Identification of wheat genes induced by *Puccinia triticina*

by

Kerri Allison Neugebauer

B.S., Kansas State University, 2011

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Department of Plant Pathology
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Abstract

Bread wheat (*Triticum aestivum* L.) is an important staple crop for 35% of the world's population. One economically important pathogen of wheat is *Puccinia triticina*, the causal agent of leaf rust, can cause up to 50% yield loss during epidemics. Despite the lack of an alternate host to complete the sexual stages, *P. triticina* still has variation within the population, which can make achieving durable resistance difficult. This study aims to gain a better understanding of the *P. triticina*-wheat interaction by identifying wheat genes that are induced by individual and multiple races. Six *P. triticina* races were evaluated on a susceptible variety of wheat at six days post inoculation. RNA was sequenced and 63 wheat genes were identified that showed varying expression in response to the six *P. triticina* races. Fifty-four wheat genes were characterized during the first seven days of infection using real-time PCR. Race specific gene expression was found in three wheat genes with race differences on *Lr2A*, *Lr2C*, and *Lr17A*. Wheat genes that had similar expression in response to all six races were also identified. Seven of the characterized genes were then silenced using RNAi hairpin constructs. The transgenic plants were molecularly characterized and inoculated with a virulent *P. triticina* race in the T_2 generation. However, the endogenous genes were not silenced and the transgenic plants maintained susceptibility. A mutation approach was also used to identify wheat genes involved in infection. A mutant population of 3780 wheat plants was created using EMS. Fifteen hundred mutants from the M_1 population were screened for plants with a different infection phenotype compared to the non-mutated control and 570 were selected. After two additional generations of selection, eight resistant mutants were obtained. The gene expression of the seven previously identified genes were evaluated and one mutant showed reduced expression of an ER molecular chaperone gene. This research uses a forward and reverse genetics approach to identify and evaluate the function

of wheat genes in the wheat-*P. triticina* interaction. Although RNAi could not determine the gene function, the knockout mutant shows that the identified genes may have a crucial role in infection.

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Approved by:

Major Professor
Harold N. Trick

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Bread wheat (*Triticum aestivum* L.) is an important staple crop for 35% of the world's population. One economically important pathogen of wheat is *Puccinia triticina*, the causal agent of leaf rust, can cause up to 50% yield loss during epidemics. Despite the lack of an alternate host to complete the sexual stages, *P. triticina* still has variation within the population, which can make achieving durable resistance difficult. This study aims to gain a better understanding of the *P. triticina*-wheat interaction by identifying wheat genes that are induced by individual and multiple races. Six *P. triticina* races were evaluated on a susceptible variety of wheat at six days post inoculation. RNA was sequenced and 63 wheat genes were identified that showed varying expression in response to the six *P. triticina* races. Fifty-four wheat genes were characterized during the first seven days of infection using real-time PCR. Race specific gene expression was found in three wheat genes with race differences on *Lr2A*, *Lr2C*, and *Lr17A*. Wheat genes that had similar expression in response to all six races were also identified. Seven of the characterized genes were then silenced using RNAi hairpin constructs. The transgenic plants were molecularly characterized and inoculated with a virulent *P. triticina* race in the T_2 generation. However, the endogenous genes were not silenced and the transgenic plants maintained susceptibility. A mutation approach was also used to identify wheat genes involved in infection. A mutant population of 3780 wheat plants was created using EMS. Fifteen hundred mutants from the M_1 population were screened for plants with a different infection phenotype compared to the non-mutated control and 570 were selected. After two additional generations of selection, eight resistant mutants were obtained. The gene expression of the seven previously identified genes were evaluated and one mutant showed reduced expression of an ER molecular chaperone gene. This research uses a forward and reverse genetics approach to identify and evaluate the function

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Dedication

To the two strongest women I have had the pleasure of knowing, my twin sister Katie McRell and my former lab-mate Jessica Rupp, whom without their presence in my life this degree would not have been possible. These ladies inspire me on a daily basis to be the best version of myself. I am constantly in awe of their confidence, intelligence, and savvy. They have lovingly guided me through the peaks and valleys of graduate school and I would be utterly lost without their advice and support.

Chapter 1 - Introduction

Wheat originated in the Middle East near the Fertile Crescent region, which consists of the present day regions of the eastern Mediterranean, southeastern Turkey, northern Iraq, western Iran, and northern Iran (Matsuoka, 2011). The progression to modern day hexaploid bread wheat began when the diploid (AA) genomes of *Triticum monococcum* and *T. urartu* diverged less than one million years ago. This led to the first allopolyploidization via hybridization event that occurred from 150,000-500,000 years ago between *T. urartu* and an unknown ancestor of the Poaceae family, most likely belonging to the diploid (SS) lineage of *Aegilops speltoides*. It is thought that this hybridization caused two events that generated the tetraploid species *T. turgidum* (AABB) and *T. timopheevii* (AAGG). The two species were domesticated as agriculture developed in the Middle East (Matsuoka, 2011). During this time, *T. turgidum* was domesticated to *T. dicoccum*, known as emmer wheat, which is thought to be the progenitor of modern durum wheat (Charmet, 2011). The second polyploidization event occurred about 10,000 years ago between the domesticated tetraploid *T. dicoccum* (AABB) and the diploid wild wheat species *A. tauschii* (DD) to result in the hexaploid species *T. aestivum* L. (AABBDD), which is known as bread wheat (Charmet, 2011). However, there is some speculation that the development of *T. aestivum* took multiple hybridization events. *T. aestivum* was first documented in southeastern Turkey 7800-8600 years ago (Matsuoka, 2011).

Bread wheat is an important crop in terms of consumption, production, and the economy. It is a staple food for 35% of the world's population and supplies 20% of the calories consumed worldwide (Scofield et al., 2005). Wheat is grown on the most acreage of any grain and is the third most abundant in grain production worldwide (Statistica, http://www.statista.com/topics/1668/wheat/). The estimated projection for global wheat

production in 2015/2016 is 26.98 billion bushels (USDA, http://www.ers.usda.gov/data-products/wheat-data.aspx - 25184). In 2015, the United States produced over 2 billion bushels of wheat, which contributed to 7.6% of the worldwide total (USDA, http://www.ers.usda.gov/data-products/wheat-data.aspx - 25184). Kansas alone produced 321.9 million bushels of wheat in 2015 and was the second highest state for wheat production behind North Dakota (Statistia, http://www.statista.com/statistics/190376/top-us-states-in-wheat-production/). The United States exported 775 million bushels of the wheat produced in 2015 (USDA, http://www.ers.usda.gov/data-products/wheat-data.aspx - 25184). The total value of the wheat production in the United States was approximately $10.2 billion in 2015, which is the lowest since 2009 after coming off a $17.4 billion high in 2012 (Statistia, http://www.statista.com/statistics/190362/total-us-wheat-production-value-from-2000/).

Because of the nature of agriculture production and the diverse conditions crop plants are grown in, plants have to endure many biotic stresses and abiotic stresses throughout each growing season. With increasing concerns about climate change, biotic stresses are going be important factors to consider for maintaining and increasing wheat production. It has been estimated that up to 50% of the yield losses in major crop plants are caused by environmental stress, while biotic stress accounts for only 10-20% of yield loss (Kamal et al., 2010). Abiotic stresses such as high temperature, drought, high salinity, mineral toxicity, low nutrient availability, and freezing temperatures can reduce grain yield, grain quality, plant survival, and biomass production (Kamal et al., 2010; Grover et al., 2001). Plants have adapted to abiotic stress by inducing stress response genes or expressing regulatory proteins such as phosphatases, protein kinases, and transcription factors that alter the plant to help it survive in harsh conditions (Qureshi et al., 2007; Sornaraj et al., 2016). Transcription factors interact with the promoter

regions of the stress response genes and are essential for inducing abiotic stress signaling pathways controlled by plant hormones such as abscisic acid (ABA), salicylic acid, and jasmonic acid (Sornaraj et al., 2016). The same signaling pathways can be activated in response to several different stresses (Kreps et al., 2002). For example, the jasmonic acid pathway is activated in response to drought, low temperature, and salinity, which all affect the uptake and availability of water (Qureshi et al., 2007; Bohnert and Sheveleva, 1998). In addition, the ABA-dependent pathway can induce stomatal closing due to reduced turgour pressure of the guard cells in response to drought and high salinity (Bohnert and Sheveleva, 1998; Schroeder et al., 2001).

Along with stress response pathways that are induced to a wide range of abiotic stressors, plants also can express genes and other pathways in response to specific stressors. Heat and drought are the two most limiting abiotic factors in wheat production. Drought can affect many plant processes including photosynthesis, protein synthesis, and solute accumulation (Qureshi et al., 2007). Heat stress negatively affects grain quality and overall yield, but extent of the damage depends on the wheat variety (Kamal et al., 2010; Spiertz et al., 2006). Temperatures over 35°C during grain filling can reduce the milling quality of wheat and cause dough weakening (Blumenthal et al., 1993). In response to high temperatures and other stressors, heat shock proteins (HSPs) are commonly expressed. HSPs function as molecular chaperones and assist with protein folding, assembly, stabilization, and degradation (Qureshi et al., 2007). High salinity is another common abiotic stressor for plants. About 20% of land used for cultivation has high salt concentrations (Zhu, 2001). High salinity alters ion concentrations of potassium and calcium, which causes nutrition imbalance, reactive oxygen species (ROS) damage, weakens cell membranes, and alters photosynthesis in the plant (Qureshi et al., 2007; Zhu, 2001). The mechanisms behind salt tolerance in the plant are extremely complex; an example of one

mechanism is the salt-overly sensitive (SOS) pathway (Qureshi et al., 2007). Some of the genes involved in salt tolerance may function to detoxify plants of ROS (Zhu, 2001). Excessive low temperatures also negatively affect crop plants. Cold stress occurs below 10°C while freeze stress occurs at or below 0°C. Freeze stress is often fatal to the plant due to freezing injury of the cell membrane caused by ice formation and dehydration in the plant cells (Qureshi et al., 2007; Thomashow, 2001). Plants can induce factors such as antifreeze proteins (AFPs), osmolyte-producing enzymes, oxidative stress scavenging enzymes, or lipid desaturases to help protect them from low temperature stress (Qureshi et al., 2007). Freezing tolerance is thought to be a multigenic trait. For example, seven sensitivity to freezing genes (*SFR*) and a family of transcription factors, CBF/DREB1, has been identified as essential to cold tolerance in *Arabidopsis* (Thomashow, 2001). Another abiotic stress plants encounter is metal toxicity. Plants react to metal toxicity by inducing general stress proteins, such as ethylene, as well as more specific proteins like peptide metal-binding ligand PC and MT proteins, which aid in the immobilization, exclusion, chelation, and compartmentalization of the metals (Qureshi et al., 2007). Finally, lack of essential mineral macronutrients or micronutrients is a yield-limiting factor in crop production (Shin et al., 2005). Specific plant effects of nutrient stress are unique to the limiting nutrient but are often exhibited as disease-like symptoms (Qureshi et al., 2007). Advances in soil testing and an emphasis on precision agriculture have made it much easier for farmers to accurately manage the levels of macronutrients and micronutrients in their soils.

Besides abiotic stresses, wheat yields are also influenced by biotic stresses such as bacteria, viruses, nematodes, and fungi. The most common bacterial diseases of wheat are bacterial leaf streak and bacterial leaf blight. *Xanthomonas campestris* pv. *translucens* Dye is the causal agent of bacterial leaf streak or stripe (BLS) and is also known as black chaff if the

infection moves to the glumes. Although significant damage rarely occurs, BLS can render wheat spikes sterile if infection is severe. Early symptoms of BLS are seen as small water soaked areas between leaf veins that progress into elongated tan streaks. Lesions can produce exudate in humid conditions that will dry into crusty granules or form a clear film (https://cropwatch.unl.edu/documents/Wheat%20Disease%20Identification.pdf; http://wheat.pw.usda.gov/ggpages/wheatpests.html#bacterialb; Duveiller et al., 1997). *Pseudomonas syringae* pv. *atrofaciens* McCulloch is the causal agent of basal glume rot and bacterial leaf blight. Symptoms begin as small, water soaked lesions that elongate into dark brown streaks as the infection progresses. The tips of the leaves can also become shredded. In humid conditions a light grey exudate can form. While yield losses due to *P. syringae* are minor, it can reduce the grain quality (http://store.msuextension.org/publications/AgandNaturalResources/MT200913AG.pdf). Tillage is the best control method as the disease survives on crop residue and grasses (http://wheat.pw.usda.gov/ggpages/wheatpests.html#bacterialb).

Three of the most common wheat viral diseases that can cause significant yield loss are *Barley yellow dwarf virus*, *Soil borne wheat mosaic virus*, and *Wheat streak mosaic virus*. Aphids vector *Barley yellow dwarf*, so the spread and size of affected areas are dependent on the feeding activity of the aphids. Symptoms vary depending on plant age at the time of infection, but the most common are stunting, poor spike development, and leaf reddening or yellowing that are more prominent at the leaf tip. Average yield loss due to *Barley yellow dwarf* is 20%, but can be much greater in epidemics (http://wheat.pw.usda.gov/ggpages/wheatpests.html#bacterialb). Secondly, *Soil borne wheat mosaic virus* infects winter wheat and is seen as yellow discoloration on the leaves after the wheat breaks dormancy in the spring. Later in the growing season, a dark

green mosaic pattern develops over the yellowing. Protozoa that live in wet soil conditions vector this virus. The third virus discussed is *Wheat streak mosaic virus*, which is vectored by the wheat curl mite and is often seen in co-infections with High plains virus and *Triticum mosaic virus*. Plants infected with wheat streak exhibit a yellow streaking that is most severe on the leaf tip (http://wheat.pw.usda.gov/ggpages/wheatpests.html#bacterialb; https://cropwatch.unl.edu/documents/Wheat%20Disease%20Identification.pdf).

Nematodes can also cause significant damage on wheat. The two species that are most prominent on wheat are cereal cyst nematode (*Heterodera avenae* Woll) and root knot nematode (*Meloidogyne naasi* Frank). The cysts caused by cereal cyst nematode appear on the roots as white nodes that turn dark brown with age and cause root damage that can lead to infection by other soil borne pathogens. The other prominent nematode on wheat, root knot nematode, causes infestations that are observed as formation of galls near the root tips. Above ground, the wheat plants can appear yellow and stunted. Both species of nematodes can cause significant yield loss, but the extent of the damage depends on the population level in the soil (http://wheat.pw.usda.gov/ggpages/wheatpests.html#bacterialb).

A few examples of fungal diseases on wheat that can cause significant yield losses are loose smut, powdery mildew, and Fusarium head blight. Loose smut, caused by *Ustilago tritici* (Pers.) Rostr., is a fungal disease that occurs in all wheat growing areas. Teliospores infect the developing kernel and eventually replace all of the floral parts, except the central stem of the spike, with black spore masses. Yield losses are dependent on severity, but symptoms rarely exceed 30% of spikes in a single location. Secondly, *Erysiphe graminis* f. sp. *tritici* DC. Em. Marchal is the causal agent of powdery mildew. Symptoms are seen as white to grey colored lesions on the leaves and leaf sheaths. As infection progresses, the lesions turn yellow and

develop chlorosis and necrosis and can cause significant yield losses. One of the most damaging fungal diseases on wheat is Fusarium head blight (*Fusarium graminearum* Schwabe). Infection can initially be seen as bleaching of individual spikelets in the head, which leads to shriveled pink or white grain. *F. graminearum* survives in soil and crop residues, so the best control methods are tillage, crop rotation, and genetic resistance (http://www.apsnet.org/edcenter/intropp/lessons/fungi/ascomycetes/Pages/Fusarium.aspx). Fusarium head blight can cause yield losses over 50% and can reduce grain quality due to the production of mycotoxins that can be harmful if ingested by domestic animals or humans (http://wheat.pw.usda.gov/ggpages/wheatpests.html#bacterialb; https://cropwatch.unl.edu/documents/Wheat%20Disease%20Identification.pdf).

One of the most impactful fungal pathogen groups historically and currently on wheat is rusts. Rusts belong to the order Uredinales in the Basidiomycetes phylum (Bolton et al., 2008). The three wheat rusts, yellow rust or stripe rust, caused by *Puccinia striiformis* f. sp. *tritici* West, leaf rust, caused by *Puccinia triticina* Eriks, and stem rust caused by *Puccinia graminis* Pers.:Pers. produce devastating epidemics on wheat and can be found in most wheat growing areas of the world (Khan et al., 2013; Derevnina and Michelmore, 2015). Wheat rusts produce multiple cycles of asexual spores in a single season and can travel long distances, which promotes epidemics if the weather conditions are favorable and susceptible varieties are present (Roelfs et al., 1992; Hulbert and Pumphrey, 2013).

Puccinia triticina Eriks (*P. triticina*) is an obligate biotrophic plant pathogen and the causal agent of leaf or brown rust on wheat. Leaf rust is the most regularly occurring wheat disease and is found wherever wheat is grown (Kolmer, 2013). Although leaf rust doesn't result in dramatic yield losses as often as stem rust or stripe rust have in recent years, leaf rust causes

the greatest annual global yield loss due to both its frequency and widespread occurrence (Huerta-Espino et al., 2011; Khan et al., 2013). Leaf rust affects grain quality by reducing grain weight and decreasing the number of kernels per head (Khan et al., 2013). Individual fields can be destroyed if the disease is severe prior to heading. Yield losses can reach over 50% during epidemics, while average yield losses during endemics are 15-20% (Appel et al., 2011; Huerta-Espino et al., 2011). From 2000 to 2004, yield losses in the United States due to leaf rust were estimated at over 3 million tons, which equates to over $350 million lost (Huerta-Espino et al., 2011). In 2005, worldwide losses due to *P. triticina* infection were estimated to be $2 billion (Scofield et al., 2005). Losses in Kansas were estimated at 13.9% in 2007, 4.7% in 2008, and 1.37% in 2009. The average yield loss due to leaf rust in Kansas over the past 10 years is 2.41% (Appel et al., 2014).

P. triticina is a macrocyclic and heteroecious rust fungi that has a complex life-cycle involving five spore stages and two unrelated hosts (Figure 1.1). The primary host is hexaploid bread wheat (*T. aestivum*) and the alternate host required for sexual recombination is meadow rue (*Thalictrum speciosissimum* L.). The life cycle begins with dikaryotic urediniospore infection on wheat (Figure 1.1E). Urediniospores will re-infect the wheat host throughout the growing season with appropriate moisture and temperature conditions. As the host matures, the urediniospores develop into black-brown colored dikaryotic teliospores (Figure 1.1F). The two haploid nuclei of the teliospores merge in karyogamy to form a diploid nucleus. The diploid nuclei then germinate and undergo meiosis to produce haploid nuclei (Figure 1.1A). Each cell produces a spikey structure called the sterigma, which each haploid nuclei move through to reach the newly formed basidiospore (Figure 1.1B). The nucleus in the basidiospores undergoes mitosis to produce single cell basidiospores with two haploid nuclei. Shortly after being formed,

the basidiospores are forced into the air from the sterigma and carried on wind currents to infect the alternate host, if present. As they infect the alternate host, basidiospores produce flask shaped structures called pycnia, which can be seen as yellow-orange pustules on the leaf surface of meadow rue (Figure 1.1C). Each pycnium structure produces haploid pycniospores and hyphae that function as male or female gametes. It is important to note that pycniospores and hyphae produced from the same pycnium are not sexually compatible, so insect and water movement disperses them. Pycniospores and hyphae of different mating types fuse to become dikaryotic mycelium in a process called plasmogamy. On the underside of the meadow rue leaf, the mycelium accumulates to form aecium (Figure 1.1D). Aecium produce dikaryotic chains of spores called aeciospores. Once aeciospores are mature, they are wind dispersed to the wheat host where they produce urediniospores (Figure 1.1E) (Kolmer, 2013; Bolton et al., 2008).

As previously stated, common hexaploid wheat (*T. aestivum*) is the primary host for teliospore and urediniospore infection. The alternate host, meadow rue (*Thalictrum speciosissimum* L.), is required for *P. triticina* to complete the sexual pycnial and aecial stages. *T. speciosissimum* L. is not native to North America. The *Thalictrum* species that are present in North America are resistant and infection is rarely found, thus *P. triticina* cannot complete the pycnial-aecial sexual stages of its life cycle (Huerta-Espino et al., 2011; Bolton et al., 2008; Kolmer, 2013). Instead, *P. triticina* infection in North America consists of urediniospores that cycle asexually on wheat throughout the growing season. *P. triticina* can over-winter as mycelium or urediniospores on winter wheat or over-summer in the southern U.S. and in Mexico on volunteer wheat to maintain a large population of inoculum which is then blown north through the wheat production areas during the growing season to the Canadian prairies. Infection

can reach Minnesota by mid-June. This yearly movement of urediniospores is known as the "Puccinia Pathway" (Kolmer, 2005; Kolmer, 2013; Bolton et al., 2008).

Leaf rust symptoms are characterized at the uredinial stage on the primary wheat host (Figure 1.2). Uredinia are orange to brown in color and ovoid to round in shape (Bolton et al., 2008). They can be found on the upper and lower surfaces of the leaf blade and leaf sheath but can also infect glumes and awns (Bolton et al., 2008; Marsalis et al., 2006; DeWolf et al., 2010). As the disease progresses, black teliospores may become visible on the underside of the leaf (Marsalis et al., 2006). Susceptible wheat varieties have no chlorosis or necrosis surrounding the pustules, while resistant varieties are characterized by hypersensitive flecks with small or medium size uredinia surrounded by chlorotic or necrotic halos (Bolton et al., 2008).

Because *P. triticina* is an obligate biotroph, it requires a living host to complete its lifecycle and is reliant on its host for structure and nutrients. Thus, the infection process carried out by the fungus is quite complicated. A urediniospore attaches to either side of the wheat leaf, imbibes water, swells, and germinates to produce a germ tube usually within 4-8 hours (Bolton, et al., 2008). The germ tube growth is controlled by a thigmotropic or touch response to the leaf surface and will elongate along the leaf surface until a stomata is reached (Hu et al., 2007; Bolton et al., 2008). The germ tube differentiates into an appressorium over the stomata within 24 hours after infection (Hu et al., 2007; Bolton et al., 2008). The stomata close in the presence of the appressorium and remain closed throughout maturity. The two nuclei from the urediniospore move into the appressorium and undergo mitosis (Bolton et al., 2008). A single infection peg, also known as a substomatal vesicle, from the appressorium enters the substomatal cavity using turgor pressure. The nuclei complete another round of mitosis and travel through the infection peg (Hu et al., 2007). Infection hyphae elongate from the substomatal vesicles, come

into contact with a leaf mesophyl cell, and then form an external haustorial mother cell (Hu and Rijkenberg, 1998). The haustorial mother cell forms 12 to 24 hours after appressorium penetration and adheres to the plant cell wall. A penetration peg forms in the host plasma membrane between the haustorial mother cell and the host cell to develop haustoria (Song et al., 2011, Voegele et al., 2003; Bolton et al., 2008). Haustoria are specialized hyphae that act as feeding cells to absorb host nutrients for pathogen growth and exchange information between the host and fungus to suppress the host defense response (Song et al., 2011; Voegele et al., 2003). The haustoria breach the host cell wall but are not actually intracellular; the haustoria and the host cytoplasm remain separated by the exhaustorial matrix, a carbohydrate rich gel layer that sits between the fungal haustorial wall and the extrahaustorial membrane (Voegele et al., 2003; Bolton et al., 2008; Szabo and Bushnell, 2001). The extrahaustorial membrane is a thickened part of the host plasma membrane that forms closely around the haustoria and serves as the interface between the host and the pathogen where nutrient uptake occurs and compatibility in the host is established and maintained by secreting pathogen effectors (Voegele et al., 2003; Bolton et al., 2008; Jonge et al., 2011).

Pathogen effectors are secreted by the haustoria. Once secreted, the effectors have to travel from the fungal haustorial cytoplasm through the haustorial plasma membrane, the haustorial wall, the extrahaustorial matrix, the extrahaustorial membrane, and finally to the host cytoplasm or host apoplast (Szabo and Bushnell, 2001). Effectors serve the purpose of altering host physiology to benefit the pathogen by suppressing host defenses, modifying host cell structure, and modifying host metabolism to create nutrient sinks (Jonge et al., 2011; Sperschneider et al., 2014; Petre and Kamoun, 2014; Song et al., 2011). Effectors also play a role in host resistance. The interaction between *P. triticina* and wheat follows the gene for gene

theory described by Flor (1955), which states that for each host resistance (R) gene product there is a corresponding avirulence (Avr) gene product in the pathogen (Flor, 1955). In a resistant reaction, a host R protein directly or indirectly recognizes a pathogen Avr gene, most of which encode effector proteins (De Wit et al., 2009). The resistant reaction is known as effector triggered immunity (ETI) and triggers plant defense responses such as hypersensitive cell death (Rafiqi et al., 2012; Mur et al., 2008; De Wit et al., 2009). Directional selection from a resistance gene will select for and identify the virulent pathogen mutants, increase their frequency in the population, and eventually cause the host resistance to break (McDonald and Linde, 2002). Plants then develop new R genes or varieties with new R genes are released to recognize the novel effectors and the co-evolutionary arms race between the host and pathogen continues (De Wit et al., 2009). Pathogen effectors are thought to be the most rapidly evolving genes in pathogen genomes (Sperschneider et al., 2014).

Despite the lack of a sexual cycle, there is still variation within the *P. triticina* population due to a high mutation frequency, host specificity, and adaptation (Liu et al., 2014). Between 50 and 70 *P. triticina* races are identified each year in North America through annual virulence surveys (Kolmer, 2013; Bolton et al., 2008). Races in the leaf rust population can be determined by observing the qualitative infection types as either avirulent or virulent on host differentials (Kolmer, 2013). The most commonly used differentials are a group of Thatcher near isogenic lines each with a single leaf rust resistance gene (*Lr* gene) developed by Dyck and Samborski (Dyck and Samborski, 1968; Bolton et al., 2008). The differentials were grouped into three host sets (Long and Kolmer, 1989) and a fourth set was added later for use in national virulence surveys in the United States and Canada (Kolmer et al., 2007) (Figure 1.3A). A fifth differential set has also been added, but was not used to classify races in this study (Kolmer and Liu, 2000;

Huerta-Espino et al., 2011). Instead of writing out the rust's infection type on each of the resistance genes in the differentials, the race is named using a letter code (Long and Kolmer, 1989) (Figure 1.3B). The naming code is made up of the letters B through T minus the vowels and each letter represents a specific pattern of virulence and avirulence on a differential set. The letters are assembled in a race name by matching the letter pattern to the response on each set of differentials (Long and Kolmer, 1989; Huerta-Espino et al., 2011). Race name length depends on how many sets of differential are used, but four or five letter race names are most common.

Because leaf rust is such a widespread and devastating wheat disease, many control strategies have been developed, such as cultural control methods, fungicide application, and genetic resistance. Each of these control strategies has benefits and negatives. Examples of cultural control strategies are crop rotation and removing volunteer wheat, which is also known as breaking the green bridge. The intent of cultural control is to reduce the amount of inoculum present. Breaking the green bridge can prevent the carryover of spores from one growing season to the next, while crop rotation can reduce the pathogen population, as leaf rust is species specific. However, these efforts do not guarantee protection against leaf rust because urediniospores can be wind-blown long distances from other infected fields (Marsalis and Goldberg, 2006). Planting early maturing winter wheat varieties to avoid spring infection may also reduce severity of infection (Roelfs et al., 1992). Another leaf rust yield loss prevention strategy that is widely used is fungicide application. Majority of the yield losses due to leaf rust are caused by infection of the flag leaf prior to flowering and grain fill. Fungicide application can be used to protect the flag leaf if severe infection of the flag leaf is suspected. Although fungicides are effective, it is not always economically sound for farmers to apply fungicides as they can be quite costly, ranging from $10 to $24/ac. Farmers are likely to profit by applying

fungicides if yields are expected to be greater than 40 bu/ac, if market prices are at least $3.00/bu, and if weather conditions are favorable for severe infection. Timing of application is essential for the fungicides to provide effective control. The best time to apply is between the last leaf emergence and complete head emergence. Triazoles and strobilurins are the two most common active ingredients in fungicides that target leaf rust. Tilt® and Propimax® are examples of fungicides that contain triazoles, while Quadris® and Headline® contain strobilurins. Some products, such as Stratego® and Quilt®, contain both active ingredients (Marsalis and Goldberg, 2006).

Genetic resistance from two classes of resistance genes is the preferred method to prevent yield losses from leaf rust (Bolton et al., 2008). Over 150 genes that provide resistance to the three wheat rusts have been identified and named (Hulbert and Pumphrey, 2013), however only seven genes have been cloned and characterized: *Yr1, Yr10*, *Yr5*, *Sr33*, *Sr35*, *Lr34*, *Lr10*, and *Lr21* (Kolmer, 2013). Majority of the genes belong to a common class of resistance genes known as major genes or race specific resistance genes that encode a nucleotide-binding site leucine-rich repeat regions (NBS-LRR). The proteins encoded by the NBS-LRR gene interact directly or indirectly with the rust effector protein in the host cytoplasm secreted from the rust haustoria to confer effector trigger immunity (ETI) (Hulbert and Pumphrey, 2013). Because the resistance genes only recognize and interact with certain pathogen effectors, they provide race specific resistance. Race specific resistance genes will select for and increase virulent races in the pathogen population. The ETI generated by major gene resistance is typically seen as hypersensitive flecks of necrosis or chlorosis surrounding urediniospores on the wheat leaf (Kolmer, 2013). Examples of leaf rust resistance genes that encode NBS-LRR proteins include *Lr10* (Feuillet et al., 2003), *Lr21* (Huang et al., 2003), and *Lr1* (Cloutier et al., 2007; Kolmer,

2013; Hulbert and Pumphrey, 2013). *Lr10* was derived from common bread wheat, while *Lr21* was identified in *Aegilops tauschii* (Bolton et al., 2008). Although most race specific resistance genes provide resistance from the seedling stage through the adult plant stage, there are other race specific resistance genes, like *Lr12, Lr13, Lr22a,* and *Lr37* that only provide resistance in the adult stages but not in seedling stages of the plant. *Lr12* and *Lr13* were found in bread wheat while *Lr22a* and *Lr37* were derived from *Ae. tauschii* and *Ae. ventricosa* respectively (Kolmer, 2013). *Sr33* and *Sr35* are also major resistance genes that confer resistance to the *P. graminis* race Ug99 (Hulbert and Pumphrey, 2013). *Sr33* was transferred from *Ae. tauschii* into bread wheat. It is also orthologous to *Mla*, a barley (*Hordeum vulgare*) gene that confers resistance to powdery mildew (*Blumeria graminis* f. sp. *hordei*) (Periyannan et al., 2013). *Sr35* was introgressed from *Triticum monococcum* (Saintenac et al., 2013). *Yr10* and *Yr5* are examples of *P. striiformis* f. sp. *tritici* resistance genes. *Yr10* is a major seedling resistance gene identified in the wheat cultivar Moro (Smith et al., 2002) and has been classified as the first full-length NBS-LRR resistance gene cloned in cereals (Frick et al., 1998). *Yr5* is another major seedling expressed resistance gene. It was originally identified in a *Triticum aestivum* subsp. *spelta* var. *album* accession on chromosome 2B (Zhang et al., 2009). *Yr5* functions as an NBS-LRR and was also identified as an orthologue of the rice bacterial blight resistance gene *Xa-1* (Smith et al., 2007).

Another class of resistance genes is adult plant resistance genes, minor genes, non-race specific genes, or quantitative traits (QTL). As opposed to major gene resistance, minor genes condition partial resistance to multiple races, also known as quantitative resistance, and is commonly expressed only in the adult plant stage (Kolmer, 2013). *Lr34* is one of the most characterized adult plant resistance genes (Dyck et al., 1966; Kolmer, 2013). *Lr34* functions as

an ABC transporter and has maintained partial adult plant resistance for over 60 years, but the level of resistance is somewhat dependent on environment and genetic background. *Lr34* is associated with a distinct leaf tip necrosis phenotype and confers a resistance reaction of smaller and fewer uredinia (Krattinger et al., 2009; Kolmer, 2013; Hulbert and Pumphrey, 2013). *Lr34* also provides slow rusting resistance to stripe rust and slow mildewing to powdery mildew as *Yr18* and *Pm38,* respectively (Ayliffe et al., 2008; Singh, 2011). *Lr67* is another example of a minor resistance gene that has been cloned. *Lr67* encodes a hexose transporter and provides partial resistance to all three wheat rusts and powdery mildew. Similar to *Lr34*, *Lr67* provides multi-pathogen resistance and displays a leaf tip necrosis phenotype (Moore et al., 2015). *Lr46* and *Lr68* are other examples of minor resistance genes but they have not been cloned or sequenced (Kolmer, 2013). *Lr46* is located on chromosome 1BL and confers resistance to powdery mildew and stripe rust as *Pm39* and *Yr29* respectively. Like *Lr34* and *Lr67*, *Lr46* is also associated with a leaf tip necrosis phenotype (Singh et al., 2011). *Yr36*, a cloned minor resistance gene, has shown resistance to all *P. striiformis* f. sp. *tritici* races to date in high temperature conditions. The *Yr36* gene encodes proteins with a S/TPK domain and a steroidogenic acute regulatory protein-related lipid transfer (START) domain (Fu et al., 2009; Hulbert and Pumphrey, 2013). *Sr2* is another non-race specific rust resistance gene. *Sr2* is completely linked with a pseudo-black chaff phenotype and a purple-black discoloration on the glume and peduncle that has reduced its popularity with breeders and farmers. There may be multiple loci involved in the *Sr2* complex but it has only recently begun to be studied by quantitative trait loci (QTL) mapping and not much is known about the other genes in the complex. The *Sr2* complex has been durable for over 50 years (Hulbert and Pumphey, 2013; Singh et al., 2011).

Minor gene resistance can be a powerful tool for breeders; minor genes may only show small signs of resistance when deployed alone, but can provide high levels of resistance when used in combination with other minor genes or major genes. Because minor genes are not race specific, they are thought to put less selection pressure on the pathogen and offer more durable resistance than major genes. For example, CIMMYT's bread wheat breeding program has shown that stacking four to five minor genes can provide near immune levels of resistance to all three rusts (Singh et al., 2011; Khan et al., 2013). Even though pyramiding major and minor genes can increase resistance and durability, deployment needs to be managed carefully. If the genes in the pyramid have previously been deployed, sequential selection can occur to overcome all of the genes in the pyramid (Hulbert and Pumphrey, 2013). Although minor genes with large effects have been durable for decades that resistance can be affected by environmental factors such as temperature, humidity, and light intensity. *Lr34* and *Lr46* have shown less resistance under high temperatures (Hulbert and Pumphrey, 2013). Minor genes can also result in undesirable phenotypes, such as the previously mentioned leaf tip necrosis associated with *Lr34* and *Lr67* and the pseudo-black chaff phenotype seen with *Sr2*. These phenotypes could hinder yield potential. Near-isogenic lines with *Lr34* have been shown to yield 5% less than the same lines without *Lr34* in multiple years of yield trials. This could be caused by less photosynthetic area from the leaf tip necrosis phenotype (Hulbert and Pumphrey, 2013).

Breeding for quantitative resistance with minor additive genes is often time consuming and difficult (Singh et al., 2011). Identification of the QTLs associated with quantitative resistance is complicated because they lack typical segregation and the phenotypic effects of a gene associated with a complex trait often result in small additive effects that are hard to phenotype (Asins, 2002; Singh et al., 2014). A single source genotype may not contain enough

minor genes to result in a visible phenotype (Singh et al., 2011). For example, identifying resistant QTLs outside of mapping populations that contain large effect minor genes such as *Lr34* and *Lr46* is difficult because the small additive effects cannot be seen (Singh et al., 2014). Mapping populations are created by crossing a parent that contains the quantitative trait and a parent that does not, then analyzing the segregating progeny to link the QTL to molecular markers. Once identified, the molecular markers can be used in marker-assisted selection (MAS) to indirectly identify the alleles associated with the quantitative trait (Asins, 2002). Markers also make introducing the identified material into related and unrelated species easier, without the drawbacks often associated with the introduction of unadapted material using conventional methods, such as sterility and linkage drag (Asins, 2002; Hulbert and Pumphrey, 2013). QTL regions are often very large, which increases the likelihood that a QTL region is linked to undesirable characteristics (Asins, 2002). However, phenotyping for single minor genes is difficult so identifying tightly linked markers is unlikely and very expensive. Many minor genes do not have markers available (Singh et al., 2011). In addition to QTLs being hard to identify, the population sizes required for selecting and fixing minor genes into elite backgrounds are very large compared to those used to select major genes and are often impractical for most breeding programs (Hulbert and Pumphrey, 2013; Singh et al., 2011).

Aside from conventional breeding for genetic resistance, utilizing transgenic technology to control wheat rusts may be a viable alternative option. Using transgenic technology, one would be able to manipulate single or multiple genes to provide broad-spectrum durable resistance to multiple races or even species of rusts, without a yield penalty, and that will remain effective in multiple environments. The target gene or genes could be either wheat or rust genes, but they should be essential for pathogenicity and therefore would require a large fitness cost for

the pathogen to overcome the resistance (Hulbert and Pumphrey, 2013). The use of transgenics for rust resistance has been demonstrated *in planta* using two distinct delivery systems. Panwar et al. (2013a, 2013b) used *Barley stripe mosaic virus* (BSMV)-induced RNAi host induced gene silencing (HIGS) and a modified *Agrobacterium tumefaciens*-mediated induced transient gene silencing (PITGS) to target three genes in *P. triticina* with predictive functions in pathogenicity, a MAPK, a cyclophilin, and a calcineurin regulatory subunit. Both gene-silencing systems effectively lowered the transcript levels of the targeted genes and resulted in a reduced *P. triticina* infection phenotype. The PITGS plants were also inoculated with *P. graminis* and *P. striiformis* and suppressed infection for both rusts was observed (Panwar et al., 2013a; Panwar et al., 2013b). Yin et al. (2014) also utilized a BSMV mediated HIGS silencing method to demonstrate the pathogenicity of a rust gene. They targeted a *P. graminis* f. sp. *tritici* gene encoding for a tryptophan 2-mono-oxygenase that is expressed in haustoria cells and may be involved in auxin production to result in suppressed infection (Yin et al., 2014).

The transgenic studies mentioned previously and the research being proposed utilizes the gene silencing mechanism of RNA interference (RNAi). RNAi is an ancient antiviral defense mechanism found in eukaryotes that has been developed into a gene silencing mechanism where specific sequences of mRNA are degraded by introducing double stranded RNA into the plant (Ali et al., 2010). RNAi was first observed in petunias in 1990 when Napoli and Jorgensen tried to deepen the purple color of the flower by overexpressing chalcone synthase (CHS), a pigment-producing gene, but the flower they produced was white instead. The expression levels of CHS were 50 fold lower than the wild type and led them to a theory about the introduced gene causing "co-suppression" (Napoli et al., 1990; Sen and Blau, 2006). Further studying this phenomenon, Guo and Kemphues (1995) demonstrated that injecting the sense or antisense of the *par-1* gene

in *Caenorhabdites elegans* resulted in suppression (Guo and Kemphues, 1995; Ali et al., 2010). Finally in a crucial breakthrough, Andrew Fire and Craig Mello proved the elicitor behind the "co-suppression" was double stranded RNA (dsRNA). In 1998, they injected *C. elegans* with a dsRNA mixture containing both the antisense and sense strands of the target gene *unc-22* to obtain a greater suppression than with just a single strand alone (Fire et al., 1998; Ali et al., 2010; Sen and Blau, 2006). Much progress has been made since on understanding the exact process of the gene silencing. RNAi is triggered when dsRNA is recognized as foreign by the host and initiates host defenses to degrade the dsRNA for sequence specific silencing. The dsRNA inserted into the host tissue is the identical sequence of the target gene. The elicitor dsRNA is recognized by the host and cleaved by an enzyme, Dicer, into 21-25 bp segments called small interfering RNAs (siRNAs). Next, the siRNAs are incorporated into the RNA-induced silencing complex (RISC). The double stranded siRNAs are unwound by a helicase and RISC is changed into its active form through an ATP dependent process. The RISC helps guide the single stranded siRNA to find its complimentary mRNA. The complementary mRNA strand is cleaved by an Argonaute protein into 22 nucleotide long fragments and is degraded. The siRNA can be reused to recognize additional sequence specific mRNA (Ali et al., 2010; Lilley et al., 2012).

Leaf rust is an economically important disease that can cause significant yield losses and additional control strategies are needed to reduce the economic loss endured by farmers. Instead of focusing on genetic resistance, this study takes a different approach to leaf rust resistance. This research aims to gain a greater understanding of the dynamics of the wheat and *P. triticina* interaction by identifying wheat genes that are induced by individual races as well as genes that are induced by multiple races. Wheat genes that are induced by individual *P. triticina* races could show that small differences in the pathogen effectors cause changes in host processes during

infection. Alternatively, wheat genes that are induced by many races could be pathogenicity factors, or host gene products that *P. triticina* has to have present in order for infection to occur. Altering these genes in the host by transgenic or mutation techniques could lead to durable resistance that would be challenging for the pathogen to overcome. As a means to test this hypothesis, the objectives of my dissertation are as follows:

- Identify differentially expressed wheat genes induced by six *P. triticina* races using RNAseq
- Characterize host gene expression throughout infection using qPCR
- Determine the effect host genes have on leaf rust infection using transgenic and mutation techniques and;
- Characterize transgenic plants at the molecular level

Figures

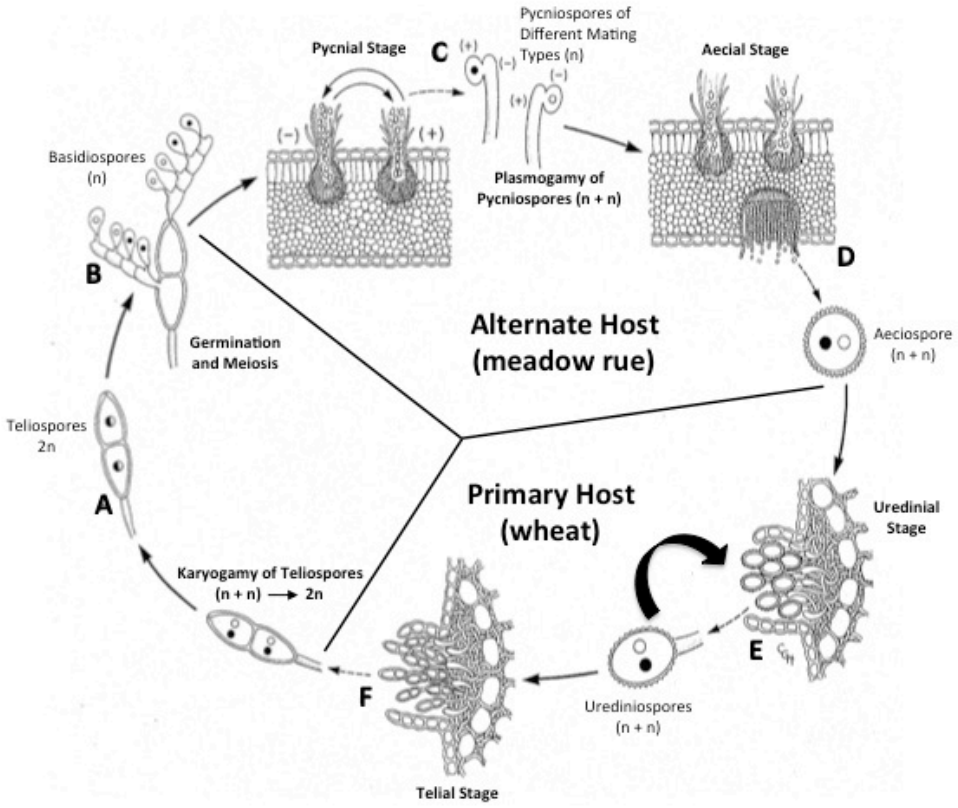


Figure 1.1 Lifecycle of *Puccinia triticina.*

P. triticina is a macrocyclic and heteroecious rust fungi with five spore types and two unrelated hosts. The primary host is wheat, while the secondary host, meadow rue, is required for the completion of the sexual stage of the life cycle. Figure adapted from Alexopoulos, C.J., Mims, C.W., and Blackwell, M.M. (1996) Introductory Mycology, 4th Edition, Wiley and Sons

Figure 1.2 *P. triticina* infection on a susceptible variety of wheat.

Since the alternate host is not present in North America, *P. triticina* infection is seen as urediniospore infection on the primary host, wheat. Uredina are orange to brown in color and can be found on upper and lower wheat leaves. Photograph was taken in March 2016 at CIMMYT in Obregon, Mexico.

A

Host Set 1	Lr1	Lr2a	Lr2c	Lr3
Host Set 2	Lr9	Lr16	Lr24	Lr26
Host Set 3	Lr3ka	Lr11	Lr17	Lr30
Host Set 4	LrB	Lr10	Lr14a	Lr18

B

Code				
B	L	L	L	L
C	L	L	L	H
D	L	L	H	L
F	L	L	H	H
G	L	H	L	L
H	L	H	L	H
J	L	H	H	L
K	L	H	H	H
L	H	L	L	L
M	H	L	L	H
N	H	L	H	L
P	H	L	H	H
Q	H	H	L	L
R	H	H	L	H
S	H	H	H	L
T	H	H	H	H

Figure 1.3 Host differential gene sets and race name code.

To determine the *P. triticina* race name, the infection is determined as high or low on host differentials, each with a single *Lr* gene. A) The sixteen differentials are broken up into four distinct host sets (Long and Kolmer, 1989; Kolmer et al., 2007). B) The quantitative infection on each host set is matched to the letter chart to determine the race name (Long and Kolmer, 1989).

Chapter 2 - Identification of wheat genes with differential expression induced by six *Puccinia triticina* races

Abstract

Puccinia triticina, the casual agent of wheat leaf rust, can cause 15-20% endemic yield losses on up to 90% of the wheat growing area. During fungal infection, the host plant recognizes proteins, secreted effectors, and other molecules, which trigger a host defense response. Changes in the pathogen effectors and strong varietal selection pressure are responsible for the rapid selection of virulent *P.triticina* races. This study aims to identify wheat genes that are differentially expressed in response to different *P. triticina* races. Six *P. triticina* races were evaluated on a single susceptible wheat variety at six days post inoculation. RNA was isolated and Cofactor Genomics preformed RNA sequencing. Based on read count differentials from the RNAseq data, 63 wheat genes were identified that showed differential expression. A time course was then conducted to further evaluate the gene expression. A single susceptible variety was inoculated with the same six *P. triticina* races and RNA was isolated from day zero through day six-post inoculation. Using real time PCR, 54 wheat genes were characterized for gene expression during the first week of infection. Three general wheat gene expression patterns were identified, including wheat genes with expression patterns indicative of pathogenicity factors, gene products that are essential for pathogen infection. In addition, two distinct expression patterns were identified that showed race specific induced wheat gene expression. An ER molecular chaperone gene showed differential expression caused by a lineage shift between *P. triticina* races in two lineage groups. This differential expression may be caused by the presence or absence of the effectors that interact with *Lr17A*. The second race specific gene expression

pattern was a race difference displayed in an alanine glyoxylate aminotransferase gene affected by *Lr2A* and *Lr2C*.

Introduction

Hexaploid bread wheat (*Triticum aestivum* L.) endures yield losses to a wide range of biotic stresses, including many diverse fungal pathogens. One of these fungal pathogens is *Puccinia triticina* Ericks (*P. triticina*), the casual agent of wheat leaf rust or brown rust. Leaf rust is widespread and infects wheat grown all over the world. Yield losses due to leaf rust can be over 50% during epidemics and 15-20% in endemic years (Appel et al., 2011; Huerta-Espino et al., 2011). Annual worldwide losses due to leaf rust have been estimated at $2 billion (Scofield et al., 2005). *P. triticina* has one of the most complex lifecycles of any fungal pathogen with five different spore types and two unrelated hosts. The alternate host, meadow rue, (*Thalictrum speciosissimum* L.) is required for leaf rust to complete the sexual phase of its life cycle. Meadow rue is not native to North America and the species that are present are resistant to leaf rust. Without sexual recombination, leaf rust reproduces asexually and infects as urediniospores on the primary host, wheat (Kolmer, 2013; Huerta-Espino et al., 2011; Bolton et al., 2008). Leaf rust is an obligate biotroph, which means it requires living tissue to complete its lifecycle (Kolmer, 2013). Because of this, wheat and *P. triticina* have a complex and intricate relationship. One aspect of their interaction can be described by the gene-for-gene theory, which states that for every host resistance gene product, there is a corresponding pathogen avirulence gene product (Flor, 1955). Most pathogen avirulence (avr) genes encode pathogen effectors, which are essential for infection and modify the host by suppressing host defenses and altering host metabolism to benefit the pathogen (De Wit et al., 2009; Jonge et al., 2011; Sperschneider et al.,

2014; Petre and Kamoun, 2014; Song et al., 2011). A compatible reaction between the host resistance protein and pathogen avirulence protein will condition resistance in the host. Host selection pressure increases the virulent races within the rust population. Even without sexual recombination, the mutations in *P. triticina* effectors give rise to many races; annual surveys identify between 50 and 70 *P. triticina* races present in North America every year (Huerta-Espino et al., 2011; Kolmer, 2013; Bolton et al., 2008). *P. triticina* races are characterized by typing the infection as high or low on a standard set of isogenic differential lines and are given a four letter name based on the reaction (Kolmer, 2013; Dyck and Samborski, 1968; Bolton et al., 2008; Long and Kolmer, 1989; Kolmer et al., 2007; Huerta-Espino et al., 2011).

There is several control strategies farmers utilize to prevent yield loss due to leaf rust. Cultural methods, such as crop rotation and controlling volunteer wheat, will help reduce the population of spores that can carryover from one season to the next. However, cultural control methods do not eliminate the threat of leaf rust, as spores are easily windblown long distances from other infected fields. Fungicides are another mechanism to prevent yield loss. Fungicides can be applied if there is potential for severe infection on the flag leaf that could lead to high yield loss. But fungicides are costly to apply and are not always an economically sound decision for the farmer (Marsalis and Goldberg, 2006). Genetic resistance is the most commonly utilized yield loss prevention strategy for leaf rust (Bolton et al., 2008). Genetic resistance can be grouped as either major gene resistance or minor gene resistance. Most major genes provide high levels of race specific resistance and encode nucleotide-binding site leucine-rich repeat regions (NBS-LRR) that interact directly with the pathogen effector protein (Hulbert and Pumphrey, 2014). Because of its race specific mechanism, major gene resistance selects for virulent races in the population, which makes durable resistance hard to obtain (Kolmer, 2013; Huerta-Espino et

al., 2011). Single major resistance genes can break 4-5 years after release (Khan et al., 2013). Minor genes condition partial resistance to multiple *P. triticina* races and can be stacked with other minor or major genes to increase the level of resistance. Unlike major genes that mostly encode NBS-LRR, minor genes encode proteins that have a wide range of functions. Although minor genes do not provide high levels of resistance like major genes, they put less selection pressure on the host and are typically more durable (Kolmer, 2013; Hulbert and Pumphrey, 2014; Khan et al., 2013). For example, *Lr34* has provided partial adult plant resistance for over 60 years (Krattinger et al., 2009; Kolmer, 2013). However, minor genes have many challenges in application. Many minor genes, such as *Lr34, Sr2,* and *Lr46*, are tightly linked with undesirable phenotypes, such as leaf tip necrosis or pseudo-black chaff and have also been associated with yield drag (Krattinger et al., 2009; Kolmer, 2013; Hulbert and Pumphrey, 2014; Singh et al., 2011). In addition, breeding with minor genes can be very challenging and time consuming due to a lack of molecular markers. Transferring these genes into elite backgrounds can also be hindered by sterility and the requirement of large population sizes (Hulbert and Pumphrey, 2014; Singh et al., 2011). Instead of relying solely on major and minor genes for resistance, an alternative is to use transgenic technologies to target genes involved in the complex interaction between *P. triticina* and wheat. This study aims to gain a greater understanding of the *P. triticina* and wheat interaction by identifying and characterizing wheat genes induced by *P. triticina* infection that could be potential targets for transgenics.

To identify wheat genes involved in *P. triticina* infection, a susceptible variety of wheat was inoculated with six *P. triticina* races. RNAseq was conducted at six days post inoculation and 63 wheat genes with differential expression in response to the six races were found. Out of the 63 genes identified with RNAseq, 54 genes were characterized with real-time PCR using a

time course. Six of the thirteen total genes that were not characterized did not have primers designed because they had retrotransposons as proposed functions, while the remaining seven genes had inefficient primers that would not yield reliable data. The time course was conducted from zero days through six days post inoculation of the same six races on a susceptible wheat variety. The expression data identified three genes with race specific gene expression and a group of sixteen genes with similar expression in response to all six races, which may be pathogenicity factors. Silencing pathogenicity factors would condition durable resistance because it requires the pathogen to alter its lifecycle. The race specific gene expression was caused by a *P. triticina* lineage shift and a difference in two groups of *P. triticina* races. The differential expression is most likely caused by the presence or absence of specific effectors that interact with host resistance genes. The wheat genes identified had a wide range of proposed functions including metabolism, photosynthesis, plant defense, and protein transport. This study successfully identified wheat genes that are induced during compatible *P. triticina* infection.

Materials and Methods

Selection of wheat cultivar and *P. triticina* races

The hard red spring wheat cultivar Thatcher (University of Minnesota, 1936) was used as an infective host for the fungus. Six races of *P. triticina* were chosen for the study: MHDS, MLDS, MJBJ, TDBG, THBJ, and TNRJ (Table 2.1). The races used are virulent on Thatcher, which only contains *Lr22A,* and have a 3+ infection type using a scale developed by Stakman et al. (1962) and Gassner and Straib (1932) and modified by McIntosh et al. (1995) (Kolmer, 2009; Stakman et al., 1962; Gassner and Straib, 1932; McIntosh et al., 1995). All six races are

commonly found in the United States. Races were obtained from Jim Kolmer of the USDA-ARS at the Cereal Disease Laboratory (Minneapolis, Minnesota).

Seedling growth, inoculation, and tissue collection

Thatcher seedlings were grown in square pans (7.5 cm^2) containing Metro Mix 360 soil medium (SunGro, Vancouver, Canada) and maintained in a Percival Intellus growth chamber at 18 °C with 16 hour day and 8 hour night cycles. Seedlings were inoculated at the two to three leaf stage. The *P. triticina* uredinospores were stored at -80 °C. For inoculation, the spores were heat shocked at 42°C for 20 minutes and 5 mg spores were suspended per ml Soltrol 170 isoparaffin solvent (Philips 66, Bartlesville, OK) and sprayed onto the plants using an atomizer and an air compressor at 40 PSI. Plants were incubated in a dark 100% humidity Percival Intellus dew chamber overnight for 16 hours at 18 °C, then returned to a growth chamber set to the conditions described above. Leaf tissue samples were collected for RNA isolation by pooling 15 plants inoculated with one of the six races, and for each subsequent race, at six days post inoculation, flash freezing in liquid nitrogen, and storing at -80 °C. Only leaf tissue with heavy symptoms of infection was collected. A Soltrol isoparaffin solvent inoculated control and a non-inoculated control were also obtained. Total RNA was isolated by randomly selecting five to six one-inch leaf pieces from each pooled sample and processed using the *mir*Vana miRNA isolation kit (AM1560, RNA Life Technologies, Carlsbad, CA) according to the manufacturer's protocol and following the recommendation to remove the miRNA enrichment step. A Nanodrop ND1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) was used to quantify the RNA.

RNA Sequencing

Total RNA was sent to Cofactor Genomics (St. Louis, MO) for RNAseq analysis, assembly, and primary analysis. The RNA was sequenced by in house protocols that were

summarized by Bruce et al. (2014) and modified as stated below. Large and small ribosomal subunit RNA (rRNA) was removed from the total RNA using the RiboMinus Eukaryote Kit (Invitrogen, Carlsbad, CA) to obtain whole transcriptome RNA. rRNA-specific biotin labeled probe was hybridized to 5 μg of total RNA at 70°C for five minutes and then removed using streptavidin-coated magnetic beads. The rRNA-free transcriptome was concentrated by ethanol precipitation. The double-stranded cDNA was treated with a mixture of T4 DNA polymerase, Klenow large fragment, and T4 polynucleotide kinases to form blunt-ended DNA. A single 'A' base was added to the 3' end of the blunt-ended DNA using Klenow fragment (3' to 5' exo-) and dATP. Paired end adaptors were ligated to the A-tailed DNA using T4-DNA ligase from the Illumina RNA-seq kit (Illumina, San Diego, CA). The adaptor-ligated cDNA underwent size selection by cutting the desired fragment from a 4-12% acrylamide gel. In-gel PCR and the Phusion High-Fidelity system (New England Biolabs, Ipswitch, MA) was used to obtain the amplified cDNA library with ideal fragment size. The amplified DNA was sequenced with the Illumina RNAseq protocol (Illumina).

cDNA Synthesis and Illumina Sequencing

Fragmentation of one ug rRNA depleted RNA occurred using the fragmentation buffer included in the Illumina RNA-seq kit (Illumina) and the resulting fragmented RNA was purified using ethanol precipitation. Fragmented RNA was primed with random hexamers then reverse transcription with Superscript II (Invitrogen) to obtain first strand cDNA. The first strand cDNA was incubated on ice for five minutes with second strand buffer, RNase Out, and dNTP from the Illuminia RNA-seq kit to acquire second strand cDNA. DNA Pol I and RNaseH was added to the reaction mix and incubated at 16 °C for 2.5 hours (Invitrogen).

Data Processing and Assembly

Reads were kept for assembly that had 80% of bases with quality scores above 20 (fastq_quality_trim –q 20 – t 30) and were longer than 30% (fastq_quality_filter –p 20 –q 80). The tools used were procured from the FASTX-Toolkit (http://hannonlab.cshl.edu/fastxtoolkit/). Reads were assembled into transcripts using the de Brujin graph-based assembler, Trinity v2011059 (Grabherr et al., 2011). Assembled transcripts were aligned against a *Triticum aestivum* L. expressed sequence tag (EST) resource using Novocraft novoalign v2.06.09 software package (www.novocraft.com/products/novoalign/) and kept in separate FASTA files. The predicted contigs were assembled in "ActiveSite", an interactive web based analysis software developed by Cofactor, which was then used to sort the assembled contigs based on read counts. Contigs with greater than two fold expression ratios were selected for expression analysis.

Expression Profiling, cDNA Synthesis, and qRT-PCR Analysis

Wheat seedlings were inoculated with six races as described above. Leaf tissue samples for real time PCR analysis was collected in a time course. Leaf tissue from 15 plants inoculated with one race was collected at each time point: 0, 1, 2, 3, 4, 5, and 6 DPI. The leaf tissue was pooled at collection and then separated into three 50 ml conical tubes. Only leaf tissue with heavy symptoms of infection was collected. The conical tubes were flash frozen in liquid nitrogen and stored at -80 °C. The tissue collection process was the same for each of the six races. A Soltrol isoparaffin solvent inoculated control and a non-inoculated control were also obtained. Total RNA was isolated by randomly selecting five to six one-inch leaf pieces from each pooled sample and processed using the *mir*Vana miRNA isolation kit (AM1560, RNA Life Technologies, Carlsbad, CA) according to the provided protocol and following the recommendation to remove the miRNA enrichment step. A Nanodrop ND1000

spectrophotometer (Thermo Fisher Scientific, Waltham, MA) was used to quantify the RNA. First strand cDNA was acquired by priming one µg total RNA with random hexamers and then reverse transcription with Superscript II (Invitrogen) according to the manufacturer's recommendations. qRT-PCR primers were designed from the assembled contigs. All primers were evaluated for dimer formation and efficiency before being used for expression data collection. The qRT-PCR conditions were as follows: 95 ℃ for 3 minutes, 40 cycles of 95 ℃ for 10 seconds, and 62 ℃ for 30 seconds. The run was completed with a melt curve: 65 ℃ to 95 ℃ heating in 0.5 ℃ increments for 5 seconds. All reactions used the Bio-Rad CFX96 Real-Time System and the Bio-Rad iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., La Jolla, CA) in a 25 µL reaction which contained 6 ul cDNA template (diluted in a 2:1 ratio), and 10pmol of each primer. Three technical replicates were obtained for all reactions. Relative expression was calculated using the ΔΔCT method (Livak and Schmittgen, 2001). The cycle threshold (CT) values were averaged and compared to the CT values of the Soltrol isoparaffin solvent inoculated control. The resulting CT value was subtracted from the CT value of the internal wheat ubiquitin (UBQ) housekeeping gene (Paolacci et al., 2009). For example: $\Delta CT_{GOI} = CT_{oil} - CT_{treatment}$. $\Delta CT_{UBQ} = CT_{oil} - CT_{treatment}$. $\Delta\Delta CT = \Delta CT_{GOI} - \Delta CT_{UBQ}$. Three biological replicates were completed for the eight candidate genes discussed in depth and two biological replicates were completed for ten candidate genes. The remaining candidate genes were only evaluated with one biological replicate.

Results

The six *P. triticina* races, MHDS, MLDS, MJBJ, THBJ, TDBG, and TNRJ, used in this study are all commonly found in the North America. No symptoms were seen on Thatcher from zero days to two days post inoculation (DPI). At three DPI, slight flecking and yellowing could

be seen on the inoculated leaves. By six DPI, more distinct flecking and yellowing was seen. Spores were present, but could not be seen on the leaf surface yet. No difference was observed in severity of infection between these races (Figure 2.1).

Thatcher was inoculated with the six *P. triticina* races stated above and tissue was collected at six DPI for RNA isolation. The RNA was sent to Cofactor Genomics for RNA sequencing. The RNAseq data generated 164,753,758 raw reads, ranging from 23,415,788 to 33,225,893 per race in the primary analysis (Table 2.2). The sequenced transcriptomes were separated into wheat associated and leaf rust associated files by aligning the assembled mRNA fragments to the leaf rust draft genome V2 of Race1 (pathotype BBBD, http://www.broadinstitute.org/scientific-community/data) and to a reference TIGR wheat EST database (available at http://www.jcvi.org). The wheat-associated file contained about 11 million to 18 million reads per race (Table 2.3). The read count data from the RNAseq analysis was presented using "ActiveSite", a web based interface that aided in sorting the RNAseq data by using a Boolean matrix of ones and zeros to select wheat gene candidates that had greater than two-fold differential in read counts in the presence of specific races. The total read counts for each mRNA fragment was required to be above 150 for selection. A total of 63 mRNA fragments were selected that met the above requirements and were thought to have differential expression induced by different *P. triticina* races (Table 2.4).

In order to further evaluate the gene expression for the 63 mRNA fragments selected from the RNAseq data in response to six *P. triticina* races, a time course was conducted during the first week of infection and gene expression was evaluated using real time PCR. Primers for real time PCR (Table 2.6) were designed from the sequences of the 63 mRNA fragments using MacVector 12.7.3 and Integrated DNA Technologies Primer Quest tool using the following

parameters: optimal Tm 58 °C, GC content 50%, primer size ranged from 18 to 24 nt, and amplicon size from 100 to 250 bp. Out of the 258 primers designed, only 69 primers gave usable data due to dimer formation and poor primer efficiency when tested on a dilution series cDNA template. The total primer number includes primers designed and used for the control gene as well as the PR genes. The ΔΔCt method was used to calculate the relative expression values for each gene and statistical analysis was performed using SAS version 9.2 (SAS Institute, Cary, NC).

In addition to the selected mRNA fragments, the expression of three pathogen response proteins, PR-1, PR-2, and PR-5, were also evaluated in this study. The purpose of characterizing already well known genes was to ensure that real time PCR could effectively detect changes in gene expression in response to a pathogen. Expression for all three PR genes was initially very low and increased rapidly until three days post inoculation. The PR gene expression then decreased sharply at four days post inoculation and increased slowly through five and six days post inoculation (Figure 2.2).

Out of 63 differentially expressed mRNA fragments identified using RNAseq, 54 were characterized using real time PCR (Table 2.5). The 54 characterized mRNA fragments were separated into seven groups based on proposed function from BLAST. The first group of wheat genes are involved in energy and metabolism. There were nine genes within this group, equating 16.67% of the total genes, including ribulose biphosphate carboxylase, chlorophyll a-b binding protein, light regulated protein, and photosystem II reaction center protein Z. The second group of genes had proposed functions in membrane function and protein transport. Eleven genes, or 20.37%, were in this group including ER molecular chaperone, alanine-glyoxylate aminotransferase, luminal binding protein, glutathione-S transferase, type 1 non-specific lipid

transfer protein precursor, multiprotein bridging factor, and cytosolic malate dehydrogenase. The third group of genes may encode stress-related proteins. Five genes, or 9.26%, were proposed to have a stress related function including universal stress protein A, PR1, heat shock protein HSP70, cysteine proteinase inhibitor WC-1, and brown planthopper susceptibility protein. The fourth group of genes was comprised of nineteen RNA binding proteins, or 35.20% of the total genes identified. There were two groups of RNA binding proteins that the genes belonged to, low temperature responsive RNA binding proteins or glycine rich RNA binding proteins. The fifth group consisted of six genes, or 11.11%, that did not have a previously identified function. The sixth group had three genes, or 5.56%, with proposed function in secondary metabolism including glutamine dependent asparagine synthetase, hydroxyhenylpyruvate dioxygenase, and ananain cysteine-type peptidase. Finally, the last group consisted of one gene that may encode an mRNA turnover 4-like protein homolog.

The expression of the 54 wheat genes found using real time PCR were grouped into three general gene expression patterns by visually assessing expression similarities and grouping those that had similar qualities. In the first pattern, the host gene responds to all six races similarly but the gene expression changed throughout infection (Figure 2.3A). F-tests for the shown ER molecular chaperone-1 showed no significance for race but were significant for day. For example, the relative expression of ER molecular chaperone-1 increased and decreased four times every other day in response to all six *P. triticina* races during the first week of infection. Genes that followed this pattern could be pathogenicity factors because the changes in gene expression are not race specific and the expression changes drastically throughout the time course in the presence of the pathogen. Sixteen genes were found to exhibit this expression pattern including five RNA binding proteins, four ER molecular chaperones, two photosystem II

reaction center proteins, one multiprotein bridging factor, one glutathione-S-transferase, two unknowns, and one universal stress protein.

The second gene expression pattern observed occurs when the host gene has different expression in the presence of each *P. triticina* race (Figure 2.3B). It appears each wheat gene in this pattern are responding to each race specifically and while there are distinct trends, it is challenging to identify a clear pattern. F-tests conducted for the glutamine dependent asparagine synthetase showed significance for race and day, which shows that the gene is being up regulated by multiple races. For instance, the expression of the glutamine dependent asparagine synthetase increases until three days post inoculation then decreases through six days post inoculation. However, the gene expression in response to each race is varied. For example, from five DPI to six DPI, the gene expression in response to MJBJ, TDBG, MLDS, and THBJ increased two to four-fold, TDBG induced a two-fold reduction in gene expression, and TNRJ induced very little change in gene expression. Thirty genes fit this expression pattern including seven RNA binding proteins, two protein transfer, one type one non-specific lipid transfer protein, one alanine-glyoxylate aminotransferase, four unknowns, three RuBisCo, two brown planthopper susceptibility proteins, one phosphoglycerate kinase, one cysteine proteinase inhibitor, two cytosolic malate dehydrogenase, one heat shock protein, two light regulated proteins, two chlorophyll a-b binding proteins, one universal stress protein, and one glutamine dependent asparagine synthetase.

The third gene expression pattern found was characterized by low host gene expression in response to infection by all six races (Figure 2.3C). F-tests conducted for the RNA binding protein was not significant for day, but showed significance for race. For example, the RNA binding protein is not expressing at zero DPI and the relative expression level only fluctuates

slightly each day until six DPI. In addition, each *P. triticina* race induced a similar level of gene expression in this low expressing pattern. This group of genes may have been misidentified by the RNAseq data that was supposed to identify genes with high expression and differential expression in response to the six *P. triticina* races. Seventeen genes exhibited this expression pattern including ten RNA binding proteins, one unknown, one protein transfer, one light regulated protein, one ananain cysteine-type protease, one RuBisCo, one ER molecular chaperone, and one mRNA turnover protein.

Two unique expression patterns were also found. The first unique pattern is a lineage shift identified by host genes with differential expression induced by races from different lineages (Figure 2.4A). Fifty *P. triticina* races found in North America have been grouped into lineages based on evaluation of single nucleotide polymorphism (SNP) data (Kolmer and Fellers, unpublished). Two of the races used (MHDS and MLDS) are a part of the North America 3 lineage (NA3), while the other four (MJBJ, THBJ, TDBG, and TNRJ) were categorized into the North America 5 lineage (NA5). The ER molecular chaperone-2 gene had two and a half times higher expression in response to races in NA3 from two through five DPI than gene expression levels in response to races in NA5. NA3 and NA5 formed separate lineages as a result of the introduction of the wheat cultivar Jagger (Kansas State University, 1994), which contains the resistance gene *Lr17A*. The differential expression could be caused by the presence or absence of the pathogen effector that interacts with *Lr17A*. However, the lineage shift was not found to be statistically significant. An RNA binding protein was also identified that had differential expression in response to the lineage shift. A wheat gene with a putative function of an alanine glyoxylate aminotransferase exhibited the second unique expression pattern, which is a race difference between the three “M” races (MHDS, MLDS, MJBJ) and the three “T” (THBJ,

TDBG, and TNRJ) races (Figure 2.4B). This gene showed about one and a half times higher expression in response to "T" races than to "M" races three DPI through six DPI. Interestingly, the alanine glyoxylate aminotransferase was not even expressing in response to "M" races 4-5 DPI. Based on qualitative infection types on wheat differentials, "T" races are virulent on *LR2A* and *LR2C* while "M" races are avirulent. Most likely, the wheat gene is responding to the presence or absence of the effector or effectors that interacts with *LR2A* and *LR2C*. The expression differences induced by the differences in races were found to be statistically significant.

Discussion

Leaf rust is an obligate biotrophic fungal pathogen that has the potential to cause significant yield losses in wheat all over the world (Kolmer, 2013; Scofield et al., 2005; Huerta-Espino et al., 2011). Since leaf rust is constantly evolving due to high mutation rates from large amounts of wind-blown spores, achieving durable resistance is quite challenging (Liu, et al., 2014; Bolton et al., 2008). Genetic resistance is the most common method used to prevent yield losses to leaf rust (Bolton et al., 2008). Although major gene resistance can provide high levels of control, its effectiveness can erode in as little as three to four years after being deployed in the field (Khan et al., 2013). Minor gene resistance has become the focus of many breeding programs and can provide more durable resistance than major genes (Singh et al., 2011). However, minor genes can be costly and time consuming to integrate into adapted cultivars (Hulbert and Pumphrey, 2013). Another approach to durable resistance of leaf rust is to disrupt critical portions of the wheat and *P. triticina* interaction using transgenic methods. For that to be a possibility, more about this complex interaction needs to be understood. This study aimed to

identify wheat genes whose expression was induced by specific *P. triticina* races and multiple races using RNAseq and real time PCR. RNAseq conducted at six DPI identified 63 genes with differential expression induced by six *P. triticina* races. Real time PCR was used to further evaluate the expression of these genes to the same six races during the first week of infection. Sixteen genes had similar expression in response to all six races and may be pathogenicity factors. Three wheat genes that had race specific expression were also identified.

Before characterizing mRNA fragments that were differentially expressed during *P. triticina* infection, the expression of three pathogenesis-related proteins (PR), PR-1, PR-2, and PR-5, were evaluated in response to *P. triticina* infection. PR proteins are induced in response to a wide variety of pathogens and are also involved in plant development. Specifically, PR-1, PR-2, and PR-5 have been shown to inhibit growth of a variety of fungi (Muthukrishnan et al., 2001). There are seventeen classes of PR proteins identified to date and each class has a specific function (Naz et al., 2014). For example, PR-2 proteins have a β-1,3- glucanase activity (Kauffmann et al., 1987) and are induced in the presence of fungi that contain β-1,3-glucans in their cell walls (Muthukrishnan et al., 2001). PR-5 functions as a thaumatin-like protein (Carr and Klessig, 1989; Hejgaard et al., 1991; Naz et al., 2014). The specific function of PR-1 is still unknown (Breen et al., 2016). The expression patterns of the PR genes showed the typical response of a plant defense gene to pathogen infection (Figure 2.2). PR-1 and PR-5 had similar expression patterns; the genes were not expressing at zero DPI, increased steadily until three DPI, decreased about four-fold at four DPI, and increased again at five-six DPI. This expression pattern was similar in response to all six races. From zero DPI through three DPI, the pathogen is establishing itself within the host. It is during this time that the appressorium is formed, enters into the host using a penetration peg, and form haustoria by three DPI. The haustoria serve as a

specialized feeding cell for the pathogen and also secrete pathogen effectors. The initial detection of the pathogen would induce plant defenses, seen as the PR-1, PR-5, and PR-2 expression increase from zero DPI thorough three DPI. The increase in expression at five-six DPI may be in response to secondary *P. triticina* infection. At this time, the pathogen is spreading through the host using hyphae and establishing more haustoria. Urediniospore formation is also occurring. The gene expression of PR-2 had the same general trend as PR-2 and PR-5, but "M" and "T" races induced differential expression at four-six DPI. It is possible that the PR-2 differential expression is due to the presence or absence of the effectors that interact with *Lr2A* and *Lr2C*.

Genes that encoded low temperature responsive and glycine rich RNA binding proteins made up 35% of the total genes putatively identified and had a range of gene expression patterns. All of the mRNA sequences from the RNAseq analysis that were putatively identified as RNA binding proteins aligned to different segments of one RNA binding protein (AGI04359.1) according to NCBI BLAST. It seems peculiar that only one gene that codes for a RNA binding protein was identified. This could imply that only a specific class of RNA binding proteins are involved in the wheat-*P. triticina* system. However, a more likely possibility that could cause this is that an incomplete wheat genome was used for alignment in the RNAseq analysis so there were a limited number of genes available to align to. RNA-binding proteins (RBP) are a group of regulatory factors that interact with the binding domains of single-stranded or double-stranded RNA throughout all post-transcriptional processes including: mRNA splicing, polyadenlyation, sequence editing, transport, mRNA stability, mRNA localization, RNA export, chromatin modification, and translation (Silverman et al., 2013; Xuan et al., 2010; Lorkovic et al., 2009). These interactions are thought to be essential for functionality, processing, and regulation of RNA that aid in plant responses to changes in environmental conditions, flower development,

floral patterning, abscisic acid signaling, circadian rhythms, and chromatin modification (Ambrosone et al., 2012; Lorkovic et al., 2009; Naqvi et al., 1998). In addition, stress activated RNA binding proteins may function as molecular chaperones and assist in the translation of stress-associated genes to help plants recover from cellular stress injuries (Ambrosone et al., 2012; Silverman et al., 2013). This has been shown in several plant species. *Arabidopsis* RBPs, *AtGRP2* and *AtGRP7*, increase seed germination rates, seedling growth, and stress tolerance of *Arabidopsis* in cold stress conditions (Silverman et al., 2013; Xuan et al., 2010). Rice RNA binding proteins, *OsGRP1*, *OsGRP4*, and *OsGRP6,* aid in seed germination and seedling growth as well stress tolerance in high temperature conditions (Xu et al., 2013; Kwak et al., 2013; Sahi et al., 2007). Tobacco RBP *NtGRP1* was up regulated during abiotic stresses such as flooding, while *NtGRP1a* and *NtGRP3* expression was induced by cold or osmotic stresses (Kwak et al., 2013). In addition, *LgGRP1* in perennial ryegrass has been shown to play a role in cold adaptation (Xuan et al., 2010).

RNA binding proteins have also been reported to be involved in plant pathogen interactions and may help regulate the plant defense system (Naqvi et al., 1998; Silverman et al., 2013). The *Pseudomonas syringae* effector protein, *HopU1*, modified *Arabidopsis* RNA-binding proteins during infection. As a result, the RBPs had a reduced ability to bind and regulate their target RNAs, which caused increased susceptibility (Silverman et al., 2013). In barley, two glycine rich RNA binding proteins, *Hvgrp2* and *Hvgrp3*, displayed increased mRNA levels in the presence of fungal pathogens *Erysiphe graminis* and *Rhynchosporium secalis* in incompatible and compatible interactions (Molina et al., 1997). In addition, the expression of the tobacco glycine rich RNA binding protein *ngRBP* was induced twenty-four hours post *Tobacco mosaic virus* infection (Naqvi et al., 1998).

Endoplasmic reticulum (ER) molecular chaperones were the proposed function of five of the characterized mRNA fragments. The sequences of the five mRNA fragments aligned to different segments of the same ER molecular chaperone (AGN94841.1), similar to the issue displayed with the RNA binding protein. There were two ER molecular chaperones with particularly interesting gene expression determined by real time PCR. The expression level of ER molecular chaperone-1 in response to all six races was the same, but the expression changed drastically every other day (Figure 2.3A). It is thought that the expression of this gene is being influenced by the pathogen during the first week of infection and therefore may be essential for successful infection of *P. triticina*. In contrast, the expression of ER molecular chaperone-2 was race specific and thought to be dependent on the presence or absence of the effectors associated with *Lr17A* (Figure 2.4A). The mechanism of ER molecular chaperones may play a role in their importance in the *P. triticina* wheat interaction. ER molecular chaperones are involved in the ensuring proper quality of proteins. Almost all secreted proteins enter the endoplasmic reticulum (ER) during or right after synthesis. When the proteins enter the ER, the ER molecular chaperones recognize mis-folded or unstable proteins and aid in correcting their orientation upon exit of the ER. Correct protein folding and maturation in the ER is essential for protein transport in the secretory pathway (Nishikawa et al., 2005). ER molecular proteins can be induced during cell stress to refold non-native proteins and are also important in regulating ER-associated degradation (Goeckeler et al., 2010).

An alanine-glyoxylate aminotransferase (EMT26999.1) was identified to have differential gene expression using RNAseq and its gene expression was further characterized using real time PCR. Real time PCR showed differential race specific expression of the alanine-glyoxylate aminotransferase during the first week of infection that may be caused by the

presence or absence of the pathogen effectors that interact with *Lr2A* and *Lr2C* (Figure 2.4B). Alanine aminotransferase (AlaAT) belongs to a pyridoxal phosphate multigene family and functions in animals, plants, yeast, and bacteria. AlaAT is an enzyme that catalyzes the transfer of an amino group from glutamate to pyruvate to form 2-oxoglutarate and alanine (Ricoult et al., 2006; Miyashita et al., 2007). AlaAT is thought to be involved in many physiological processes throughout the life cycle of plants. AlaAT regulation has been associated with responses to low-oxygen stress, carbon stress, and nitrogen stress in many plant species. For example, AlaAT was induced during hypoxia in barley, maize, soybean, and Arabidopsis (Miyashita et al., 2007) and is necessary for seed and seedling germination of *Medicago truncatula* in hypoxia conditions (Ricoult et al., 2006). In addition, AlaAt was up regulated by light and nitrogen stress in *Panicum miliaceum* leaves and up regulated during recovery from nitrogen stress in maize (Miyashita et al., 2007). Although AlaAT has not been previously characterized in response to pathogens, it may be functioning as a stress response to *P. triticina* infection. *P. triticina* could force the host plant into carbon and nitrogen stress as it accumulates plant nutrients for its own growth. This could be an example of specific *P. triticina* races interacting with and utilizing wheat's resources differently to achieve successful infection.

This study identified and characterized one mRNA fragment with the putative function of glutamine-dependent asparagine synthetase (AAU89392.1). The expression level of this gene was different in the presence of each *P. triticina* race (Figure 2.3B). Asparagine synthetases are important factors in the recovery of glutamate and glutamine in non-leguminous plants such as sunflower, maize, rice, sorghum, soybean, and *Arabidopsis*. They also have many roles in plants including nitrogen assimilation and long distance transport of nitrogen (Ohashi et al., 2015; Gaufichon et al., 2013; Gaufichon et al., 2010). For example, *Arabidopsis* asparagine synthetase

AtASN1 mobilizes nitrogen during germination and leaf senescence, while *AtASN2* is important for nitrogen accumulation and distribution in abiotic and biotic stress conditions. *OsAS1* in rice is responsible for the synthesis of asparagine and the assimilation of nitrogen in the roots Interestingly, a tomato asparagine synthetase, *ASN1*, was induced by bacterial pathogens (Gaufichon et al., 2010; Ohashi et al., 2015; Gaufichon et al., 2013). Similar to AlaAT, the asparagine synthetase's role in nitrogen distribution could be important for leaf rust nutrient accumulation.

When compared to the gene expression found using real time PCR, the RNAseq analysis only had 13% similarity at six DPI. For example, real-time PCR identified 32 host genes with no differential expression in the presence of multiple races at six DPI, while the RNAseq analysis concluded that those 32 genes had a two fold or greater expression difference induced by different races. In addition, the race or races that RNAseq analysis determined to induce the highest gene expression in genes with differential expression were not the same as in the real time PCR data. This low prediction accuracy could be attributed to several reasons. The RNAseq data was aligned to the TIGR wheat EST database (available at http://www.jcvi.org) and not a complete sequence. Secondly, the analysis was done using a beta version of software. In addition, there was a lack of replicates that were sent for sequencing. However, even the wheat genes that did not have race specific differential expression according to the real time PCR data were very useful in understanding the pathogen-host interaction.

There have been numerous studies that have previously evaluated wheat gene expression in a compatible reaction with wheat rusts. Wang et al. (2009) used cDNA AFLPs to identify transcript-derived fragments (TDF) that were differentially expressed throughout the first seven days of *Puccinia striiformis* f. sp. tritici infection. Seventy-four transcript-derived fragments

were identified, sequenced, and categorized by function. Similar to results found in this study, they found RuBisCo and chlorophyll a-b binding protein to be down-regulated during the early stages of infection and identified ten up-regulated TDFs that were associated with signal transduction functions (Wang et al., 2009). Using an Affymetrix Wheat GeneChip, Coram et al. (2010) identified 73 transcripts induced by *Puccinia striiformis* f. sp. tritici in a compatible reaction. Transcript accumulation peaked at 24 hours after infection. Of the transcripts found, 25 transcripts were defense-related, six were involved in signal transduction, eight were involved in metabolism, seven transcripts were in protein and carbohydrate transport, 19 were specific to biotrophic interaction transcripts, four had functions related to electron transport, and 25 transcripts were of unknown function (Coram et al., 2010). In addition, Bozkurt et al. (2010) identified 42 probe sets that were up regulated and one probe set that was repressed in a compatible *Puccinia striiformis* f. sp. tritici wheat interaction. Majority of the probe sets identified had functions related to plant defense, while nine of the probe sets functioned in carbohydrate metabolism (Bozkurt et al., 2010).

In order to stay ahead in the arms race between *P. triticina* and wheat, a greater understanding of the interaction between the host and pathogen is needed. This study aimed to identify wheat genes whose expression was induced by *P. triticina* and to characterize the expression of these genes during the first week of infection. A total of sixty-three wheat genes that had differential expression were identified with RNAseq. The gene expression of fifty-four of the wheat genes was further evaluated with a time course using real time PCR. Three wheat genes with race specific expression were identified. These genes may be evidence that the variance in *P. triticina* effectors initiates a different interaction with endogenous wheat genes that don't function as typical resistance genes but instead function in vital plant processes. This

could provide much needed insight into the wheat-*P. triticina* interaction and the role pathogen effectors play in infection. In addition, sixteen wheat genes were identified that showed similar expression in response to multiple races. This group of genes could be essential for *P. triticina* infection. Both groups of genes need further study and characterization to demonstrate these findings *in planta*. Transgenic methods will be used to silence selected host genes to determine their effect on *P. triticina* infection. If the wheat genes were essential for *P. triticina* infection, the pathogen's fitness cost for overcoming the resistance would be very high, thus the resistance would be durable.

Figures and Tables

Race Designation	**Avirulent /** Virulent
MHDS	***Lr2a, Lr2c, Lr9, Lr24, Lr3ka, Lr11, Lr30, Lr18 /*** *Lr1, Lr3a, Lr16, Lr26, Lr17, LrB, Lr10, Lr14a*
MLDS	***Lr2a, Lr2c, Lr16, Lr24, Lr26, Lr3ka, Lr11, Lr30, Lr18 /*** *Lr1, Lr3a, Lr9, Lr17, LrB, Lr10, Lr14a*
MJBJ	***Lr2a, Lr2c, Lr9, Lr26, Lr3ka, Lr11, Lr17, Lr30, LrB, Lr18 /*** *Lr1, Lr3a, Lr16, Lr24, Lr10, Lr14a*
TDBG	***Lr9, Lr16, Lr26, Lr3ka, Lr11, Lr17, Lr30, LrB, Lr14a, Lr18 /*** *Lr1, Lr2a, Lr2c, Lr3a, Lr10, Lr24*
THBJ	***Lr9, Lr24, Lr3ka, Lr11, Lr17, Lr30, LrB, Lr18 /*** *Lr1, Lr2a, Lr2c, Lr3a, Lr16, Lr26, Lr10, Lr14a*
TNRJ	***Lr16, Lr26, Lr17, LrB, Lr18 /*** *Lr1, Lr2a, Lr2c, Lr3a, Lr9, Lr24, Lr3ka, Lr11, Lr30, Lr10, Lr14a*

Table 2.1 Reactions to *Lr* genes for the six *P. triticina* races used in the study.

Races are determined based on their infection type on a set of isogenic differential lines. The descriptions of the interaction with each *Lr* gene in the differentials are shown above. The *Lr* genes listed in bold text condition a low infection type for the race, while the genes listed in underlined text condition high infection.

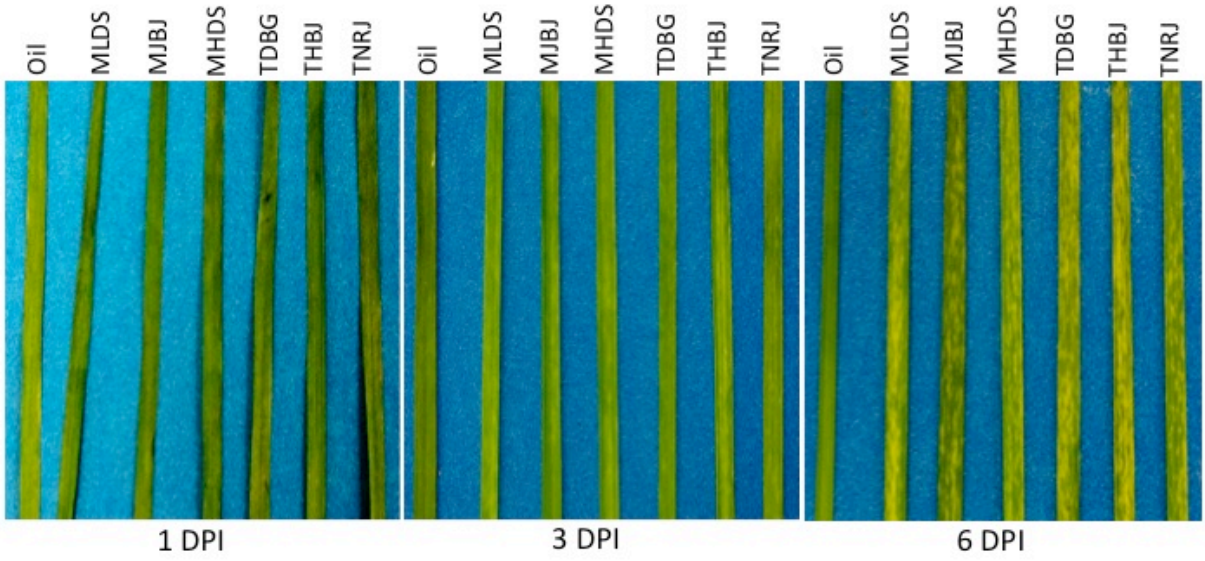


Figure 2.1 Symptoms of six *P. triticina* races on Thatcher at 1, 3, and 6 DPI.

Oil is a Soltrol inoculated control. Symptoms cannot be seen at 1 DPI. By 3 DPI yellow flecking has developed. At 6 DPI, more distinct yellow flecking can be seen. Urediniospores are present but are below the wheat leaf surface.

Primary Analysis of RNAseq		
Sample	Illumina Raw Reads	Base Pairs
MLDS	25,556,420	3,066,770,400
MHDS	26,419,162	3,170,299,440
MJBJ	23,415,788	2,809,894,560
TDBG	27,731,985	3,327,838,200
THBJ	33,225,893	3,987,107,160
TNRJ	28,404,510	3,408,541,200
Total	164,753,758	19,770,450,960

Table 2.2 Primary analysis of RNAseq

Number of raw read generated by Illumina sequencing for plants infected with each race used in the study using standard parameters and paired end 60bp reads before assembly.

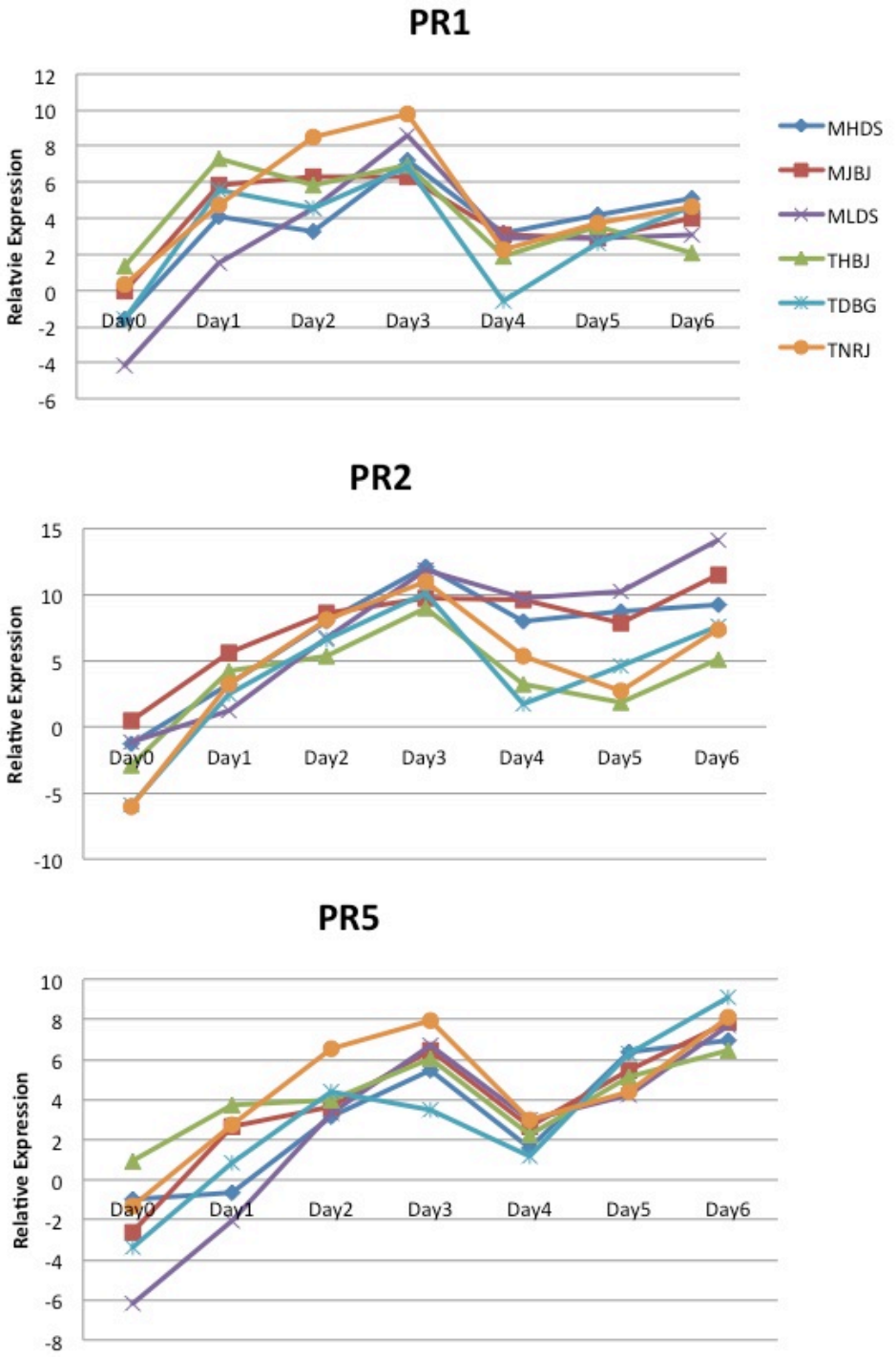


Figure 2.2 Relative expression of three PR genes in response to six *P. triticina* races.

The gene expression of PR genes in response to pathogens is well characterized and the gene expression found is typical. The genes are not expressing when the pathogen is not present at 0 DPI, then expression increases greatly through 3 DPI as the pathogen establishes itself in the host by obtaining plant nutrients and secreting pathogen effectors. The gene expression increase from

4 DPI through 6 DPI could be caused by secondary pathogen infection due to pathogen hyphae spreading through the host and the formation of urediniospores.

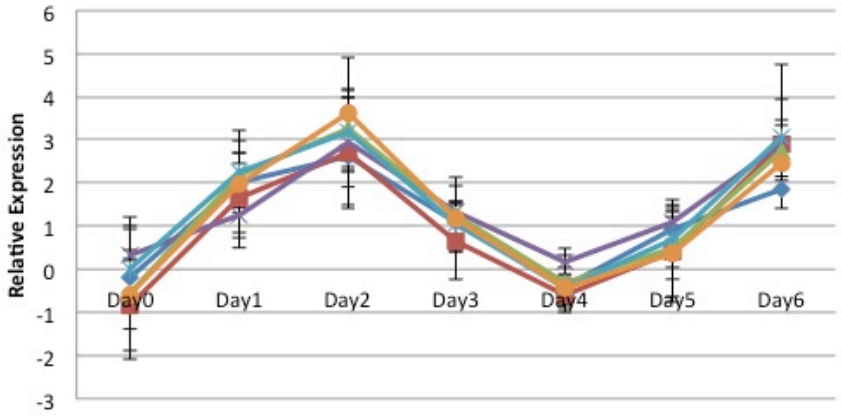
Secondary Analysis of RNAseq Data		
Sample	Illumina Aligned Reads	Base Pairs
MLDS	11,758,504	1,411,020,480
MHDS	11,466,255	1,375,950,600
MJBJ	11,105,663	1,332,679,560
TDBG	13,660,759	1,639,291,080
THBJ	18,754,321	2,250,518,520
TNRJ	16,118,395	1,934,207,400
Total	82,863,897	9,943,667,640

Table 2.3 Secondary analysis of RNAseq data

For each sample the number of raw reads that aligned to the TIGR wheat EST database (available at http://www.jcvi.org) using Novocraft novoalign v2.06.09 software package is reported.

A

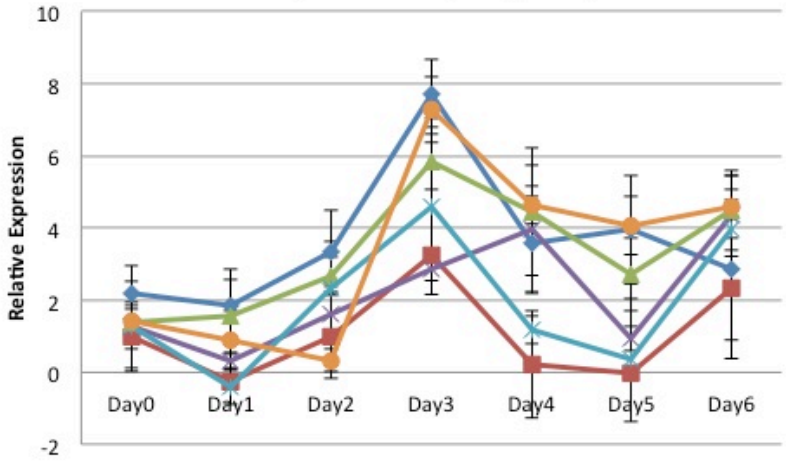
ER Molecular Chaperone-1



5 RNA binding protein
4 ER molecular chaperone
2 photosystem II reaction center protein
1 multiprotein bridging factor
1 glutathione-S-transferase
2 unknown
1 universal stress protein
16 total

B

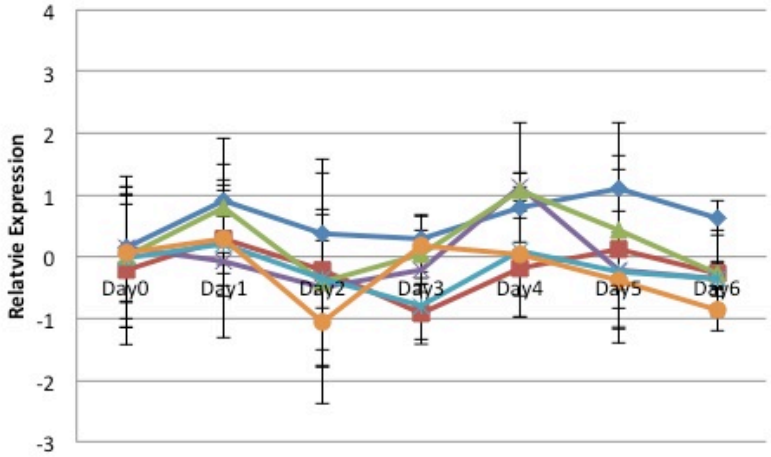
Glutamine Dependent Asparagine Synthetase



8 RNA binding protein
2 protein transfer
1 type 1 non-specific lipid transfer protein
1 alanine-glyoxylate aminotransferase
4 unknown
3 RuBisCO
2 brown planthopper susceptibility protein
1 phosphoglycerate kinase, choloplastic
1 cysteine proteinase inhibitor
2 cytosolic malate dehydrogenase
1 heat shock protein
2 Light regulated proteins
2 chlorophyll a-b binding protein
1 universal stress protein
31 total

C

RNA Binding Protein-2



8 RNA binding protein
1 unknown
1 protein transfer
1 light regulated protein
1 ananian
1 RuBisCo
1 ER molecular chaperone
1 mRNA turnover protein
15 total

Figure 2.3 Three general expression patterns and proposed function for wheat genes in each group.

A) The wheat gene expression is similar in response to all six races, but the gene expression changes throughout infection. This gene could be induced or suppressed by the pathogen influencing the gene expression. B) The wheat gene expression is varied in response to each race, but a clear pattern cannot be determined. The gene expression peak at three DPI could be caused by the formation and function of haustoria secreting effectors and obtaining nutrients from the host. C) The wheat gene does not appear to be induced by *P. triticina* infection. The gene could be an escape from the RNAseq data or the gene expression could be suppressed by the pathogen. The standard error bars represent the standard error based on the biological replicates of each race.

Sum of Read Counts	Log Ratio	Boolean search string	mRNA Fragment Number	MLDS	MHDS	MJBJ	TDBG	THBJ	TNRJ
344.44	4.01	010000	3318	75.18	153.51	18.07	76.87	9.50	11.31
189.6	3.35	000100	4596	11.31	26.31	36.83	86.14	20.59	8.42
858.1	4.83	110100	3428	183.27	338.22	69.68	202.94	52.07	11.92

Table 2.4 Example of ActiveSite.

ActiveSite is an interactive web based platform Cofactor used to assemble the RNAseq data to enable the user to set parameters and sort through the data. Log ratio was set for above two and describes the fold difference in read counts for each mRNA fragment in response to each race. The Boolean search string used a matrix of '1' and '0' to enable the user to display mRNA fragments that had higher read counts in response to a particular race or races. The "summary of read counts field" was set to a total read count value for each mRNA fragment of above 150. The fields for each of the six races showed the read counts of the mRNA fragments in response to the specified race.

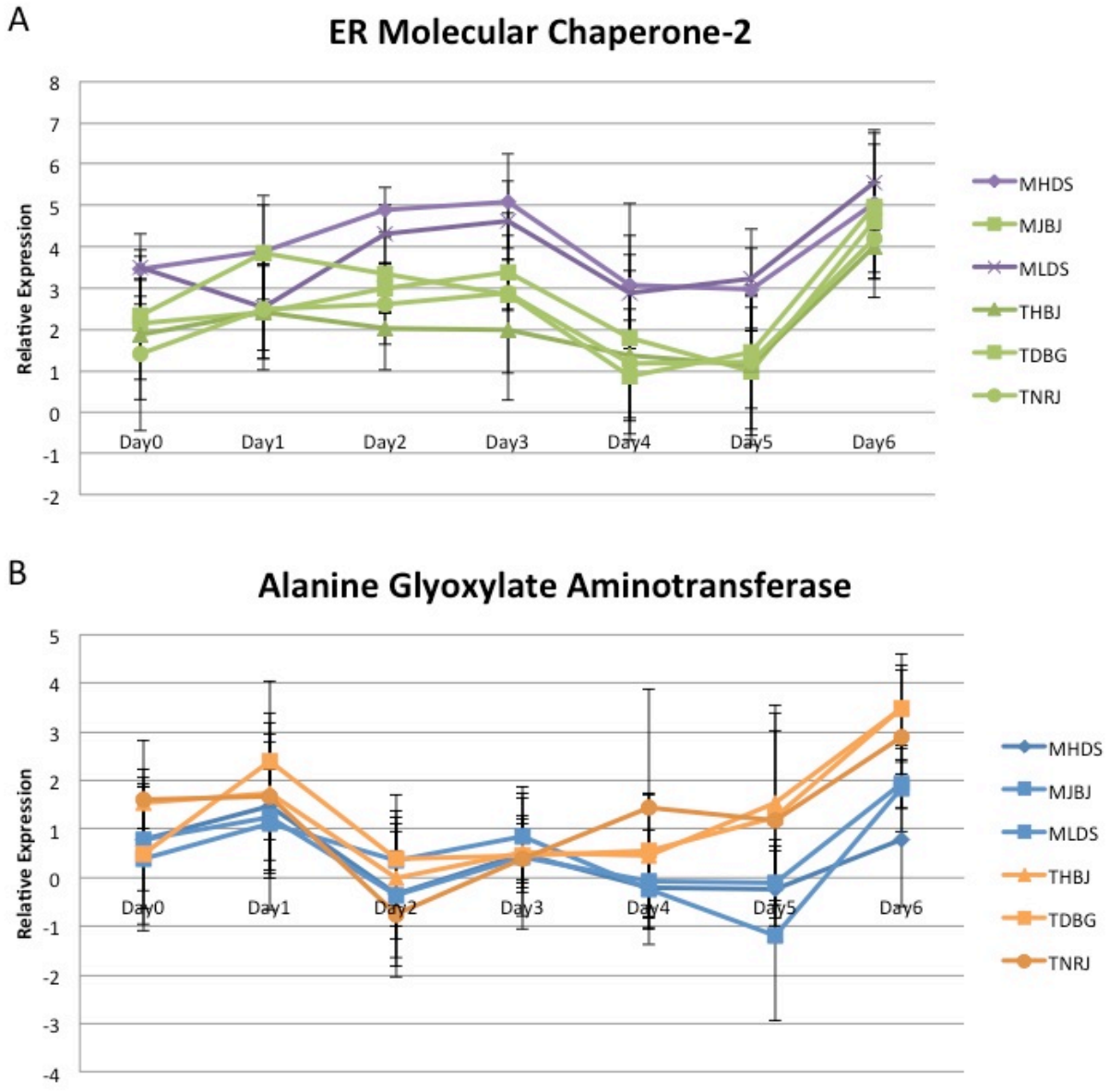


Figure 2.4 Two unique expression patterns.

Both examples show race specific induced differential gene expression. The errors bars show the standard error from three biological replicates of each race. A) Lineage shift: The wheat gene has differential expression induced by races from NA3 and NA5 lineages 2 DPI through 5 DPI. The differential expression is most likely due to the presence or absence to the effector that interacts with *Lr17.* B) Race differences: The wheat gene has differential expression induced by "M" and "T" races 4 DPI through 6 DPI. The differential expression may be caused by the presence or absence of the effectors that interact with *Lr2A* and *Lr2C.*

General Function	Number of mRNA Fragments	Specific Functions
Energy and Metabolism	9 (16.67%)	ribulose biphosphate carboxylase, chlorophyll a-b binding protein, light regulated protein, and photosystem II reaction center protein Z
Protein Transport	11 (20.37)	ER molecular chaperone, alanine-glyoxylate aminotransferase, luminal binding protein, glutathione-S transferase, type 1 non-specific lipid transfer protein precursor, multiprotein bridging factor, and cytosolic malate dehydrogenase
Plant Stress/Defense	5 (9.26%)	universal stress protein A, PR1, heat shock protein HSP70, cysteine proteinase inhibitor WC-1, and brown planthopper susceptibility protein
RNA Binding Proteins	19 (35.20)	Low temperature responsive and glycine rich
Unknown	6 (11.11%)	Unknown
Secondary Metabolism	3 (5.56%)	glutamine dependent asparagine synthetase, hydroxyhenylpyruvate dioxygenase, and ananian
Replication	1 (1.85%)	mRNA turnover 4-like protein

Table 2.5 Summary of proposed functions of the 54 wheat genes characterized using real time PCR.

The genes were grouped into seven general groups based on proposed function.

Primer Name	Sequence
16780 F	TGGGATTGGTCCTATCAG
16780 R	CAGGTCACTTGAAACACG
959 F	AACTGGAGGGAGTGAATG
959 R	AACATCTCGGAACCTACG
959 F	AAGTGTTATCGCTCGCCTC
959 R	TCATTCATAGCCAACGGG
13973 F	CGGTAACAAGTAACACGG
13973 R	AACTGGAGGGAGTGAATG
1519 F	AACTGGAGGGAGTGAATG
1519 R	AACAGGTAACACGGAACG
1519 F	AACAGGTAACACGGAACG
1519 R	AACATCTCGGAACCTACG
777 F	AACTGGAGGGAGTGAATG
777 R	GACGGTAACAAGTAACACG
24701 F	ATCATAGACGAGTCAGCG
24701 R	ACCAGGAGTAGTTTGGAG
13975 F	AACTGGAGGGAGTGAATG
13975 R	AACATCTCGGAACCTACG
13984 F	AACTGGAGGGAGTGAATG
13984 R	AACATCTCGGAACCTACG
15100 F	AACTGGAGGGAGTGAATG
15100 R	AACATCTCGGAACCTACG
15153 F	TCTGGCTACCGTTAGATG
15153 R	CACATTAGGGACCACTTTG
19930 F	AACATCTCGGAACCTACG
19930 R	AACTGGAGGGAGTGAATG
1192 F	AACTGGAGGGAGTGAATG

1192 R	GAAGCATAGCGAACAGAG
13985 F	AACTGGAGGGAGTGAATG
13985 R	ACATCTAACGGTAGCCTG
15148 F	AACTGGAGGGAGTGAATG
15148 R	ACATCTAACGGTAGCCTG
20213 F	GAAGCATAGCGAACAGAG
20213 R	AACTGGAGGGAGTGAATG
20525 F	GAACAGTAACACGGAACG
20525 R	AATGGAGGGAGTGAATGC
12547 F	CTTATCTCCACAGGGTAAAC
12547 R	GGAACCACAAGAATCCTTAG
4596 F	GCCGCATCTTACAAACAAC
4596 R	TCTTCACAAAGCCAGTGG
4231 F	GTCCAACTGACTAACTGC
4231 R	CCTTGTGAGAACTATGAGG
3426 F	ACAGCCTCCATTAGAAGC
3426 R	ATCATCCAGACCATCTCC
3428 F	AACATCTCGGAACCTACG
3428 R	AACTGGAGGGAGTGAATG
955 F	CTTCGGCAATGTGTTCAG
955 R	ACAACGAGGTGCTGTTTC
222 F	TCCCTTAGTGGAATCACGGC
222 R	AGTTCACACCCTTGCGGATG
4010 F	CTCTGTGGCTTACAAGAATG
4010 R	GTACGGTACTCCTTGATTGA
2862 F	CTTGTGGCTTCAGACTTCTA
2862 R	ATACATGTAGCCCATCAAGG
955 F	CTTACTGCATCCATCTTCCT

955 R	GGACGCTAATATACACCTACTG
16104 F	TACTAAGACTGAGGAAGTCG
16104 R	GAGGATTTGATGGTAGCTTG
16104 F	CCATCAGGAGAAGCAAATAC
16104 R	GGTAGCTTGGAGGATTAGAA
22994 F	GGCCAACAGACTAATAACAG
22994 R	CGAGGAGACATGGATAATTG
9694 F	GTGATATCTGCCAAATCGGA
9694 R	GAGTTCAGACCCATGCTTAG
7068 F	ATGTTGTAGGCGTAGTTCTC
7068 R	CTGACAAGGAGGAGATTGAG
1911 F	CTACCAAACCAGCAAGTAAC
1911 R	GACCTCTGCTGAGAATAAGA
1911 F	GTAAGGTGTTGGGTTAAGTC
1911 R	ATAGGTACTCCCTCTCCTTC
PR1 F	CGGGAATATCATTGGACAGA
PR1 R	CGATTAGGGACGAAAGACTA
PR2 F	GGATGTTGCTTCCATGTTTG
PR2 R	ATGGATTGCACACTCATAGG
PR5 F	CTACCAGATCACCTTCTGTC
PR5 R	GCGGCTGTAATATGACAATG
16209 F	GCTGTTATTAGTCTGTTGGC
16209 R	GCATCATCTTTCCTTCATCC
16209 F	TTTCACCACCTACCAGGACC
16209 R	TTCAGGATACCGTTGGCGTC
2283 F	CATTTGATTCTGCGTGAGC
2283 R	TTGATGACGAGGAGCAAC
24701 F	ATCATAGACGAGTCAGCGG

24701 R	ACGACCAGGAGTAGTTTGG
16208 F	TGACTTCGCTCTGAGGAGTG
16208 R	TCCAGGACTTTGAGGGTGAG
15083 F	AACTGGAGGGAGTGAATG
15083 R	AACATCTCGGAACCTACG
3588 F	CTCCGCAAGTACAAGAAGAAC
3588 R	TTGATGAGGCACTCGTACAC
3318 F	CTCTTATTCTCAGCAGAGGT
3318 R	AAGACGAACGAATTGATGAC
20525 F	CGGAGCGAGATCTAGGATAC
20525 R	GCAAATGGAGGGAGTGAATG
3692 F	GAAAGAAGGTCATGCAGATAG
3692 R	CACCTCAAATTCTCGGAATAA
2593 F	CCCTTCGAATGTGGATTTAC
2593 R	CCTGCTGGTCTTATCTACTC
612 F	AGAGAGGGAATTCTTGAGAT
612 R	CGTACATCAGGGAACTACTA
3503 F	CATCAGGAGCAAACTTATCA
3503 R	GAGGGTTGAGGAAGATAAAC
38 F	GTCAACAACAACATCCTCAC
38 R	CCCTCGCATATATACCTCTC
3429 F	TCGTACAAACACAGATCATAC
3429 R	GCCTAAACTACGAGTTGAAA
3429 F	ATCACACTGGTGTTGTATATG
3429 R	GGCCTATGTATGATTGTCTTT
23621 F	ACTTGATTGTCCTGGTTTAC
23621 R	GAAGATTGCAGATGTGATGA
23621 F	CCTGGTTTACTATCGGTAATCT

23621 R	TGATGACATCTCCAGCAATC
25668 F	TTAAAGTTCTCCCATGGTTTC
25668 R	GCTGAAGAAGCAAATTGTTG
15606 F	AGAAGTACGACTTCGACAAC
15606 R	CCAGATACCCAAGATGAGTG
14659 F	GGCCAACAGACTAATAACAG
14659 R	CTGTAGATGGTTTGGAAAGG
12574 F	GAATAATCTGAACGTGGTGTA
12547 R	CCTGAACATTAGTCTGTGTG
12574 F	GCGCTATTATATATGGTGGATT
12574 R	AGCTCGGATGAAGTTTAATG
15093 F	CTGGCCAGTTATCCTAGTTAC
15093 R	CTGAACATCTCGGAACCTAC
2562 F	AAGGAATGGAAAGGAAGGAT
2562 R	CAGGGATAGATGAGCAAACT
3480 F	CAACTCTGACATTGCTTTGG
3480 R	CCGATAGCATTCACATACGA
3480 F	GAGGAGGTCAAGAAGGAGTA
3480 R	CCAAAGCAATGTCAGAGTTG
15093 F	AACTGGAGGGAGTGAATGG
15093 R	AACATCTCGGAACCTACGC
3480 F	ACTGGAGGGAGTGAATGGTG
3480 R	AGCCTGAACATCTCGGAACC
16207 F	TCCAGGTCTTTGAGGGTGAG
16207 R	CTTTCTCGTTCGTGATGGTG
16301 F	CACCATCATCCAGACCATCTC
16301 R	GCGAATCCACAAGAAGAAAG
Ubq F	GCACCTTGGCGGACTACAACATTC

Ubq R	GACACCGAAGACGAGACTTGTGAACC

Table 2.6 Primer sequences for real-time PCR primers.

The number in the primer name column corresponds to the number of the mRNA fragment was designed for. The "F" is the forward primer and the "R" is the reverse primer of the primer pair.

Chapter 3 - RNAi mediated silencing of endogenous wheat genes for resistance to *Puccinia triticina*

Abstract

Puccinia triticina, the casual agent of wheat leaf rust, can cause significant yield losses over the global wheat growing area. Yield losses in the United States due to leaf rust were estimated to cause a $350 million loss for farmers from 2000-2004. The ten year average yield loss in Kansas due to leaf rust is 2.41%. A greater understanding of the *P. triticina* wheat interaction would aid in obtaining durable leaf rust resistance that is needed to reduce the dramatic yield and economic losses farmers have to endure. To evaluate a portion of the *P. triticina*-wheat interaction, a transgenic approach was taken in this study to determine the effect of specific wheat genes on *P. triticina* infection. In Chapter 2, wheat genes with differential expression induced by six *P. triticina* races were identified and the gene expression was evaluated during the first week of infection. Based on the putative gene function and the gene expression, seven wheat genes were selected to silence using an RNAi approach. The genes were cloned in an RNAi hairpin vector and wheat embryos were transformed using particle bombardment. Transgenic plants were recovered from each construct and the resulting plants were molecularly characterized through three generations to confirm the presence of the hairpin construct and target gene insert using PCR. A bioassay was conducted in the T_2 generation where the plants were inoculated with a virulent *P. triticina* race and scored. Small differences compared to the non-transgenic control were observed in plants obtained from six of the constructs. Real time PCR determined that the hairpin constructs were not able to silence their respective endogenous host gene. Although this study could not confirm the function of the

seven identified wheat genes in *P. triticina* infection, they could potentially be targets for future gene editing efforts based on the gene expression profiles found in Chapter 2.

Introduction

Hexaploid bread wheat (*Triticum aestivum* L.) is a host for a wide range of fungal diseases. One fungal disease that infects wheat is *Puccinia triticina* Eriks*,* the causal agent of leaf rust. Leaf rust is the most regularly occurring wheat disease and is found in all wheat growing regions of the world (Kolmer, 2013). Leaf rust has been infecting wheat for thousands of years. It most likely originated in the Fertile Crescent region and travelled to North America with the cultivation of wheat in the early 17th century (Bolton et al., 2008). Since then, great advances have been made to understand the biology of the pathogen. *P. triticina* is an obligate biotrophic pathogen that requires living tissue from its two hosts to complete its full life cycle. Wheat is the primary host and meadow rue (*Thalictrum speciosissimum* L.) is the alternate host required for *P. triticina* to complete its sexual life cycle as basidiospores, pycniospores, and aeciospores. However, susceptible meadow rue is not present in North America, so *P. triticina* typically cycles asexually on wheat as urediniospores. Despite the lack of a sexual cycle, *P. triticina* still has variation within the population that can make achieving durable resistance challenging. As a result, leaf rust can cause significant yield losses for farmers (Bolton et al., 2008; Kolmer, 2013). The annual global yield loss has been estimated at $5 billion (Scofield et al., 2005). While yield losses during endemics range from 15-20% (Huerta-Espino et al., 2011), entire fields can be lost if the infection is severe prior to heading (Appel et al., 2011). To combat this significant economic loss, genetic resistance and the application of fungicides are commonly used to control leaf rust. Both approaches have their drawbacks. Fungicides are effective but can be

economically inefficient especially when grain prices are low (Marsalis and Goldberg, 2006). Genetic resistance can also be effective although single major resistance genes are often overcome within a few years of release (Khan et al., 2013). Minor resistance genes, such as *Lr34,* have proved to be durable for many years and can provide high levels of resistance when used in conjunction with other major or minor resistance genes. However, minor genes are challenging to identify and utilize due to often being located in non-adapted material and a lack of genetic markers (Kolmer, 2013; Hulbert and Pumphrey, 2013; Singh et al., 2011). Clearly, new approaches for leaf rust control are needed.

A new approach that is being explored for durable leaf rust resistance is utilizing transgenic methods, such as post-transcriptional gene silencing (PTGS). PTGS is a natural phenomenon in plants that is part of a defense network against viruses and has been manipulated to use as a mechanism to regulate gene expression. Plants recognize short segments of double-stranded RNA (dsRNA) that are then used for sequence specific degradation (Kamthan et al., 2015). PTGS or transcriptional gene silencing (TGS) can also initiate co-suppression in which the homologous gene products are also silenced (Kamthan et al., 2015; Kusaba et al., 2004). This system of gene silencing can be triggered using silencing mechanisms such as RNA interference (RNAi). RNAi is a gene silencing mechanism in which dsRNA triggers the degradation of sequence specific messenger RNA (mRNA) to reduce translation and overall gene expression (Ali et al., 2010; Kamthan et al., 2015; Saurabh et al., 2014; Katoch et al., 2013). RNAi has been used for many aspects of plant improvement, including developing resistance to abiotic and biotic stressors, increasing plant nutritional content, altering the plant biomass, increasing grain yield, decreasing allergens or toxins, creating male sterility, formation of seedless plants, and increasing the shelf life of fruits and vegetables (Kamthan et al., 2015; Saurabh et al., 2014). To

achieve targeted gene silencing, an RNAi hairpin construct containing a homologous sequence of the target gene is transformed into the plant. An enzyme called Dicer recognizes the dsRNA and cuts it into 21-24 nt pieces of double stranded small interfering RNAs (siRNAs). The siRNAs are incorporated into the RNA-Induced Silencing Complex (RISC) and unwound into single strand RNA. The antisense siRNA acts as a guide to locate the targeted transcript using base pairing, then enzymes in the RISC degrades the target mRNA into 22 nt long fragments or blocks translation to silence the targeted endogenous gene (Jagtap et al., 2011; Ali et al., 2010).

RNAi gene silencing has been used to generate transgenic plants that are resistant to fungi in previous studies. In one example, RNAi was used to silence the wheat homolog of the barley gene, *mlo*, to produce wheat that is resistant to *Blumeria graminis* f. sp. *tritici* (Riechen, 2007). Another group obtained a significant increase in *Nicotiana tabacum* resistance to *Phytophthora parasitica* var. *nicotianae* compared to the controls by silencing the glutathione *S*-transferase enzyme (Hernandez et al., 2009; Jagtap et al., 2011). In another study, the potato homologs of the *Arabidopsis* syntaxin-related 1 gene (*StSYR1*) and soluble N-ethymaleimide-sensitive factor adaptor protein 33 (*StSNAP33*) were silenced. The transgenic potatoes were challenged with *Phytophthora infestans* and *StSYR1*-RNAi plants showed increased resistance (Eschen-Lippold, 2012). Although RNAi has not been used to confer resistance to any of the wheat rusts, several studies have used virus-induced gene silencing (VIGS) to transiently target wheat or rust genes to evaluate their roles in rust infection (Zhang et al., 2013; Panwar et al., 2013; Zhang et al., 2012; Yin et al., 2011; Scofield et al., 2005; Zhou et al., 2007).

Leaf rust is a devastating disease that causes great economic loss to farmers; thus there is a need for alternative control methods to be identified. One alternative is utilizing transgenic approaches to further unravel the wheat-*P. triticina* interaction and identify genes that could be

targeted to obtain durable resistance. As previously described in Chapter 2, sixty-three wheat genes were identified that had differential expression in response to six *P. triticina* races and the expression of fifty-four of the identified wheat genes was further characterized during the first week of infection. Most of the wheat genes' expression in response to *P. triticina* was visually categorized into three general expression patterns. Three wheat genes were identified with race specific gene expression. The characterized wheat genes had a wide range of proposed functions involved in plant processes such as metabolism, protein transport, photosynthesis, and plant defense. Seven wheat genes were selected to silence using RNAi based on gene expression and proposed function: three RNA binding proteins, an alanine-glyoxylate aminotransferase, two ER molecular chaperones, and a glutamine-dependent asparagine synthetase. To ensure adequate representation of the data, genes from each of the three-expression pattern groups and the genes with race specific gene expression were chosen. The selected wheat genes were cloned into an RNAi hairpin vector and integrated into wheat calli through particle bombardment. Transgenic plants were obtained from all seven constructs and molecularly characterized for the presence and expression of the hairpin construct and gene of interest through three generations. In the T_2 generation, a bioassay was conducted to determine if the silenced wheat genes were influential on *P. triticina* infection. However, there was little visual difference between the non-transgenic controls and the transgenic plants. Although the construct and the gene insert was successfully integrated into the plants, the hairpin constructs did not silence the endogenous wheat genes. Further research could utilize new gene editing technologies, such as CRISPR-Cas9, to effectively silence the endogenous wheat genes.

Materials and Methods

Cloning and Plasmid Construction

Forward and reverse primers were designed by inputting the unique assembled transcript fragment derived *Triticum aestivum* mRNAs (based on GenBank release 163) for seven wheat genes into the MacVector software (MacVector Inc., Apex, North Carolina). Primers were selected that had product sizes of 200-400 bp and a Tm of 50-65 °C. The sequence "CACC" was added to the 5' end of each forward and reverse primer (Table 3.1) for cloning purposes. The PCR master mix used for each reaction is as follows: 10pm forward primer, 10 pm reverse primer, 0.8mM dNTP, 25mM $MgCl_2$ (Sigma Life Sciences, St. Louis, MO), 10X PCR Buffer (Sigma Life Sciences, St. Louis, MO), 1.25 U Taq DNA Polymerase (Sigma Life Sciences, St. Louis, MO), and 200ng DNA template. The conditions used for amplification are: denaturation at 92 °C for 3 minutes, 34 cycles of extension 92 °C for 1 minute, 60 °C for 2 minutes, and 72 °C for 2 minutes, final extension at 72 °C for 10 minutes, and final hold at 6 °C. All reactions were completed on a PTC-200 Peltier Thermal Cycler. Samples were run on a 1% agarose gel containing 0.033ng/mL ethidium bromide. The gel was run in an electrophoresis box with 1X TAE buffer (50X 242g TRIS base, 57.1ml acetic acid, 100mL 0.5M EDTA pH 8.5) at 120V for 20 minutes. Gels were placed on a UV light box for band visualization and pictures were taken using a digital camera and Kodak 1D image analysis software. The PCR products underwent pENTR directional TOPO Cloning (Invitrogen, Carlsbad, CA) chemical transformation using One Shot chemically competent *E. coli* cells (Invitrogen, Carlsbad, CA) according to the manufacturers instructions. The resulting bacteria culture was spread onto LB media plates with kanamycin (50ug/mL) using sterilized glass beads. Plates were incubated overnight at 37 °C. Resulting colonies were picked and transferred to tubes with 2mL LB media with kanamycin

(50ug/mL) and left on a shaker at 37 °C overnight. DNA was isolated from the cultures using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) following the manufactures protocol. The constructs were digested with the restriction enzyme *Mlu*I (New England BioLabs, Ipswich, MA) and then sequenced using Sanger sequencing to ensure the insert was present (Kansas State University DNA Sequencing and Genotyping Facility). The pENTR constructs were then cloned into RNAi vector pANDAmini (Miki et al., 2004; Miki et al., 2005) by combining 2ul LR Reaction Buffer (Invitrogen, Carlsbad, CA), 300ng pANDAmini vector, 200ng pENTR clone, and 2ul LR Clonase Enzyme Mix (Invitrogen, Carlsbad, CA) and incubating at 25 °C overnight. The ligation was transformed into DH5α competent cells by mixing 1ng of the ligation in the competent cells and incubating on ice for 20 minutes. The cells were heat shocked for 30 seconds at 42 °C and immediately transferred to ice. 250ul LB was added and the cells were incubated at 37 °C for 45 minutes in a shaker. The cells were spread on LB with ampicillin (100mg/ml) plates using sterilized glass beads. The clones were digested with *EcoRI* and sequenced with Sanger sequencing (Kansas State University DNA Sequencing and Genotyping Facility) to ensure the insert was present and in the correct orientation.

Plant Transformation and Tissue Culture

The spring wheat variety Bobwhite (CIMMYT, 1984) was used for transformation. Immature seeds were collected ten to fourteen days post anthesis from plants grown under standard growth chamber conditions. The immature seeds were sterilized in a solution of 20% v/v sodium hypochlorite (6%) and 0.04% Tween-20 solution for twenty minutes on a shaker. The seeds were rinsed with ddH_2O five times. The immature embryos were excised from the seeds and plated on callus induction medium CM4 (Zhou et al., 1995). The plates were put in a dark cabinet at room temperature for one week. The scutellum were then checked for callus

development under a microscope and transferred to a fresh CM4 plate. The plates were dried in a laminar flow cabinet for thirty minutes without the petri plate lid to plasmolyze the cells in preparation for particle gun bombardment. Particle gun bombardment was done according to the protocol described by Anand et al. (2003a, b). Embryos were co-bombarded with the pANDAmini vector containing one of the seven host gene fragments and the vector pAHC20 which contains the *bar* gene (Christenson and Quail, 1996) that confers resistance to the herbicide ammonium glufosinate, also known as Liberty™. The remaining tissue culture process and media used is described in Anand et al. (2003a, b).

Molecular Characterization of Transgenic Plants

After selection and regeneration, the plants that developed sufficient amounts of root and shoot growth were transferred to peat pots and grown in an enclosed high humidity translucent box in a growth chamber at 18 °C. Every few days, the lid of the box was opened slightly to slowly adjust the plants to normal growing conditions. Plants that survived the transition were transplanted to one gallon pots and grown in a growth chamber at 18 °C. Approximately one week after transplanting, the plants were tested for resistance to the herbicide ammonium glufosinate, also known as Liberty™. One leaf on each plant was painted with a 0.2% v/v Liberty™ solution (AgroEvo USA, Wilmington, DE) using cotton balls. Plants were checked for resistance one week after painting. Plants without necrotic leaves had 10 mg leaf tissue collected and genomic DNA was isolated using the E.Z.N.A. Plant DNA Kit (Omega Bio-tek, Norcross, GA) according to manufacturers instructions. PCR was used to detect the presence of the hairpin constructs. Each PCR reaction contained 200ng genomic DNA, 10pmole forward primer, 10 pmole reverse primer (Table 3.1), 0.8mM dNTP, 25mM $MgCl_2$ (Sigma Life Sciences, St. Louis, MO), 10X PCR Buffer (Sigma Life Sciences, St. Louis, MO), 1.25 U Taq DNA Polymerase

(Sigma Life Sciences, St. Louis, MO), and 200ng DNA template. The amplification conditions were denaturation at 92 °C for 3 minutes, 34 cycles of extension 92 °C for 1 minute, 60 °C for 2 minutes, and 72 °C for 2 minutes, final extension at 72°C for 10 minutes, and final hold at 6 °C. All reactions were completed on a PTC-200 Peltier Thermal Cycler. The PCR products were run on 1.0% agarose gel with 0.033ng/mL ethidium bromide added for staining. The gel was run in an electrophoresis box with 1X TAE buffer (50X 242g TRIS base, 57.1ml acetic acid, 100mL 0.5M EDTA pH 8.5) at 120V for 20 minutes. Gels were placed on a UV light box for band visualization and pictures were taken using a digital camera and Kodak 1D image analysis software. Tissue was collected from all T_1 generation plants by putting 10-12 cm^2 leaf tissue in 1.5mL plastic collection tubes. Leaf tissue was ground using liquid nitrogen and genomic DNA was isolated using the CTAB method described by Sambrook and Russell (2001). The T_2 generation was processed using a high throughput method. A 3.96mm steel bead (Abbott Ball Company, West Hartford, CT) was placed into each tube of the plate. Leaf tissue was collected (4-8cm^2) and from each plant and placed in 96-1.1 mL collection plates (USA Scientific, Ocala, FL). After collection, the plates were flash frozen in liquid nitrogen and stored in an -80 °C freezer. The tissue was homogenized using a TissueLyser (Qiagen, Valencia, CA) for 2 minutes at 30 Hz. Genomic DNA was isolated using the BioSprint 96 DNA Plant Kit (Cat. No. 941558) according to the manufacturer's instructions (Qiagen, Valencia, CA). The genomic DNA was suspended in ddH2O and stored at -20 °C.

Transgene expression was evaluated by extracting total RNA. Leaf tissue was collected (10-12cm^2) and processed using the *mir*Vana miRNA isolation kit (AM1560, RNA Life Technologies, Carlsbad, CA) according to the manufacturer's instructions and following the recommendation to remove the miRNA enrichment step. A Nanodrop ND1000

spectrophotometer (Thermo Fisher Scientific, Waltham, MA) was used to quantify the RNA. To obtain first strand synthesis, 1ug RNA was mixed with random hexamers, then reverse transcription with Superscript RTII (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. The resulting cDNA was used as a template for reverse transcription PCR (rt-PCR) reactions. Expression of the hairpin construct and reduced expression of the endogenous gene was determined by using cloning insert primers and primers designed outside of the insert (Table 3.1).

Real-time PCR (qPCR) was used to determine the relative gene expression of the target genes. The cDNA described above was used as a template for the qPCR reactions. Primers were designed using the unique assembled transcript fragment derived *Triticum aestivum* mRNAs (based on GenBank release 163) for each gene. All primers were evaluated for dimer formation and efficiency before used for expression data collection. The qPCR conditions were as follows: 95 °C for 3 minutes, 40 cycles of 95 °C for 10 seconds, and 62 °C for 30 seconds. The run was completed with a melt curve: 65 °C to 95 °C heating in 0.5 °C increments for 5 seconds. All reactions used the Bio-Rad CFX96 Real-Time System and the Bio-Rad iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., La Jolla, CA) in a 25 μL reaction which contained 6ul cDNA template (diluted in a 2:1 ratio), and 10pmol of each primer. Three technical replicates were obtained for all reactions. The Ct value for the target was compared to the Ct value of a wheat ubiquitin housekeeping gene (Paolacci et al., 2009) and a nontransgenic control using the ΔΔCt method (Livak and Schmittgen, 2001).

Bioassay

Bobwhite transgenic seedlings were grown in a greenhouse with 16-h day/8-h night, at 18 °C. The seedlings were inoculated at the two- to three-leaf stage. The leaf rust urediniospores

were stored in an -80 °C freezer. The spores were heat shocked at 42 °C for 20 minutes and 5mg spores were suspended per mL Soltrol 170 isoparaffin solvent (Philips 66, Bartlesville, OK) and sprayed onto the plants using an atomizer and an air compressor at 40 PSI. Plants were incubated in a dark 100% humidity Percival Intellus dew chamber overnight for 16 hours at 18 °C, wall temperature at 3.5 °C and water temperature at 40 °C then returned to the greenhouse. Plants were scored 10-14 days post inoculation using a 0-4 rating scale developed by Stakman et al. (1962) and Gassner and Straib (1932) and modified by McIntosh et al. (1995) (McIntosh et al., 1995; Stakman et al., 1962; Gassner and Straib, 1932).

Results

Seven wheat genes, three RNA binding proteins, an alanine-glyoxylate aminotransferase, two ER molecular chaperones, and a glutamine-dependent asparagine synthetase, were selected for gene silencing based on putative gene function and gene expression induced by *P. triticina* infection found in Chapter 2. Cloning primers (Table 3.1) were designed from the mRNA fragment sequences generated from RNAseq assembly (see Chapter 2). Fragments from each of the seven genes were amplified using the cloning primers in a PCR reaction. The PCR products were cloned into the pENTR vector using directional TOPO Cloning and sequenced. The pENTR vectors were then cloned into the RNAi hairpin construct pANDAmini and sequenced to ensure the insert was present and in the correct orientation.

The alanine glyoxylate aminotransferase and all three RNA binding proteins were cloned into pENTR and pANDAmini in the correct orientation with few failed attempts or empty vectors. However, this was not the case when cloning every fragment. It took many attempts to obtain clones that contained inserts from both ER molecular chaperones and the glutamine-dependent asparagine synthetase, but eventually pANDAmini clones with inserts in the correct

orientation were obtained. In addition, cloning was attempted for more than seven genes. Fragments from a heat shock protein (AAB99745.1), an unnamed protein product (CDM81404.1), and an additional RNA binding protein (AGI04359.1) could not be amplified using PCR. An ER molecular chaperone (AGN94841.1) and a chlorophyll a-b binding protein (XP_003523016.2) were successfully cloned into pENTER, but could not be cloned into the pANDAmini construct in the correct orientation to form the hairpin. Eventually, there were seven pANDAmini constructs obtained; alanine glyoxylate aminotransferase, three RNA binding proteins, two ER molecular chaperones, and a glutamine-dependent asparagine synthetase.

After the seven gene fragments were successfully cloned into the RNAi hairpin vectors, each gene along with the selectable marker *bar* gene that confers resistance to the herbicide ammonium glufosinate was co-bombarded into immature Bobwhite wheat embryos. The bombarded embryos were taken through the tissue culture process, selected on media that contained glufosinate, and the putative transgenic plants were regenerated. All of the T_0 plants were tested with the herbicide Liberty™ at the two- to three-leaf stage as an initial selection for plants that putatively contained the *bar* gene. DNA was extracted from all *bar* resistant plants and evaluated by PCR analysis for the presence of the *bar* gene and the hairpin construct. The transgenic plants that contained the hairpin construct and gene insert were grown to seed and harvested independently. For each T_0 transgenic plant that contained the gene of interest, six seeds were planted for the T_1 generation. Plants were labeled with their bombardment number and an addition of a number one through six. Leaf tissue was collected at the two to three leaf stage, DNA was isolated, and PCR was used to confirm the presence of the hairpin construct and gene insert (Figure 3.1). Plants that contained the transgene were selected, selfed, and harvested separately. Positive plants were continued to the T_2 generation and confirmed to have the hairpin

construct and gene insert using PCR (Figure 3.2). All the T_2 plants then underwent a bioassay, where they were inoculated with a virulent *P. triticina* race to determine if the silenced genes had an effect on *P. triticina* infection. Plants were scored on a 0-4 Stakman scale with '0-1' being resistance, '2' moderately resistant, '3' susceptible, and '4' being very susceptible. None of the transgenic plants scored below a '2'. Some plants showed small differences compared to the non-transgenic controls, such as flecking and smaller pustules at the leaf tip (Figure 3.3). Those plants had leaf tissue collected four to five weeks after planting and RNA was isolated (Table 3.3). Reverse transcriptase PCR was used to evaluate hairpin construct expression and endogenous gene expression (Figure 3.4). The endogenous gene expression was also quantified using real time PCR (Figure 3.5). Plants that contained the hairpin construct and gene insert were selected, selfed, and harvested individually.

A 203 bp segment (Table 3.2) of the ER molecular chaperone-1 (AGN94841.1) was cloned in the RNAi hairpin construct pANDA-mini. Particle bombardment occurred in nine independent experiments on 1450 wheat callus. Regeneration resulted in 69 putative transgenic plants. The plants were tested for the *bar* gene by screening with the herbicide glufosinate. Ten plants putatively contained the *bar* gene and underwent PCR with genomic DNA to test for the presence of the transgene. Six plants were found to contain the vector construction. For each of the six events, six seeds were planted in the T_1 generation and all of the plants were characterized with PCR to determine which plants contained the transgene. Event 1 resulted in four plants that contained the transgene; Event 2 had two positive plants; Event 3 contained three plants; Event 4 resulted in two plants; Event 5 had four plants; and Event 6 resulted in two transgene positive plants. Out of the 36 total plants for the ER molecular chaperone-1 construct, seventeen plants contained the transgene. Due to a lack of seed, only fifteen plants from five events were

continued on to the T_2 generation where up to twenty seeds per plant were planted. PCR was used to determine that Event 1 had a total of twenty plants and fourteen contained the transgene. A total of 44 plants were analyzed from Event 3 and 27 contained the transgene. Event 4 had a total of 28 plants and 21 contained the transgene. A total of 80 plants were analyzed from Event 5 and 62 were positive for the transgene. For Event 6, 40 plants were analyzed and 19 contained the hairpin construct and the gene insert. In the T_2 generation, a total of 212 plants were analyzed and 143 plants were confirmed to have the transgene. A bioassay was conducted in the T_2 generation to select plants that had fewer leaf rust symptoms than the non-transgenic control. Event 1 resulted in one plant that showed differences; Event 3 contained two plants; Event 4 resulted in three plants; Event 5 resulted in fourteen; and Event 6 resulted in five plants, for a total of 25 plants with phenotypic differences (Table 3.3). The plants that showed differences were analyzed using rt PCR to determine if the construct and the endogenous gene were expressing. For all of the plants, the hairpin construct was not expressing and the endogenous gene had not been silenced.

For the ER molecular chaperone-2 gene (AGN94841.1), a 221 bp segment was cloned into the hairpin construct. Seven independent particle bombardment experiments were conducted on 975 wheat embryonic callus and 75 putative transgenic plants were regenerated. The plants were screened for glufosinate resistance and nine plants were found to be resistant. PCR was conducted and five plants contained the hairpin construct and gene insert. Six seeds per each of the five events were planted in the T_1 generation for a total of 30 plants. Each of the 30 plants underwent PCR analysis to determine the presence of the transgene. No plants from Event 1 contained the transgene; Event 2 resulted in five plants that contained the transgene; Event 3 had four plants with the transgene; Event 4 had four plants with the transgene; and Event 5 contained

three plants with the transgene, for a total of sixteen plants with the transgene. Plants from Event 4 were not continued to the T_2 generation due to a lack of seed, but the other three events were continued to the T_2 generation by planting up to twenty seeds per plant. The plants were analyzed using PCR for the presence of the hairpin construct and gene insert. Event 2 had a total of 42 plants evaluated and 26 contained the transgene; Event 3 had a total of 28 plants and 16 were found to have the transgene; and Event 5 had 26 plants evaluated and 18 contained the transgene, for a total of 96 plants analyzed and 60 plants with the transgene. The bioassay found only two plants, one from Event 2 and the other from Event 5, with a phenotypic infection difference from the non-transgenic control (Table 3.3). Both of these plants had hairpin constructs that were not expressing and the endogenous genes were still expressing.

A 240 bp segment of the alanine-glyoxylate aminotransferase (EMT26999.1) was cloned into the pANDAmini vector. Particle bombardment was conducted in six independent experiments on 850 wheat embryonic callus resulting in 25 putative transgenic plants. The plants were screened for the *bar* gene with the herbicide glufosinate and four plants were found to be resistant to the herbicide. The plants were tested for the presence of hairpin construct and gene insert using PCR and two plants were positive. The two events were continued to the next generation and six seeds were planted per event, for a total of 12 plants. PCR was conducted in the T_1 generation to determine the presence of the transgene. Event 1 had four plants that contained the transgene while Event 2 did not have any positive plants. Since this gene had race specific induced gene expression, up to 31 seeds were planted for each event so the plants could be evaluated with multiple races, as this gene showed race specific gene expression in the presence of “M” and “T” leaf rust races. Out of 105 plants, 94 were gene of interest positive. After the bioassay, five plants were determined to have phenotypic differences (Table 3.3). The

plants were challenged with virulent “M” and “T” races but there were no differences in symptoms. Although all five of the plants’ hairpin constructs were expressing, the endogenous gene was also still expressing.

A 268 bp segment of the glutamine-dependent asparagine synthetase gene (AAU89392.1) was cloned in the hairpin construct and transformed in 1200 wheat embryonic callus in eight independent experiments. Seventy-nine putative transgenic plants were regenerated and eleven plants were found to be resistant to glufosinate. PCR analysis found ten plants contained the hairpin construct and gene insert. The ten events were continued to the T_1 generation by planting six seeds for each event. Each plant underwent PCR analysis to confirm the presence of the transgene. Event 1 resulted in one plant with the transgene; Event 2 did not have any plants with the transgene; Event 3 had five plants that contained the transgene; Event 4 resulted in two plants with the transgene; Event 5 had four plants with the transgene; Event 6 did not have any plants with the transgene; Event 7 had three plants with the transgene; Event 8 resulted in five plants that contained the transgene; Event 9 had four plants with the transgene; and Event 10 had two plants with the transgene for a total of 60 plants evaluated and 26 with the transgene. The plants that contained the transgene were continued onto the T_2 generation by planting up to 20 seeds per plant. Each plant was then analyzed for the presence of the hairpin construct and gene insert using PCR. Event 1 had 20 plants and all 20 were positive for the transgene; in Event 3, 98 total plants were evaluated and 80 were confirmed to have the transgene; Event 4 had 40 total plants and 28 contained the transgene; in Event 5, 80 plants were evaluated and 50 were determined to have the transgene; Event 7 had 44 total plants and 26 had the transgene; in Event 8, 98 plants were evaluated and 78 were found to contain the transgene; Event 9 had 78 plants and 68 had the transgene; and Event 10 had 20 plants and 9 contained the

transgene for a total of 478 plants evaluated and 359 which contained the transgene. After the bioassay, Event 1 had one plant; Event 3 had three plants; Event 4 had three plants; Event 5 had one plant; Event 7 had two plants; Event 8 had three plants; Event 9 had two plants; and Event 10 had one plant that showed phenotypic differences for a total of 16 plants (Table 3.3). Rt PCR confirmed the expression of the hairpin construct for all of the plants and the expression of the endogenous host gene for all plants except the plant from Event 1. However, real time PCR showed the endogenous host gene expression level was greater than the control for the plant from Event 1.

A 217 bp segment of the glycine rich RNA binding protein-1 (AGI04359.1) was cloned into the pANDAmini vector. The construct was transformed into 925 wheat embryonic callus explants in six independent experiments. Forty-one putative transgenic plants were regenerated and screened with glufosinate. Five plants were resistant to glufosinate and four plants contained the hairpin construct and the gene insert. The four events were continued to the T_1 generation by planting six seeds per event and all 24 plants were evaluated with PCR. Event 1 had three plants that were positive for the transgene; in Event 2, five plants were confirmed to contain the transgene; Event 3 had four plants with the transgene; and Event 4 did not have any plants that contained the transgene for a total of twelve plants with the transgene. These plants were continued to the T_2 generation by planting up to 20 seeds per plants and tested for the presence of the hairpin construct and gene insert using PCR. In Event 1, 54 plants were tested and 37 were found to contain the transgene; Event 2 had 22 plants and 11 had the transgene; and Event 3 had 34 plants and 28 contained the transgene for a total of 110 plants evaluated and 76 found to contain the transgene. The bioassay determined that five plants had phenotypic differences and all five plants were from Event 1 (Table 3.3). Results from rt PCR analysis confirmed that four

out of the five plants hairpin constructs that were expressing but the endogenous wheat genes in all five plants were not silenced.

A 277 bp segment of the glycine-rich RNA binding protein-2 (AGI04359.1) was cloned in the hairpin construct and transformed into 925 wheat embryonic callus through six independent particle bombardment experiments. Sixty putative transgenic plants were regenerated and seven plants were found to putatively contain the *bar* gene and hairpin construct after glufosinate screening and PCR, respectively. The seven events were continued to the T_1 generation by planting six seeds per events. Each plant was evaluated using PCR to confirm the presence of the hairpin construct and gene insert. In Event 1, four plants contained the transgene; Event 2 had one plant that had transgene; Event 3 and Event 4 had no plants with the transgene; in Event 5, four plants were confirmed to have the transgene; Event 6 did not have any plants with the transgene; and Event 7 had one plant that contained the transgene, for a total of 42 plants evaluated and only 10 plants from four events with the transgene. All ten plants were continued to the T_2 generation by planting up to 20 seeds per plant. In Event 1, a total of 72 plants were evaluated and 49 contained the transgene; Event 2 had 22 plants evaluated but no plants had the transgene; 76 plants from Event 5 were evaluated and 23 contained the transgene; and Event 7 had 20 plants evaluated but none contained the transgene for a total of 188 plants evaluated and 72 with the transgene. The bioassay found that six plants had phenotypic differences; five of the plants were from Event 1 and the other plant was from Event 7 (Table 3.3). For the five plants from Event 1, rt PCR determined the hairpin construct was expressing but the endogenous gene was not silenced. The one plant from Event 7 did not contain an expressing hairpin construct and the endogenous gene was expressing as well.

A 288 bp segment of the glycine rich RNA binding protein-3 (AGI04359.1) was cloned in the pANDA-mini hairpin construct. Six independent particle bombardment experiments were conducted on 925 wheat embryonic callus and thirty putative transgenic plants were regenerated in tissue culture. Five plants were resistant to glufosinate and all five plants contained the hairpin construct and gene insert. The five events were continued to the T_1 generation by planting six seeds per event. Event 1, Event 2, Event 3, and Event 4 did not have any plants that contained the transgene. In Event 5, two plants had the transgene. This event was continued to the T_2 generation, where 40 plants were evaluated and nine plants contained the transgene. However, the bioassay did not show any differences between the transgenic plants and the non-transgenic control, so expression analysis was not conducted.

Discussion

Leaf rust is a fungal disease that infects all wheat growing areas of the world and can cause significant yield loss. In 2007, a leaf rust epidemic caused 13.9% yield loss in Kansas, while the average yield loss due to leaf rust in Kansas for the last 10 years is 2.41% (Appel et al., 2014). The most common strategy to prevent losses due to leaf rust is genetic resistance. This strategy isn't always effective as single major gene resistance can break down in as little as three to four years after cultivar release (Khan et al., 2013). Minor gene resistance is more durable, but can be time consuming to transfer to elite material where linkage drag is often an issue (Hulbert and Pumphrey, 2013). Fungicides are also utilized as a prevention strategy, but can be an economically poor decision for farmers if wheat prices are low or if the timing of the application is too early or late (Marsalis and Goldberg, 2006). Because it has been so challenging to prevent yield losses caused by leaf rust, new methods of resistance should be explored. In this study, a transgenic approach was taken to target specific wheat genes and gain a greater understanding of

the *P. triticina*-wheat interaction. Seven wheat genes were cloned into hairpin constructs for sequence specific gene silencing using RNAi: three RNA binding proteins, two ER molecular chaperones, an alanine-glyoxylate aminotransferase, and a glutamine-dependent asparagine synthetase. The genes were selected based on gene expression data and putative gene function. Genes from all three general expression groups were selected along with genes that showed race specific gene expression to try to best represent the data. Because of the findings in Chapter 2, the selected wheat genes were hypothesized to play a role in *P. triticina* infection and were selected to determine the specificity and importance of their function in the host pathogen interaction. If the silenced wheat genes were essential for *P. triticina* infection, the transgenic plants would condition durable resistance because of the high fitness cost for the pathogen to overcome the resistance. Transgenic plants were obtained from all seven constructs in the first generation and were continued through two additional generations where they underwent molecular characterization for presence of the transgene and the expression of the hairpin construct and endogenous gene. The plants were also challenged with *P. triticina* in the last generation, scored, and selected. The transgenic plants did not score below a '2' on the Stakman rating scale, but did display small signs of resistance, such as flecking and smaller pustules on the leaf tip. Expression analysis showed the endogenous wheat genes were not completely silenced. The gene expression suppression may have led to the partial resistance phenotype. This approach to pathogen resistance is not novel, however reported studies for rust resistance has only utilized transient expression assays (Zhang, et al., 2013; Panwar et al., 2013; Zhang et al., 2012; Yin et al., 2011; Scofield et al., 2005; Zhou et al., 2007) instead of creating stable transgenic plants.

The T_0 generation resulted in multiple events that contained the hairpin construct and gene insert for their respective genes. The T_1 and T_2 generations also underwent PCR analysis to confirm the presence of the hairpin construct. Expression analysis conducted in the T_2 generation found that regardless of expression of the hairpin construct, the endogenous host gene was not completely silenced. Because of this, there was very little variation in infection symptoms compared to the susceptible non-transgenic controls and no plants were totally resistant. Some of the plants exhibited smaller pustules and flecking at the leaf tip, but this phenotype was also seen in some of the non-transgenic controls. This could be attributed to an internal plant defense mechanism or plant response to variance in environmental conditions in the greenhouse. A virulent *P. triticina* race was used in the bioassay to ensure that genetic resistance did not cause any altered symptoms that could be confused for resistance due to the silencing of the gene of interest. Alternatively, the leaf tip resistant symptoms displayed by the transgenic plants are similar to adult plant resistance responses. The slight suppression of gene expression generated by RNAi may have provided partial resistance. This type of resistance is extremely difficult to detect and to score, thus a larger number of plants need to be evaluated. Testing the transgenic plants at multiple plant growth stages, especially in the adult stage where the greatest level of this resistance is typically seen, will also aid in determining if the plants have partial resistance.

There are several factors that may have contributed to the lack of effectiveness of the RNAi silencing in this study. Particle bombardment often leads to multiple insertions and copies of the transgene, which can hinder the ability of the hairpin construct to silence the gene of interest. High copy number can result in lower expression of the transgene and cause DNA methylation (Matzke et al., 2000). In addition, RNAi often results in a knockdown of the target gene expression due to partial and unstable silencing instead of generating a complete gene

knockout (Kusaba et al., 2004; Puchta and Fauser, 2014). The lack of stability of RNAi silencing in wheat may be due to a dosage effect. Wheat plants that are homozygous have a higher accumulation of siRNAs and a stronger reduction in expression of the gene of interest than in heterozygous plants (Travella et al., 2006). The transgenic plants in this study are in early generations and are still undergoing segregation. Other factors that contribute to the effectiveness of gene silencing are GC content of the gene of interest region, hairpin melting temperatures, DNA methylation, and chromatin modification (Fu et al., 2007; Reynolds et al., 2004; Hammond et al., 2000). A factor that could impact the effectiveness of RNAi silencing that is specific to wheat is the ability to silence all of the copies of the target gene. Since bread wheat is a hexaploid, it has a high rate of gene duplication and typically has up to three homologues that are functionally redundant and are usually expressed (Fu et al., 2007; Travella et al., 2006). If the target sequence doesn't contain conserved regions of the gene family, it may not be able to silence all of the homologues simultaneously. Some studies in wheat have shown that RNAi can silence all three homologues and even paralogues with unique constructs, (Travella et al., 2006; Uauy et al., 2006) but there have also been reports of exceptions where the silencing of homologues has failed even when gene inserts with high sequence identity are used in the construct (Yue et al., 2007; Regina et al., 2006). Due to the lack of sequence data for wheat, it can be challenging to determine the conserved regions shared among homologues.

Although the endogenous wheat genes were not effectively silenced, the gene expression and putative functions found in Chapter 2 indicate that the selected target genes are induced by *P. triticina* infection and could be important for pathogen development. RNA binding proteins are involved in post-transcriptional gene regulation, can help plants regulate RNA involved in plant responses to changing environmental conditions, and aid in the translation of stress

associated genes (Ambrosone et al., 2012; Silverman et al., 2010; Lorkovic et al., Naqvi et al., 1998; Xuan et al., 2010). RNA binding proteins also regulate the plant defense system in response to pathogens. For example, an effector from *Pseudomonas syringae* altered *Arabidopsis* RNA binding proteins and prevented them from regulating their target RNAs (Silverman et al., 2013). Barley and tobacco RNA binding proteins were induced by fungal pathogens *Erysiphe graminis* and *Rhynchosporium secalis* and *Tobacco mosaic virus,* respectively (Molina et al., 1997; Naqvi et al., 1998). In this study, RNA binding proteins may be regulating the plant responses to the altered internal environment caused by *P. triticina* infection.

ER molecular chaperones are located in the ER lumen and ensure the quality of secreted proteins by binding unfolded or partially folded protein. They also transport proteins across the ER membrane. Binding protein (*BiP*) and glucose-regulated protein (*GRP94*) are ER chaperones that are members of the heat shock protein family and have increased expression during plant stress. *BiP* has been found to reduce drought-induced leaf senescence in soybeans and tobacco, while *GRP94* is up regulated during powdery mildew infection of barley (Nishikawa et al., 2005; Gupta and Tutei, 2011; Goeckeler et al., 2010). The ER molecular chaperones in this study may be induced in response to the plant cell stress caused by leaf rust infection.

Alanine-glyoxylate aminotransferase (AlaAT) is an enzyme that catalyzes the transfer of an amino group from glutamate to pyruvate to form 2-oxoglutarate and alanine. AlaAT is up regulated in response to low-oxygen stress, carbon stress, and nitrogen stress in many plants (Ricoult et al., 2006; Miyashita et al., 2007). Specifically, AlaAT is induced during hypoxia in barley, maize, soybean, and *Arabidopsis* and is necessary for germination of *Medicago truncatula* in hypoxic conditions (Ricoult et al., 2006). AlaAT is also up regulated by light and nitrogen stress in *Panicum miliaceum* and up regulated by nitrogen stress in maize (Miyashita et

al., 2007). In this study, AlaAT could be responding to *P. triticina* causing a sink for nutrients like carbon and nitrogen and putting the plants under stress.

Glutamine-dependent asparagine synthetase is involved in nitrogen assimilation, long distance transport of nitrogen, and glutamate and glutamine recovery in plants such as sunflower, maize, rice, sorghum, soybean, and *Arabidopsis* (Ohashi et al., 2015; Gaufichon et al., 2013; Gaufichon et al., 2010). *Arabidopsis* asparagine synthetase *AtASN1* is responsible for nitrogen movement during germination and leaf senescence, while *AtASN2* plays a role in nitrogen accumulation and distribution in abiotic and biotic stress conditions. *ASN1* in tomato has increased expression due to bacterial pathogens (Gaufichon et al., 2010; Ohashi et al., 2015; Gaufichon et al., 2013). *OsAS1* is important for the synthesis of asparagine and assimilation of nitrogen in rice roots (Ohashi et al., 2015). Like AlaAT, the glutamine-dependent asparagine synthetase may be induced during *P. triticina* infection due to the pathogen altering nitrogen accumulation and distribution.

In this study, a transgenic approach was taken towards obtaining resistance to *P. triticina* and to gain a greater understanding of the *P. triticina*-wheat interaction. RNAi was used to silence seven genes in the host whose gene expression was induced by *P. triticina* infection. The wheat genes were cloned in an RNAi hairpin construct and transformed in embryonic callus using particle bombardment. Transgenic plants were obtained for each construct and molecularly characterized through three generations for the presence of the transgene and expression of the hairpin construct and endogenous wheat gene. A bioassay was conducted to determine if the silenced genes conditioned resistance to *P. triticina*; however, the endogenous wheat genes were not completely silenced and the transgenic plants maintained susceptibility and only showed slight signs of resistance. Although the gene silencing was not effective, the gene candidates

evaluated are likely to be genes involved in *P. triticina* infection based on the proposed gene function and gene expression found in Chapter 2. In future endeavors, gene-editing techniques such as CRISPR-Cas9 could be implemented to re-evaluate the targets. CRISPR-Cas9 can be designed for higher gene specificity than RNAi and results in a complete gene knockout rather than just a knockdown. CRISPRs can also be used to multiplex and simultaneously target multiple genes or multiple gene copies, which would be very useful in a hexaploid species (Kim et al., 2014). However, there is a strong possibility that completely silencing the wheat genes with CRISPR-Cas9 may be lethal to the plant because the targeted wheat genes are involved in essential plant functions.

Based on putative function, an additional candidate for silencing using gene editing is the brown planthopper susceptibility gene. Although the specific susceptibility gene identified in this study has not been characterized for molecular function, other brown planthopper resistance genes have. *Bph14* (Du et al., 2009), *BPH18* (Ji et al., 2016), and *BPH26* (Tamura et al., 2014) have been identified to code for coiled coil nucleotide binding site leucine rich repeat (CC-NBS-LRR) proteins and *BPH9* (Zhao et al., 2016) codes for an NBS-LRR protein. *Bph14* activates the salicylic acid pathway, while *BPH9* induces both the salicylic acid and jasmonic acid signaling pathways in rice (Du et al., 2009; Zhao et al., 2016). *BPH26* confers resistance by inhibiting insect feeding in the phloem (Ji et al., 2016). The NBS domains of *BPH9*, *Bph14*, and *BPH18* have slightly different sequences from what is usually seen in NBS-LRR proteins; for example, *BPH18* codes for two NBS domains (Ji et al., 2016; Du et al., 2009; Zhao et al., 2016). Genes that confer resistance to brown planthoppers but do not encode NBS-LRR are *BPH29* and *Bph3*. *BPH29* is a recessive resistance gene that codes for a B3 DNA-binding domain and activates the salicylic acid pathway (Wang et al., 2015). *Bph3* is a locus that contains three genes encoding

plasma membrane-localized lectin receptor kinases and provides broad-spectrum resistance to many rice insect pests (Liu et al., 2015; Ji et al., 2016). Two genes that could be additional gene-silencing candidates based on gene expression patterns have putative functions as a chloroplast ribulose-1, 5-bisphosphase carboxylase activase (RuBisCO) and an ananain cysteine-type protease. Both of these genes have a similar gene expression pattern induced by leaf rust infection. At three and four DPI, the genes have low expression and then at five and six DPI the gene expression reduces drastically by four times. The gene expression is similar in response to all six races. While most of the characterized genes showed an increase in gene expression at five and six DPI, these two genes appear to be suppressed by *P. triticina* infection.

Figures and Tables

Primer Name	Sequence (5' to 3')	Product Size	Description
16207 F	CACCATCCAGGTCTTTGAGGGTG	203 bp	Amplification of 16207 fragment in RNAi vector
16207 R	CACCTTCTCGTTCGTGATGGTG		
16209 F	CACCTTTCACCACCTACCAGGACC	221 bp	Amplification of 16209 fragment in RNAi vector
16209 R	CACCTCTTCTCAGACTTGCCCGTG		
955 F	CACCAGATACAGCACTCCACAG	240 bp	Amplification of 955 fragment in RNAi vector
955 R	CACCAGTTGGCTTGAGCAGTAG		
2283 F	CACCGTTCAATCTCGTTACTGGC	268 bp	Amplification of 2283 fragment in RNAi vector
2283 R	CACCAACTCCTGGATACCGTAGG		
20213 F	CACCGAAGCATAGCGAACAGAG	217 bp	Amplification of 20213 fragment in RNAi vector
20213 R	CACCAACTGGAGGGAGTGAATG		
15148 F	CACCACTGGTTCGGTTCGTTAG	288 bp	Amplification of 15148 fragment in RNAi vector
15148 R	CACCTCGTTGACGGTGATGTTG		
13984 F	CACCGTTCGTTAGGGTTTAGTAGC	277 bp	Amplification of 13984 fragment in RNAi vector
13984 R	CACCTCGTTGACGGTGATGTTG		
gusF1	ATCTCTTTGATGTGCTGTGCC	216 + GOI	Used with gene specific primers to determine the presence of the GOI
gusR2	GTATCAGTGTGCATGGCTGG	353 + GOI	
16207 F	CCATCACGAACGAGAAA	454 bp	Used in RT PCR to amplify 16207 fragment outside of insert
16207 R	TCTCACCCTAGTAACCC		
955 F	TGGTGGAATAGTGGAATTAG	267 bp	Used in RT PCR to amplify 955 fragment outside of insert
955 R	CCGAAGGTGTTGAAGTAG		

16209 F	GAGGACAAGAAGGTGAAG	473 bp	Used in RT PCR to amplify 16209 fragment outside of insert
16209 R	CGGCCAACAGACTAATAA		
2283 F	CAGGAGTTACACGATCTC	260 bp	Used in RT PCR to amplify 2283 fragment outside of insert
2283 R	CTCACGCAGAATCAAATG		
13984 F	CCGTTGGCTATGAATGA	226 bp	Used in RT PCR to amplify 13984 fragment outside of insert
13984 R	CCGGTTAAGAGGTTGTAA		
20213 F	GGATCGGGTAACTAGGATA	254 bp	Used in RT PCR to amplify 20213 fragment outside of insert
20213 R	CGCGGATAAACAGACAA		
16207 F	TCCAGGTCTTTGAGGGTGAG	203 bp	Real time PCR primers to detect endogenous gene
16207 R	CTTTCTCGTTCGTGATGGTG		
16209 F	GCTGTTATTAGTCTGTTGGC	114 bp	Real time PCR primers to detect endogenous gene
16209 R	GCATCATCTTTCCTTCATCC		
955 F	CTTACTGCATCCATCTTCCT	86 bp	Real time PCR primers to detect endogenous gene
955 R	GGACGCTAATATACACCTACTG		
2283 F	CATTTGATTCTGCGTGAGC	120 bp	Real time PCR primers to detect endogenous gene
2283 R	TTGATGACGAGGAGCAAC		

13984 F	AACTGGAGGGAGTGAATG	162 bp	Real time PCR primers to detect endogenous gene
13984 R	AACATCTCGGAACCTACG		
20213 F	GAAGCATAGCGAACAGAG	217 bp	Real time PCR primers to detect endogenous gene
20213 R	AACTGGAGGGAGTGAATG		
Ubq F	GCACCTTGGCGGACTACAACATTC		Real time PCR primers for housekeeping gene
Ubq R	GACACCGAAGACGAGACTTGTGAACC		

Table 3.1 Primers and descriptions of uses in this study.

Primer pairs are noted as "F" for forward and "R" for reverse.

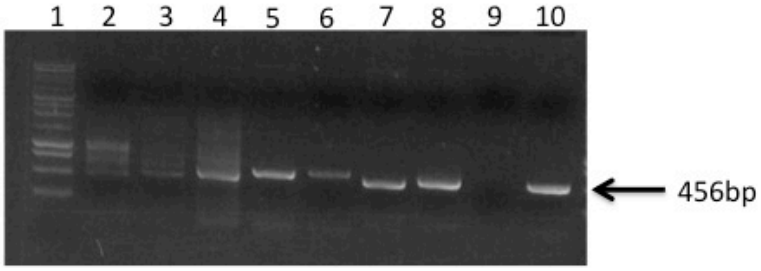


Figure 3.1 PCR analysis on gDNA of T_1 RNAi transgenic wheat.

GUS forward (gus F1) and insert R primers for each gene were used. Lane 1: Marker, Lane 2: ER-1, Lane 3: ER-2, Lane 4: GDAS, Lane 5: RBP-3, Lane 6: RBP-2, Lane 7: RBP-1, Lane 8: ALAT, Lane 9: non-transgenic Bobwhite, Lane 10: Plasmid

Construct	Sequence	Fragment Size
ER-1	AGCGCAGCATGACCAAGGACTGCCGTCTTCTCGGCAAGTTCGACCTTTCTGGCATTCCCACAGCTACCAGGGGCACT CCTCAGATCGAAGTCACCTTCGAGGTTGACGCCAACGGTATCCTGAACGTGAAGGCGGAGGACAAGGGCACAGGC AAGTCGGAGAAGATCACCATCACGAACGAGAAAGGGCGCCTGAGCCAGGAG	203bp
ER-2	AGCAGACCACCGTCTTTATTCCGNTCTTTGAGGGTGAGCGCAGCATGACCAAGGACTCCCGTTTTCTCGGCAAGTTC GACCTTTCTGGCATTCCCCCAGCTCCAAGGGGCACTCCTCAGATTGAAGTCACCTTCGAGGTTGACGCCAACGGTAT CCTGAACGTGAAGGCTGAGGACAAGGGCACGGGCAAGTCTGAGAAGATCACCATCACCAACGAGAAG	211bp
ALAT	TTCTGTATCTGAATCATGCAATTGCCGACTTTGCTGAGGCTCTGGCGGCCAAAATGCCCGGTGATCTGAAGGTTGTT TTCTTCACAAATTCTGGCACGGAGGCAAATGAGCTTGCACTGATGATCGCTCGGCTTTACACTGGTTCCCATGACATT ATTTCACTGAGGAATGGATACCATGGGAATGCAGCTGCAACAATGGGTGCTACTGCTCAAGCCAACTGGAAATTTA ATGTTGTCC	240bp
GDAS	AAGTCACTTTACTCGTACCGTAATGCATGATCCCGTGACCAGACACTTGGTCACTTTGAGCTCATTATGATGATGCAT TACCGAGTGGGCCCAGAGATACCTCTCCGTCATACGGAGTGACAAATCCCAGTCTTGATCCGTGTCAACCCAACAAA CACTTTCGGAGATACCCGTAGTATACCTTTATAGTCACCCAGTTATGTTGTGACGTTTGGTACACCCAAAGCACTCCT ACGGTATCCAGGAGTTACACGATCTCATGGTCTGC	268bp
RBP-1	CGGGGACGGAACAGTAACACGGAACGGTAGCAGCGTCACATCTAACGGTAGCCTGAACATCTCGGAACCTACGCG ACGGAGCGAGATCTAGGATACTCGGGAGCGAGCGAGCGATAACATTTTGGTAGACAGGTAAAAACGGATCGGGTA ACTAGGATAACTGGCCAGCGAGGGGCCCCAGCATTCACTCCCTCCAGTTGCCGCCGCCGGAGCCGCC	217bp
RBP-3	GGTTTAGTAGTGGGGAAGAGGAGGAAGAATGGCGGACGTCGAGTACCGCTGCTTCGTCGGCGGCCTCGCCTGGGC CACCGACGACCAGTCCCTCCAGAACGCCTTCTCCAAGTACGGCGACGTCATCGACTCCAAGATCATCACTGACAGGG AGACGGGCCGTTCCCGCGGGTTCGGGTTCGTCACCTTCGCGTCGGACGAGGCGATGCGCCAGGCGATCGAGGCCA TGAACGGCCAGGACCTGGACGGCCGCAACATCACCGTCAACGAGGCCCAGTCCCGCCGCTC	288bp
RBP-2	GGGGAAGAGGAAGGCATGGCGGACGTCGAGTACCGCTGCTTCGTGGGCGGCCTCGCCTGGGCCACCGACGACCA GTCCCTCCAGAACGCCTTCTCCAAGTACGGCGACGTCATCGACTCCAAGATCATCACTGACAGGGAGACGGGCCGTT CCCGCGGGTTCGGGTTCGTCACCTTCGCGTCGGACGAGGCGATGCGCCAGGCGATCGAGGCCATGAACGGCCAGG ACCTGGACGGCCGCAACATCACCGTCAACGAGGCCCAGTCCCGCCGCTCCG	277bp

Table 3.2 Sequence of the fragments cloned into the RNAi pANDAmini hairpin vector for plant expression.

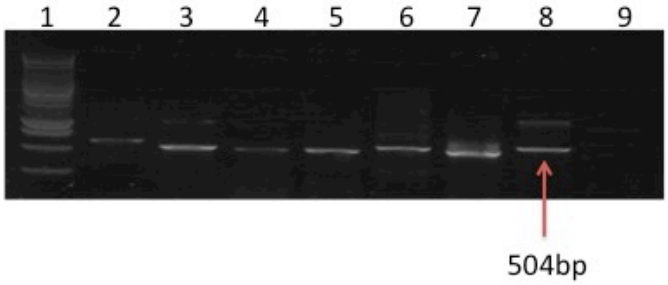


Figure 3.2 PCR analysis on gDNA of T_2 RNAi transgenic wheat.

GUS forward (gus F1) and insert R primers for each gene were used. Lane 1: Marker, Lane 2: RBP-2, Lane 3: RBP-1, Lane 4: ER-1, Lane 5: ER-2, Lane 6: GDAS, Lane 7: ALAT, Lane 8: RBP-3, Lane 9: non-transgenic Bobwhite

Infection with *Puccinia triticina*: T_2 Generation			
Event	Total Plants	GOI +	Resistant to *P. triticina*
RNA Binding Protein-1			
Event 1 3040	54	37	5
Event 2 3242	34	28	0
Event 3 3212	22	10	0
RNA Binding Protein-2			
Event 1 2730	72	49	5
Event 2 2816	76	23	1
Event 3 3122	20	0	0
Event 4 4054	30	0	0
ER Molecular Chaperone-1			
Event 1 4303	80	62	7
Event 2 4310	40	19	2
Event 3 3966	20	14	1
Event 4 3968	44	27	2
Event 5 4218	28	21	3
ER Molecular Chaperone-2			
Event 1 4249	26	18	1
Event 2 3977	42	26	1
Event 3 3979	28	16	0
Alanine Glyoxylate Aminotransferase			
Event 1 2733	103	92	5
Glutamine Dependent Asapragine Synthetase			
Event 1 4062	80	50	1
Event 2	98	80	3

4063			
Event 3 4069	20	20	1
Event 4 4101	40	28	3
Event 5 4308	44	26	2
Event 6 4359	97	78	2
Event 7 4360	78	68	2
Event 8 4425	20	9	1

Table 3.3 Results of *Puccinia triticina* bioassay of T_2 by showing resistance based on phenotypic scores.

A bioassay was conducted in the T_2 generation by inoculating with a virulent race of leaf rust and scoring the plants on a 0-4 Stakman scale. The resistant plants displayed symptoms slightly different from the non-transgenic control, such as flecking and smaller pustules on the leaf tip. Plants were also tested for the presence of the hairpin construct using PCR and are shown in the GOI+ column. Plants from each construct, except the RNA binding protein-3, and event are represented.

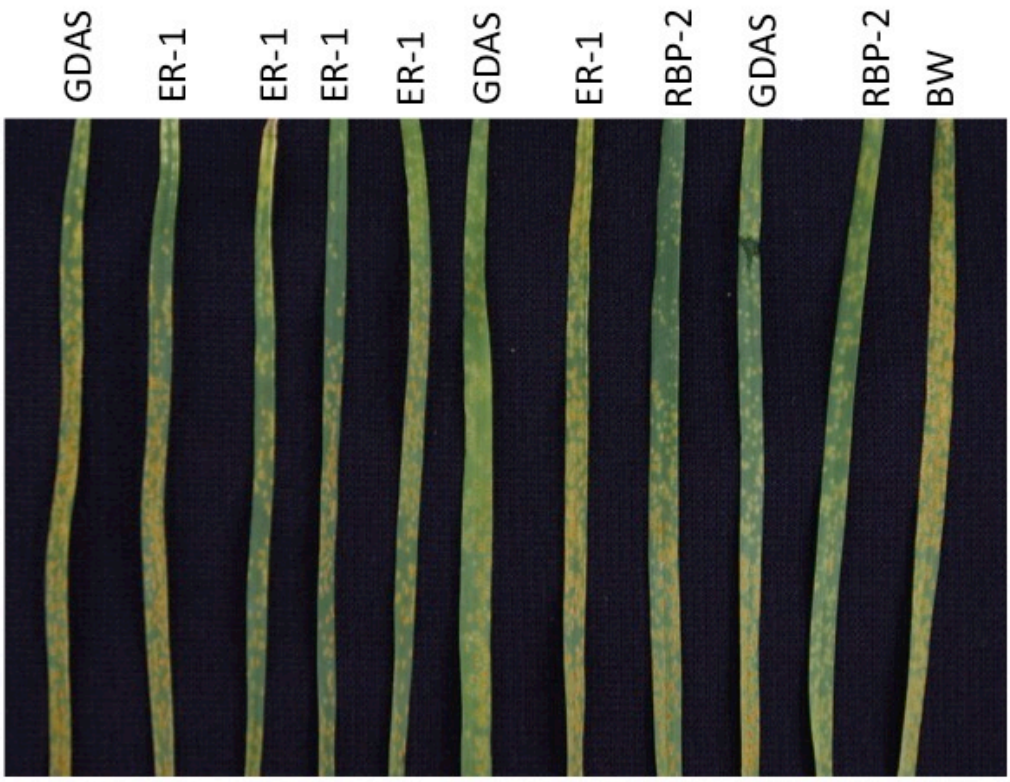


Figure 3.3 T_2 plants showing *P. triticina* infection symptoms from the bioassay .

Plants were inoculated with a virulent race of leaf rust and scored 10-14 DPI on a 0-4 Stakman scale. "BW" is non-transgenic Bobwhite used as a positive control. The transgenic plants were not resistant on the Stakman scale, but some had altered symptoms from BW such as flecking or smaller spores on the leaf tip.

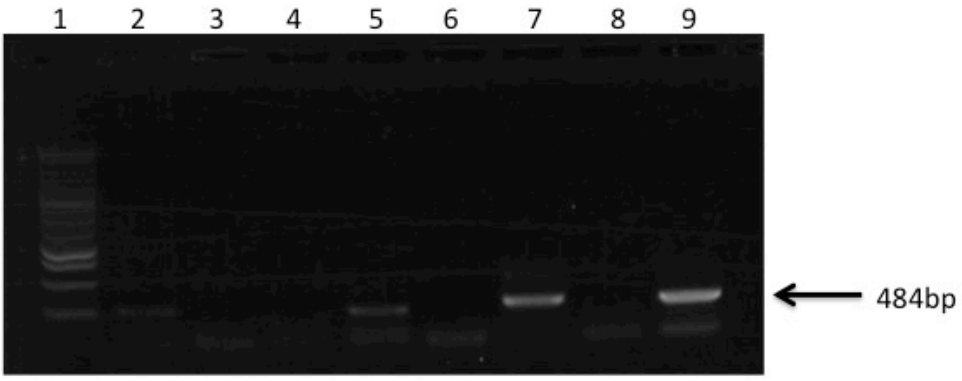


Figure 3.4 rt PCR analysis of T_2 generation cDNA with GUS forward (gusF1) and insert reverse primers were used.

Lane 1: Marker, Lane 2: RBP-1, Lane 3: RBP-2, Lane 4: ER-2, Lane 5: ALAT, Lane 6: ER-1, Lane 7: GDAS, Lane 8: non-transgenic Bobwhite, Lane 9: plasmid (BW) Some of the hairpin constructs were expressing in individual plants (Lane 1, 5, 7) while others were silenced (Lane 3, 4, 6)

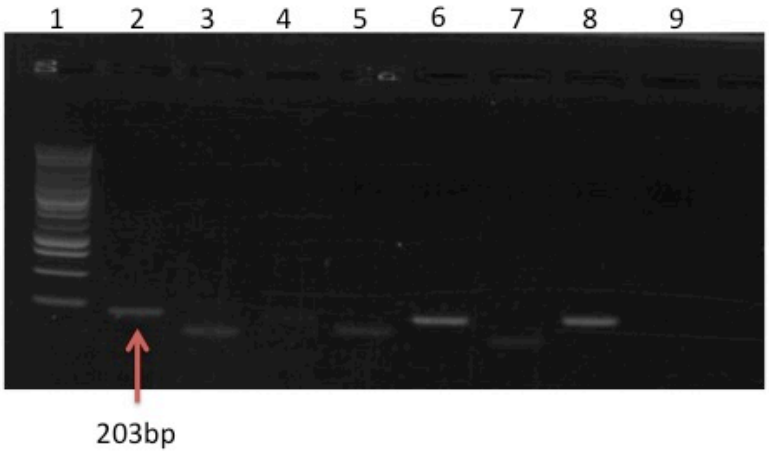


Figure 3.5 rt PCR analysis on cDNA from the T_2 generation with primers designed outside the gene fragment insert.

Lane 1: Marker, Lane 2: ER-1, Lane 3: ALAT, Lane 4: GDAS, Lane 5: RBP-2, Lane 6: RBP-1, Lane 7: ER-2, Lane 8: non-transgenic Bobwhite Lane 9: Plasmid. RNAi did not silence the endogenous genes.

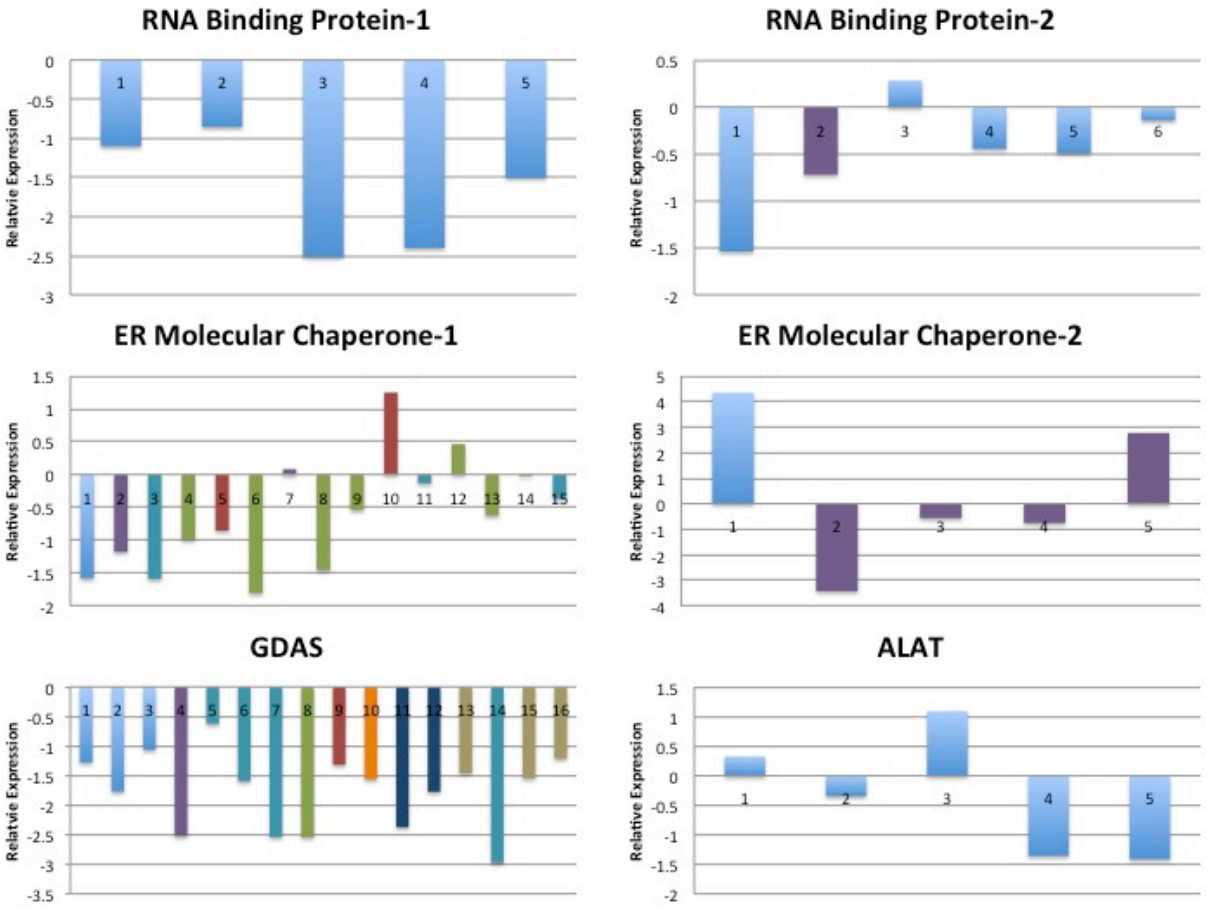


Figure 3.6. Detection of down-regulation of targeted endogenous genes.

qPCR analysis on cDNA from T_2 plants with small infection phenotypic differences from the non-transgenic control with primers designed outside the gene fragment insert. Each graph shows the endogenous gene expression level on individual T_2 plants for a particular gene. The bars represent a different T_2 plant. Bars of the same color represent T_2 plants from the same transgenic event. Although suppressed, there were no endogenous genes that were silenced.

Chapter 4 - Resistance to *Puccinia triticina* in wheat generated by EMS mutation

Abstract

As the population of the world continues to increase at a drastic rate, there is an ever-pressing need to produce more food on less land. One way to achieve crop improvement is by increasing the genetic diversity through mutagenesis. Mutagenesis is a versatile technique that has been used in plant research for decades and has maintained popularity through new techniques such as TILLING. Breeding programs utilize mutagenesis in forward genetic studies for improvement of specific agronomic traits. Mutagenesis also has applications in reverse genetics studies for applied research. Mutations can be generated using radiation or chemical mutagens. Chemical mutagens, such as ethyl methanesulfonate (EMS), can generate a high density of point mutations in a wide range of crop species, regardless of ploidy level, genome size, and amount of sequence data. In this study, EMS was used to create a mutant population of a *P. triticina* susceptible variety of hexaploid bread wheat. Out of the initial M_1 population of 3780 mutants, 1500 M_2 mutants were inoculated with a virulent *P. triticina* race. Five hundred and seventy mutants with phenotypic differences compared to the non-mutated control were selected. After two additional generations of selection, eight mutant lines were identified that were resistant to *P. triticina*. The eight resistant mutants were evaluated for the expression of the seven wheat genes identified in Chapter 2 and silenced in Chapter 3 using real time PCR. One of the resistant mutants had decreased expression for ER molecular chaperone-1. This may indicate that a functioning ER molecular chaperone-1 is required for an aspect of *P. triticina* infection. Additional research is needed to confirm the resistant mutant is a knockout for the ER molecular chaperone-1 and to identify the mutated genes in the other seven resistant mutants.

Introduction

With the world's population expected to reach 9 billion people by 2050, there is a pressing need to produce more food on less land (FAO, 2009). To keep up with this rapid population growth, the production of cereal crops has to increase 50% by 2030 (Foresight: The Future of Food and Farming, 2011) and 70% by 2050 (FAO, 2009). One factor limiting the ability to increase food production is the lack of genetic diversity among crop varieties (Chen et al., 2014). Increasing the genetic diversity has been a proven strategy for advancement in all major food crops (Parry et al., 2009). One method to facilitate genetic diversity is to utilize the natural genetic diversity from wild relatives. While wild relatives have been used successfully in breeding programs to bring in novel agronomic important traits, they can also be challenging to work with. Transferring genes from even a closely related wild relative to an adapted cultivar often causes linkage drag that introduces undesirable agronomic traits into the germplasm (Elkot et al., 2015). Certain "pre-breeding" strategies such as marker-assisted introgression can aid in the precise transfer of genes and reduce linkage drag; however the necessary molecular markers are time and resource intensive to develop due to the complexities of the wheat genome (Elkot et al., 2015; Allen et al., 2011).

Another method to increase the genetic diversity and develop improved crop plants is mutagenesis. Mutagenesis has remained a popular tool for decades and has a wide range of applications in many facets of crop improvement. One such application of mutagenesis is in breeding programs. Mutagenesis has been used in breeding programs for the last 60 years (Botticella et al., 2011). The FAO/IAEA program has estimated that over 3,200 crop varieties with traits obtained through mutagenesis have been released in the last 40 years (Chen et al.,

2014) and over 150 are bread wheat varieties (Sestili et al., 2010). One application of mutagenesis in breeding programs is in forward genetic screens to generate plants with desirable agronomic traits, such as grain quality, nutrient use efficiency, stress tolerance, herbicide resistance, insect resistance, and pathogen resistance. The improved material can then be adopted into the breeding pipeline and incorporated into varieties being developed for commercial release (Chen et al., 2014; Parry et al., 2009). Mutagenesis has remained an important resource in crop breeding and genetics for several reasons. Mutagenesis can create changes throughout the entire genome of an organism, regardless of ploidy level and without the need of extensive sequence data. There is also no need to work with un-adapted land races as any cultivar can be used as the starting material, even elite varieties (Parry et al., 2009). In addition to applications in breeding programs with forward genetic studies, mutagenesis can also be used for basic research in reverse genetic studies. With sequence data becoming cheaper and faster to obtain, there has been an increased need to link gene sequence to gene function. Other reverse genetics methods besides mutagenesis include RNAi, transposons, and T-DNA insertion; some of the methods are preferable in certain research applications. For example, RNAi can target alleles of single genes at once, while mutagenesis generally only targets a single copy of a gene (Parry et al., 2009). However, there are many advantages that mutagenesis has over RNAi, transposons, and T-DNA insertion. Mutants are not considered transgenic; this makes conducting field trials easier and eliminates the expensive and time consuming deregulation process if the variety is going to be commercially released. Secondly, RNAi requires the plant to undergo transformation, which limits the crop species that can utilize the technology. RNAi is also considered to knockdown gene expression rather than a cause a complete gene knockout that can be achieved using mutagenesis. T-DNA and transposon insertion also requires efficient plant transformation.. In

addition, transposon insertions may be challenging to implement in breeding programs because of the types of mutations they generate (Chen et al., 2014).

There are several methods used to generate mutations. One of the first methods used to induce mutations was applying radiation via X-rays. This was first done in *Drosophila* and later in plants such as barley and maize (Muller, 1930; Stadler, 1932). Irradiation was thought to alter the organism by breaking or rearranging the chromosomes or changing individual genes (Stadler, 1932). A second method to induce mutations is chemical mutagenesis. There are three classes of mutations that result from the point mutations induced by chemical mutation. Nonsense mutations are caused when an amino acid codon is changed to a stop codon. Missense mutations result when a single base change alters the codon so it codes for a different amino acid. Silent mutations occur when a single base of a codon is changed, but it does not alter the coded amino acid (McCallum et al., 2000b). Chemical mutagenesis, which can be done with ethyl methanesulfonate (EMS), sodium azide, or *N*-methyl-*N*-nitrosourea (MNU), is still widely used today because it creates a high mutational density of a wide range of mutations in many organisms. EMS is one of the most commonly used chemical mutagens. EMS induces a high frequency of single base changes or point mutations even in polyploid plants. The point mutations generated with EMS are primarily C/G to T/A changes (Henikoff and Comai, 2003; McCallum et al., 2000a), and thus the GC content of an organism can have an effect on the mutation density (Uauy et al., 2009). EMS induces a larger proportion of nonsense mutations than MNU (Parry et al., 2009). Genome size does not seem to impair the effectiveness of EMS; a similar gene mutational density has been estimated in *Arabidopsis* and in maize (Henikoff and Comai, 2003).

As powerful and useful as chemical mutagenesis is, no technique is without challenges. One of the most difficult aspects of using a chemical mutagen is achieving the appropriate dosage. Different plant species may require different dosages of the chemical to achieve appropriate levels of toxicity. In addition, variability in mutation rate is common even when different batches of the same seed are produced under identical conditions. Too high of a dosage rate will cause high rates of sterility, while a low dosage will result in a low mutation density and require additional screening and larger population sizes to identify the desired phenotypes (Henikoff and Comai, 2003). Diploid organisms can be particularly challenging to achieve an appropriate dosage level, as even low levels of the mutagen can cause sterility. On the other hand polyploid species, such as wheat, have a very high tolerance due to the redundancy of essential genes. Thus, larger mutant populations are required in diploid organisms than polyploidy to obtain a population with high mutation frequency (Parry et al., 2009). Another major challenge with chemical mutagenesis is sifting through a large mutant population to identify individual plants with novel or desirable phenotypes. To cause added difficulty in identifying mutations in polyploid species, mutations in a single copy of a homolog do not always generate a visible phenotype due to genetic redundancy (Chen et al., 2014; Parry et al., 2009; Botticella et al., 2011). To expedite the identification process, McCallum et al. (2000a) developed a reverse genetics method called TILLING (Targeting Induced Local Lesions In Genomes). In this method, point mutations induced by chemical mutagenesis are identified in pooled DNAs and sequenced (Henikoff and Comai, 2003). TILLING can detect multiple missense and nonsense mutations in target regions and can even identify individual plants with heterozygous recessive mutations (Chen et al., 2014; Parry et al., 2009). TILLING is most useful in identifying mutations that cause partial or complete loss of function rather than identifying mutations that

lead to overexpression or suppression of multiple genes (Slade et al., 2005). Since the mutant population is generated using chemical mutagenesis, TILLING can be used for any organism that can be mutated in a high frequency, regardless of genetic resources, homozygosity level in the population, genome size, or ploidy level (McCallum et al., 2000a; McCallum et al., 2000b; Chen et al., 2014). TILLING has been used in many crop plants including bread wheat, durum wheat, pea, rice, maize, barley, soybean, sorghum, potato, peanut, oat, and tomato (Chen et al., 2014; Parry et al., 2009).

The initial TILLING protocol developed by McCallum et al. (2000a) utilized an EMS generated mutant *Arabidopsis* population. The mutant DNA was pooled and the region of interest were amplified using PCR. Heteroduplexes were formed between wild type and mutant fragments using denaturation and annealing, and the mutations were identified using a denaturing high-performance liquid chromatography (DHPLC) machine, which detects base pair changes in the heteroduplexes. A mismatch between the wild type and mutant fragments is seen as a peak in a chromatogram and allows for the mutated individual to be identified and sequenced (McCallum et al., 2000a; McCallum et al., 2000b). However, the DHPLC method was difficult to scale up for high throughput experiments, so an alternative low cost detection technique was developed for large-scale experiments. Colbert et al. (2001) developed an alternative mutant identification method using enzymatic digestion with the endonuclease CEL-1, gel electrophoresis, and the LI-COR gel analyzer system. CEL-1 is a plant-specific extracellular glycoprotein from celery that cleaves DNA at the 3' end of the mismatch and can detect all types of mutations. In the high throughput method, a CEL-1 solution is added to pooled DNA. The resulting cleaved fragments are amplified using PCR and labeled with two fluorescently labeled primers. Denaturing polyacrylamide gel electrophoresis (denaturing PAGE)

separates the different fluorescents and LI-COR DNA scanners are used to generate a gel image to visually detect and determine the exact base position of mutations. Alternative visualization methods without the use of fluorescent primers, such as non-denaturing polyacrylamide gel or agarose gel electrophoresis, can be used instead of the costly denaturing PAGE and LI-COR system (Colbert et al., 2001; Chen et al., 2014). Along with different visualization methods, various groups have identified other mismatch cleavage enzymes that can be used instead of CEL-1, such as Endo-1, which has been used with great success in wheat populations. Endo-1 was obtained from *Arabidopsis* and is closely related to CEL-1 (Parry et al., 2009).

Wheat research can benefit greatly from using mutagenesis and TILLING to modify specific genes that control agronomic important traits. The genome of bread wheat is 16000 megabases, which is 140 times the size of the *Arabidopsis thaliana* genome and five times the size of the human genome. Its large size, high copy level, and high repetitive DNA content of 83% can make using traditional functional genomics and reverse genetics methods challenging. However, mutagenesis and TILLING has proved successful in wheat, which makes it an invaluable tool in polyploid crop improvement (Slade et al., 2005; Uauy et al., 2009). Even though TILLING and mutagenesis is a great genetic resource for polyploids, it may require extra efforts than when working with other crop species with simpler genomes. In wheat, it is often necessary to identify single mutations in each of the A, B, and D genome homologs and cross the plants over several generations to create a triple mutant (Chen et al., 2014; Uauy et al., 2009). In addition, individual mutated plants may carry a large amount of background mutations. These may reduce the overall agronomic performance of the plant, so it is important to remove the background mutations if the material is going to be used in a breeding program. Making backcrosses of the mutant plant to the non-mutant starting plant material for two to three

generations will reduce the amount of background mutations (Uauy et al., 2009; Botticella et al., 2011).

A lot of the early work applying TILLING to wheat has targeted starch content. Amylose and amylopectin are two glucose polymers that make up starch and have large effects on the properties of flour. High and low amylose flour have specific uses. Low amylose starches are best suited for high quality pasta and bread, frozen foods, and high strength glues and paper products, while high amylose starch could have benefits in the cereal industry as it is digested in the body similar to high fiber foods (Sestili et al., 2010; Botticella et al., 2011; Slade et al., 2005). Slade et al. (2005) used TILLING to screen for mutations in *waxy* genes encoding granule-bound starch synthase I (*GBSSI*), which are responsible for amylose synthesis in storage tissues. Partial *waxy* wheat has one or two functional *GBSSI* genes and produces starch with intermediate levels of amylose, while fully functioning *waxy* starches found in maize and rice consist mostly of amylopectin and very little amylose. TILLING was used to identify 196 new alleles of the *waxy* genes in hexaploid wheat and 50 new alleles in a tetraploid wheat TILLING population (Slade et al., 2005). Botticella et al. (2011) used TILLING to identify mutations in the three homologs of starch branching enzyme *lla* genes (*SBElla*), which are involved in amylopectin synthesis. Single null homologous mutants were pyramided and the resulting double null mutant had an increase in amylose content by 21% compared to the control (Botticella et al., 2011). Simarily, Sestili et al. (2010) targeted two groups of synthase genes, one group encoded the starch synthase II (*Spg-1*) and the other group encoded the previously discussed *waxy* proteins (*Wx*) (Sestili et al., 2010). Altered phenotypes in many other wheat traits have been identified using TILLING such as carotenoid content (Colasuonno et al., 2016), grain width (Simmonds et al., 2016), flowering (Chen et al., 2014), and vernalization (Chen and Dubcovsky,

2012; Acevedo-Garcia et al., 2016). Although TILLING is a popular method for mutant identification, there are examples of wheat mutants that have been identified through other means. Feiz et al. (2009) used EMS to develop a wheat mutant population. The M_2 population was screened for new alleles of *Pina* and *Pinb*, two genes that make up the *Hardness* (*Ha*) locus that impact grain texture. Eighteen new alleles were found by phenotyping the M_2 population for grain hardness and confirmed by sequencing. F_2 populations were developed with four of the mutant alleles by backcrossing to the non-mutant background variety (Feiz et al., 2009). Secondly, Henry et al. (2014) used exome capture and a bioinformatics pipeline called MAPS (mutations and polymorphisms surveyor) to identify mutations in EMS mutant populations of rice and tetraploid wheat (Henry et al., 2014).

Along with using TILLING and mutagenesis to generate and identify mutants with improved agronomic qualities, these methods can also be used for developing disease resistance in crop plants. Hoffman et al. (2009) identified ethylene insensitive mutants in a mutant population of soybean. These mutants displayed less severe disease symptoms of *Pseudomonas syringae* pv. *glycinea* and *Phytophthora sojae* compared to the non-mutated controls, but more severe symptoms to *Septoria glycines* and *Rhizoctonia solani* (Hoffman et al., 1999). Uauy et al. (2009) used TILLING to identify loss of function mutations in a *Wheat Kinase Start 1* (*WKS1*) gene that resulted in susceptibility to *Puccinia striiformis* f. sp. *tritici* (Uauy et al., 2009). TILLING can also be used to create mutants in susceptibility genes to condition disease resistance (Acevedo-Garcia et al., 2016). The definition of a susceptibility factor is a plant protein that is required for the host to be susceptible to a pathogen (Eckardt, 2002; Eichmann et al., 2010). A loss in function of the plant protein will result in decreased susceptibility, while overexpression of the protein will cause the plant to be more susceptible (Eichmann et al., 2010).

Obligate biotrophic pathogens, such as *P. triticina*, are reliant on their host for certain metabolic processes, thus loss of host factors involved in these processes may impact host susceptibility. The pathogen would have to evolve and alter key points of its biology in order to regain function. This makes resistance generated from loss of susceptibility factors very durable (Huckelhoven et al., 2013). One of the best-characterized susceptibility factors is the barley protein *mildew locus o* (*mlo*). *mlo* is required for susceptibility to *Blumeria graminis* f.sp. *tritici* and loss of function results in non-race specific resistance. While the exact processes are not known, *mlo* is thought to prevent the fungus from penetrating the host via hydrogen-peroxide accumulation (Eichmann et al., 2010). A recent study conducted by Acevedo-Garcia et al. (2016) used a TILLING approach to select for partial loss of function alleles of *TaMlo*, the wheat ortholog of *mlo*. Mutations were identified in each of the three homologs. Triple and even some double mutant lines showed increased resistance to powdery mildew (Acevedo-Garcia et al., 2016).

This study takes a forward genetics approach to *P. triticina* resistance. *P. triticina* is an obligate biotrophic fungal pathogen that can cause significant yield losses for wheat farmers. In this study, EMS was used to develop a mutant population of a susceptible variety of bread wheat. The population was screened for individuals with increased resistance or susceptibility to a virulent *P. triticina* race. In the M_4 generation, eight individuals were selected that displayed a resistant phenotype. The eight individuals were analyzed for gene expression of the seven wheat genes identified in Chapter 2 and silenced using RNAi in Chapter 3. One of the resistant mutants showed decreased expression of the ER molecular chaperone-1. This shows that although the RNAi silencing was not effective, the genes identified in Chapter 2 may be essential for *P. triticina* infection. Further research needs to be done to identify the wheat genes that were

mutated in the resistant individuals and to confirm the mutation of the ER molecular chaperone-1.

Materials and Methods

Mutagenesis and Population Development

Seeds were mutated according to a protocol given by Michael Pumphrey (personal commun.). Three hundred seeds of the hard red spring wheat cultivar Thatcher (University of Minnesota, 1936) were soaked in 100ml ddH_2O for eight hours on a shaker at 75 rpm, with the water changed every two hours. The water was drained and replaced with 75ml 0.3-0.45% v/v EMS- ddH_2O solution (Sigma Life Sciences, St. Louis, MO) and the seeds were soaked on a shaker for sixteen hours. The EMS solution was drained and the seeds were rinsed for two hours under running H_2O. The seeds were planted in root trainer trays containing Metro Mix 360 soil medium (SunGro, Vancouver, Canada) and grown in a greenhouse under standard conditions. The M_1 population of 3780 plants was harvested individually. For the M_2 generation, 1500 lines were planted in root trainers containing Metro Mix 360 soil medium. Seedlings were grown to the two-three-leaf stage and inoculated with *P. triticina* race BBBD. The spores were heat shocked in a water bath at 42°C for 20 minutes. 5 mg of spores were suspended per ml Soltrol 170 isoparaffin solvent (Philips 66, Bartlesville, OK) in an atomizer. The suspension was sprayed onto the seedlings with an air compressor at 40 PSI. The seedlings were placed in an 18 °C dark 100% humidity Percival Intellus dew chamber overnight for 16 hours, then returned to the greenhouse. At 10-14 days post inoculation, the plants were scored using a 0-4 rating scale developed by Stakman et al. (1962) and Gassner and Straib (1932) and modified by McIntosh et al. (1995) (McIntosh et al., 1995; Stakman et al., 1962; Gassner and Straib, 1932). Five hundred seventy mutants that displayed infection symptoms greater or less than the non-mutated control

were transplanted to a gallon-sized pot with Metro Mix 360, grown to seed, and harvested individually. The 570 lines were planted for the M_3 generation and inoculated with leaf rust as described above. One hundred fourteen mutant lines were selected, transplanted to gallon pots, and harvested individually. In the M_4 generation, the 114 lines were planted and evaluated for resistance to leaf rust. Out of the 114 lines, eight of the most resistant lines were selected for further evaluation.

Characterization Using qPCR

For each of the eight selected M_4 lines, 10-12cm^2 of leaf tissue was collected, flash frozen in liquid nitrogen and processed using the *mir*Vana miRNA isolation kit (AM 1560, RNA Life Technologies, Carlsbad, CA) according to the manufacturer's instructions and following the recommendation to remove the miRNA enrichment step. The RNA was quantified using a Nanodrop ND1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). One ug RNA was mixed with random hexamers to obtain first strand synthesis, and then reverse transcription with Superscript RTII (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. The resulting cDNA was diluted in a 2:1 ratio and used as a template for real-time PCR (qPCR) to determine the relative expression of the target genes identified in Chapter 2 and silenced in Chapter 3. The primers were designed using the unique assembled transcript fragment derived *Triticum aestivum* mRNAs (based on GenBank release 163) for each gene. After design, the primers were checked for dimer formation and efficiency. Each 25µL reaction contained 6µL cDNA template and 10pmol of each primer. The qPCR conditions are as follows: 95°C for 3 minutes, 40 cycles of 95°C for 10 seconds, and 62°C for 30 seconds. At the end of a run, a melt curve was determined by heating 65°C to 95°C in 0.5°C increments for 5 seconds. All reactions were conducted with the Bio-Rad CFX96 Real-Time System and the Bio-Rad iQ SYBR Green

Supermix (Bio-Rad Laboratories, Inc., La Jolla, CA). Each reaction was run in triplicate. A wheat ubiquitin housekeeping gene (Paolacci et al., 2009) and the non-mutated Thatcher were used in the ΔΔCt method to calculate gene expression (Livak and Schmittgen, 2001). Ct values over 30 were considered unreliable and indicative of non-expression. Any values over 30 were considered 30 so the ΔΔCt could be calculated.

Results and Discussion

Leaf rust is an obligate biotrophic fungus that can cause up to 50% yield losses on wheat during epidemics (Appel et al., 2011). Using genetic resistance is the preferred control strategy, but it is not always effective (Bolton et al., 2008). Major gene resistance puts strong selection pressure on virulent isolates of the pathogen and is associated with the boom and bust cycle (Huerta-Espino et al., 2011). Minor genes can be as effective as major genes if used in conjunction with other resistance genes and are more durable because they are not race specific. However, stacking genes should be done with great consideration, especially if the genes have been previously deployed. Minor genes can also be very challenging to integrate into adapted varieties (Hulbert and Pumphrey, 2013; Singh et al., 2011). Utilizing transgenics is another method that can be used for leaf rust control. Panwar et al. (2013) used a *Barley stripe mosaic virus*-mediated host induced gene silencing (BSMV-mediated HIGS) and a modified *Agrobacterium* system to target three predicted pathogenicity genes, MAPK, cyclophilin, and a calcineurin regulatory subunit, that resulted in suppressed symptoms of all three wheat rusts. Yin et al. (2014) also utilized a BSMV-mediated HIGS system to target tryptophan 2-mono-oxygenase, a gene expressed in haustoria cells, to reduce *P. graminis* infection. Transgenics can be used to target genes essential for pathogenicity that would require a large fitness cost for the pathogen to overcome and result in durable broad-spectrum resistance. However, transgenics are

time consuming and challenging to generate, especially in wheat. Regulations, which can make field trials challenging and the deregulation process long and expensive also hinder the application of transgenics.

Another approach to achieve durable disease resistance is mutagenesis. This study utilized a forward genetics mutation approach to identify individual plants with changes in susceptibility to *P. triticina*. The chemical mutagen EMS was used to create a mutant population of the susceptible wheat variety Thatcher. Each generation was inoculated with a virulent *P. triticina* race and plants with altered infection phenotypes compared to the non-mutated Thatcher were selected. In the M_4 generation, eight resistant mutants were selected and RNA was isolated. The expression levels of the seven genes identified in Chapter 2, three RNA binding proteins, two ER molecular chaperones, alanine glyoxylate aminotransferase, and glutamine dependent asparagine synthetase, were determined using real time PCR. One mutant showed decreased expression of ER molecular chaperone-1.

Soaking seeds of the susceptible hard red spring wheat cultivar Thatcher in the chemical mutagen EMS developed a mutant population. Kill curves were used to optimize the concentration of EMS to 0.3% to 0.45% in order to achieve the desired ~50% germination rates. Mutated plants from the kill curves with the appropriate germination rate were also used in the initial population. After the seeds were soaked in EMS and rinsed, the seeds were planted in large root trainers using forceps. A multitude of abnormal agronomic phenotypes was observed in the M_1 generation of the population. For example, some of the mutated plants were stunted, albino colored, had waxy thick leaves, abnormal spike configuration, variation in the presence and density of awns, or thin brittle leaves. There was variation seen in the color, size, and amount of seed produced. A high rate of sterility was also observed. The mutants that produced

seed were arbitrarily assigned a number from 1-3780 and harvested individually. The first 1500 mutant lines were continued to the M_2 generation. Fifteen seeds for each mutant line were planted into large root trainers and were inoculated with leaf rust at the two-leaf stage. Plants were scored 10-14 days later using a 0-4 Stakman scale. A range of infection phenotypes was observed compared to the non-mutated control, from increased susceptibility to resistant. Some mutants also displayed increased necrotic or chlorotic reactions associated with infection. Altered agronomic phenotypes and sterility were also observed in the M_2 generation. The M_2 generation resulted in 570 mutants with infection phenotypes different than the non-mutated controls and each plant was transplanted to gallon-sized pots, grown to seed, and harvested individually. The 570 selected lines were continued to the M_3 generation by planting 15 seeds per line in large root trainers. The mutants were inoculated at the two-leaf stage and scored 10-14 days post inoculation. The plants were scored and selected on the same criteria as in the M_2 generation and 114 mutants were selected, transplanted to gallon-sized pots, and harvested individually. In the M_4 generation, the 114 mutants were planted (15 seeds per line), inoculated with *P. triticina*, and scored. This generation of selection was used to identify mutants that primarily showed high levels of resistance and eight mutant lines were selected: 483, 1022, 1111, 1144, 1182, 1191, 1226, and 1268 (Figure 4.1). On the 0-4 Stakman scale, all of the mutants scored between 0-1 ratings. Segregation for the resistant phenotype was still observed in the M_4 generation. The resistant mutants had some variation in their phenotype; mutant 483 showed necrotic lesions, mutant 1022 had small lesions and leaf tip necrosis, and mutant 1191 displayed leaf chlorosis.

To characterize the eight resistant mutants for expression of the seven wheat genes identified in Chapter 2 and silenced in Chapter 3, real time PCR was conducted. Fifteen plants

per resistant line were planted and inoculated at the two-leaf stage. The mutant seedlings were scored 10-14 days post inoculation and tissue was collected from the plants showing resistance. RNA was isolated and cDNA was used as a template for real time PCR. The same primers that were used to characterize gene expression in Chapter 2 and used to test the efficiency of the RNAi on the endogenous host genes in Chapter 3 were used in the real time PCR reactions. The relative expression values were calculated using the ΔΔCt method with the non-mutated Thatcher and the housekeeping gene ubiquitin serving as controls (Figure 4.2). As can be seen in the figure, the relative expression level of the RNA binding protein-1 was decreased slightly for mutants 483, 1022, 1111, 1144, 1182, 1191, and 1268. Positive expression was maintained for 1226. The expression level of the RNA binding protein-2 was positive for 483, 1144, 1191, 1226, 1268, but was decreased slightly for 1022, 1111, and 1182. The expression level of the RNA binding protein-3 was positive for 483, 1022, 1144, 1182, 1191, 1226, and 1268 and barely negative for 1111. All of the resistant mutants maintained expression of the ER molecular chaperone-2. The glutamine dependent asparagine synthetase expression was positive for 483, 1022, 1111, 1144, and 1191, while expression was suppressed in 1182, 1226, and 1268. The alanine glyoxylate aminotransferase was expressing in all of the resistant mutants. Although the expression of some of the genes was suppressed, it was at such small values that the genes are most likely not mutated. However, the expression of the ER molecular chaperone-1 in mutant 1022 was at such low levels, it indicated that this gene may be mutated (Figure 4.3). The 1022 mutant had a raw expression (Ct value) of the ER molecular chaperone-1 over 30; Ct values over 30 are typically considered to be unreliable and designate non-expression of the gene. If the ER molecular chaperone-1 is silenced in resistant mutant 1022, it could imply that this gene is a susceptibility gene, in which reduced expression conditions resistance in the host. EMS typically

generates point mutations, which would be difficult to mutate all copies of the gene. A gene knockout could be possible if the mutation was in a non-coding region such as the gene promoter or a transcription factor. Further research is needed to confirm if mutant 1022 is a knockout mutant for ER molecular chaperone-1. If the mutation is confirmed, another factor to consider is how many functioning copies of the gene are present in non-mutated wheat and how many copies of the gene are functioning in the mutant.

The gene expression for the ER molecular chaperone-1 was evaluated in response to six races over the first seven days of infection in Chapter 2. The gene showed similar expression in response to all six races, but the expression was up-regulated four times from day zero to day two, down-regulated four times from day two to day four, then up regulated four times again from day four to day six post inoculation. Because of the fluctuations in gene expression in response to all six races, the ER molecular chaperone-1 may be a gene that is required for *P. triticina* infection. Furthermore, the mechanism of ER molecular chaperones in plants could influence its role on *P. triticina* infection. Secreted proteins enter the ER after synthesis. Proteins that are not folded correctly or are unstable, as well as non-native proteins, are recognized upon entry and are corrected by ER molecular chaperones to ensure the proteins are able to be transported through secretory pathways (Nishikawa et al., 2005; Gupta and Tutei, 2011; Jansen et al., 2012). ER chaperones, such as binding protein (*BiP*) and glucose-regulated protein (*GRP94*), have heightened expression during plant cell stress (Goeckeler et al., 2010; Gupta and Tutei, 2011). *BiP*, a member of the heat shock protein family HSP70, is thought to refold newly synthesized proteins that are not folded correctly. *BiP* plays a role in reducing drought-induced leaf senescence in soybean and tobacco. *GRP94* is also characterized in heat shock protein family HSP90 and has been shown to be up regulated in response to powdery mildew infection

in barley (Gupta and Tutei, 2011). In the *P. triticina* wheat interaction, the ER molecular chaperone-1 may be serving as a plant stress response gene. An example of a susceptibility factor that functions in the ER is *BAX inhibitor-1*, which is an endoplasmic reticulum negative cell death regulator protein that inhibits stress-related cell death in yeast, animals, and plants (Huckelhoven et al., 2013; Babaeizad et al., 2009; Eichmann et al., 2010). When overexpressed in barley, *HvBI-1* suppresses host defense responses to powdery mildew and supported penetration of powdery mildew into barley cells (Babaeizad et al., 2009). Transgenic silencing of *HvBI-1* using RNAi conditioned plants with lower levels of susceptibility compared to the non-transgenic controls (Eichmann et al., 2010). This shows that *HvBI-1* is required for susceptibility of barley and like *mlo*, it is negatively associated with penetration resistance through powdery mildew induced hydrogen peroxide burst at sites of fungal penetration (Babaeizad et al., 2009; Eichmann et al., 2010).

In this study, a mutational approach was taken to obtaining *P. triticina* resistance and identifying wheat genes that are critical for *P. triticina* infection. A mutant population of a susceptible wheat variety was created using the chemical mutagen EMS. The population was continued to the M_4 generation and was screened for *P. triticina* resistant mutants in each generation. In the M_4 generation, eight resistant mutants were selected and the expression of the seven wheat genes identified in Chapter 2 was evaluated. The resistant mutant 1022 was not expressing the ER molecular chaperone-1 and may be a knockout mutant for this gene. In order to determine if 1022 is a knockout mutant of ER molecular chaperone-1, 5' race needs to be conducted on the gene region and the resulting product should be sequenced. Southern blots could be done to determine how many copies of the gene are functioning in a non-mutant and the 1022 mutant. To order to identify the mutated genes in the additional resistant mutants, RNAseq

should be conducted. After the genes are identified, ideally a mutant for each homologous gene copy would be identified and crossed over several generations to generate a triplicate mutant. Then, all of the resistant mutants could be screened against other *P. triticina* races and other wheat rusts to determine how broad spectrum the resistance is. Fungal staining could also be done to show where the fungal growth is being stopped.

Figures

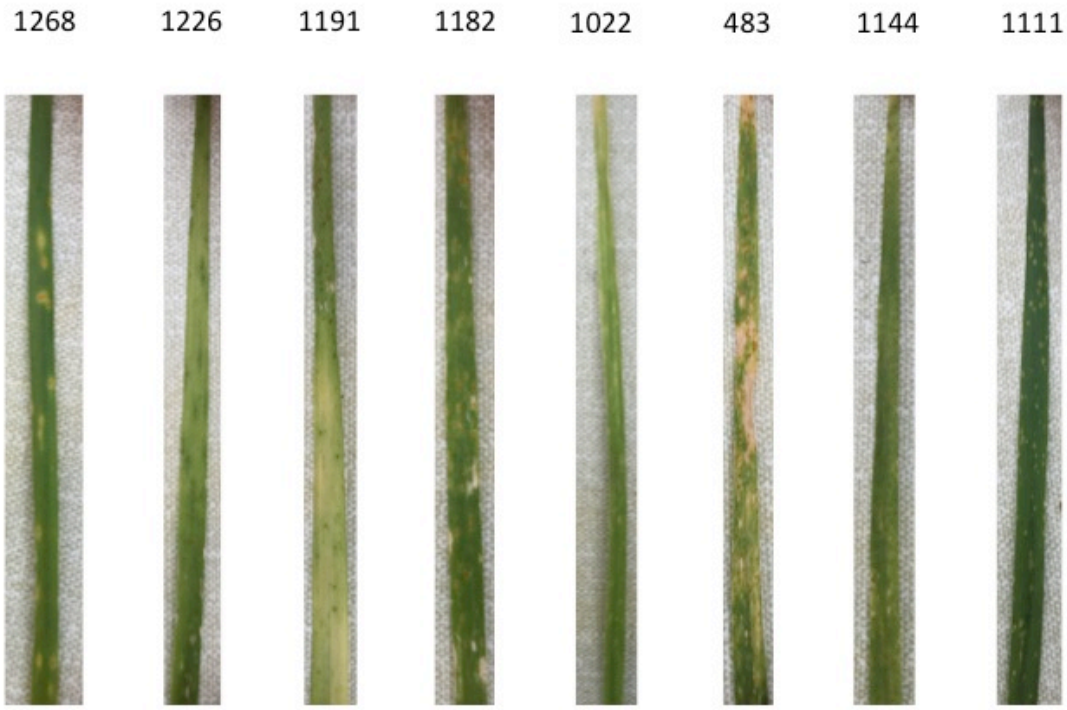


Figure 4.1 Phenotype of resistant M_4 lines 10 days post infection.

Mutants have different resistant phenotypes; some have necrotic and chlorotic lesions while others have suppressed spore formation.

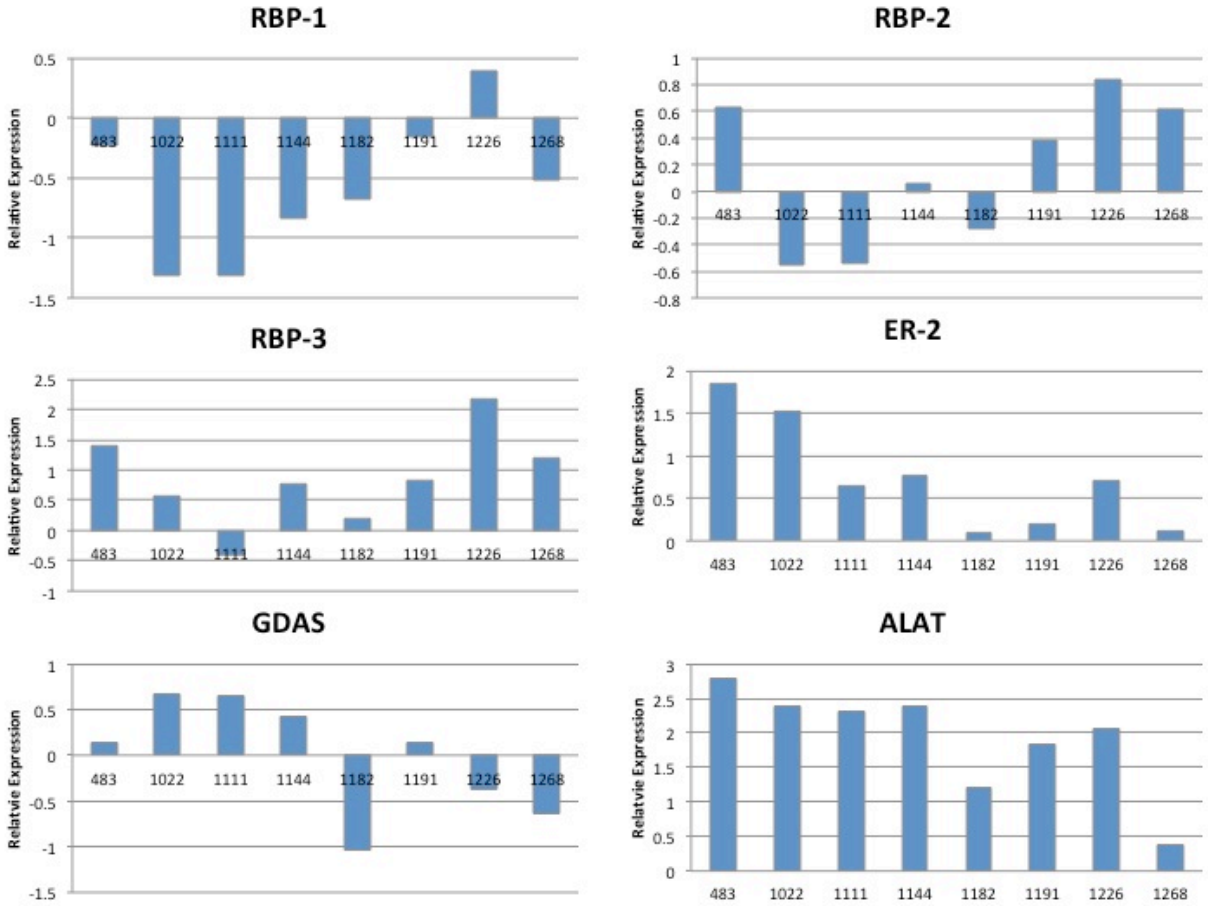


Figure 4.2 Real time PCR gene expression data showing the expression of the genes previously identified in the resistant mutants.

Real time PCR was performed on cDNA of individual mutants. Each graph shows the gene expression level of a particular wheat gene for the eight resistance mutants. Although some of the mutants showed suppressed gene expression levels, the genes are most likely still functioning.

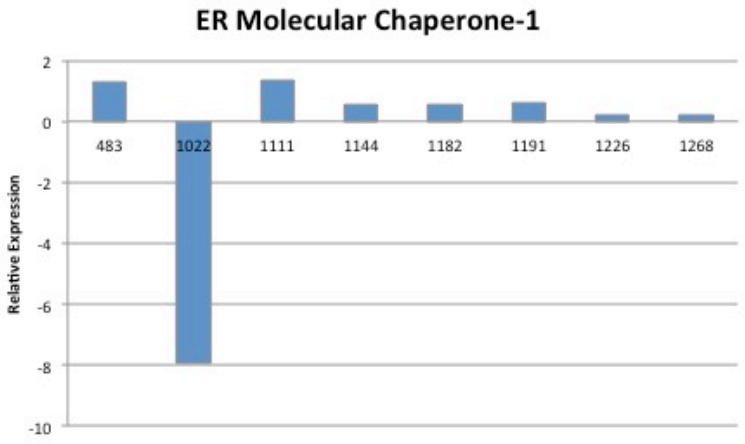


Figure 4.3 Real time PCR data showing the reduced ER molecular chaperone-1 expression in mutant 1022.

Real time PCR was performed on cDNA of individual mutants. The graph shows the gene expression level of the ER molecular chaperone-1 for the eight resistance mutants. Mutant 1022 has greatly reduced expression for the ER molecular chaperone-1. This gene may be knocked out in mutant 1022.

Chapter 5 - Summary

This work has used several distinct approaches to evaluate disease resistance and to gain a greater understanding of the *P. triticina* wheat interaction. Chapter 2 used RNAseq to identify 63 wheat genes that had differential gene expression in response to six *P. triticina* races. The expression of the identified genes was further evaluated in a time course using real time PCR to characterize the expression of 54 wheat genes during the first week of *P. triticina* infection. The putative functions of the characterized genes were varied with roles in plant defense, protein transport, replication, photosynthesis, and nutrient distribution. Genes with race specific induced gene expression and genes that maintained similar expression levels in response to all six *P. triticina* races were identified. Race specific gene expression may indicate that different pathogen effectors can induce differential expression of certain wheat genes. Wheat genes that had similar expression levels induced by all six races could be classified as a pathogenicity factor, a protein that is required for the successful infection of a pathogen. Silencing a pathogenicity factor in the host could condition durable broad-spectrum resistance because the pathogen would have to alter an aspect of its lifecycle to overcome the resistance in the host. Based on the gene expression during the first week of infection and the putative gene function, seven wheat genes were chosen to determine if the gene had significant function in the *P. triticina* wheat interaction. To test this hypothesis, a reverse genetic approach was taken through the use of the transgenic silencing technique RNAi. Gene fragments were cloned into an RNAi hairpin vector and transformed into embryonic callus using particle bombardment. Transgenic plants were obtained from each construct and molecularly characterized for the presence of the transgene through three generations. A bioassay was conducted in the T_2 generation by

inoculating the transgenic plants with a virulent *P. triticina* race and scoring the plants. There were little phenotypic differences between the infected transgenic plants and the infected non-transgenic controls. The small differences observed were seen mostly as smaller pustules and flecking on the leaf tip. The expression of the hairpin construct and the endogenous wheat gene was evaluated using rt PCR and real time PCR. Even in plants that the hairpin construct was expressing, the endogenous host gene was not silenced. RNAi is considered a gene knockdown rather than a gene knockout technology and this issue may be heightened in a hexaploid with multiple homologous copies. Alternatively, a forward genetics approach to *P. triticina* resistance was taken in Chapter 4. A mutant population of a susceptible wheat variety was generated using EMS. The population underwent three generations of selection to identify eight mutants that were resistant to a virulent race of *P. triticina*. The resistant mutants were evaluated for the expression of the seven genes that were silenced in Chapter 3. One resistant mutant had low gene expression for the ER molecular chaperone-1 and may be a knockout mutant for the gene. Compared to the small effects seen in the transgenic plants, the mutants showed much greater levels of resistance. This is comparable to the levels of resistance seen in plants with major and minor resistance genes. It is possible that the suppression in gene expression from RNAi conditioned partial resistance to leaf rust while the knockout in gene expression conditioned total resistance. The partial resistance is much harder to detect, especially with the relatively small population sizes of twenty plants per line used to screen the transgenic plants. To further evaluate this hypothesis, larger population sizes should be used. The plants should also be inoculated and scored at multiple plant stages, especially at the adult plant stage where this type of resistance is most commonly expressed. Although RNAi was not able to determine the role of the seven wheat genes in the *P. triticina* interaction, the mutant population showed that the identified genes

might be good targets for future endeavors with other technologies, such as CRISPR-Cas9. This work also demonstrates the utility of using transgenic methods for basic research studies and then applying what was found in non-transgenic methods, such as mutagenesis.

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