminole Junior College (1986 - 1987)

PROFESSIONAL PRESENTATIONS:

Oral Presentation at the 14th Annual Graduate Symposium, Oklahoma State University (2003)

Poster Presentation at the 13th Annual Graduate Symposium, Oklahoma State University (2002)

Keynote Speaker for the National Honors Society Banquet at Stroud High School, Stroud, Oklahoma (2002)

Oral Research Presentation for the Advanced Placement Biology Students from Stroud High School at Oklahoma State University (2002)

Poster Presentation at the 12th Annual Graduate Symposium, Oklahoma State University (2001)

Poster Presentation at the Molecular Plant Biology Conference: Overview of Research Programs in Molecular and Cellular Plant Biology at the Samuel Roberts Noble Foundation, Oklahoma State University and University of Oklahoma, in Oklahoma City (2000)

Oral Research Presentation at the Great Plains Cereals Biotechnology Consortium Research Symposium, in Kansas City, Missouri (2000)

Poster Presentation at the XVI International Botanical Congress. St. Louis, Missouri (1999)

Poster Presentation at the Current Topics in Plant Biochemistry, Physiology and Molecular Biology Symposium on Plant Hormones: Signaling and Gene Expression. University of Missouri-Columbia (1999)

Oral Research Presentation for the Texas Academy of Science (1995 - 1996)

Poster Presentation at the VII National Congress on Plant Biochemistry and Molecular Biology, First Joint Mexico-United States Symposium on Agrobiology, Molecular Physiology and Biotechnology of Crops Important for Mexican Agriculture, in Cocoyo, Mexico (1995)

Poster Presentation at the American Society of Plant Physiologists Convention in Charlotte, North Carolina (1995)

PUBLICATIONS:

Spollen, W., LeNoble, M. E., Samuels, T. D., Bernstein, N., Sharp, R. (2000) Abscisic acid accumulation maintains maize primary root elongation at low water potentials by restricting ethylene production. Plant Physiology (Rockville) 122: 967-976.

Samuels T D. Kucukakyuz K. Rincon Zachary M. (1997) Aluminum partitioning patterns and root growth as related to aluminum sensitivity and aluminum tolerance in wheat. Plant Physiology (Rockville) 113(2): 527-534.